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Glypican-4 Is an FGF2-Binding Heparan Sulfate Proteoglycan Expressed in Neural Precursor Cells

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ABSTRACT **FGF2 is a crucial mitogen for neural precursor cells in the developing cerebral cortex. Heparan sulfate proteoglycans (HSPGs) are thought to play a role in cortical neurogenesis by regulating the action of FGF2 on neural precursor cells. In this article, we present data indicating that glypican-4 (K-glypican), a GPI-anchored cell surface HSPG, is involved in these processes. In the developing mouse brain, glypican-4 mRNA is expressed predominantly in the ventricular zone of the telencephalon. Neither the outer layers of the telencephalic wall nor the ventricular zone of other parts of the developing brain express significant levels of glypican-4, with the exception of the ventricular zone of the tectum. In cultures of E13 rat cortical precursor cells, glypican-4 is expressed in cells immunoreactive for nestin and the D1.1 antigen, markers of neural precursor cells. Glypican-4 expression was not detected in early postmitotic or fully differentiated neurons. Recombinant glypican-4 produced in immortalized neural precursor cells binds FGF2 through its heparan sulfate chains and suppressed the mitogenic effect of FGF2 on E13 cortical precursor cells. The spatiotemporal expression pattern of glypican-4 in the developing cerebral wall significantly overlaps with that of FGF2. These results suggest that glypican-4 plays a critical role in the regulation of FGF2 action during cortical neurogenesis.**

Key words: heparan sulfate proteoglycan; glypican-4; K-glypican; FGF2; neural stem cells; cortical neurogenesis

INTRODUCTION

The vast majority of cortical neurons are generated from neural precursor cells that reside in the ventricular zone of the embryonic brain. The proliferation and differentiation of neural precursor cells are regulated by various growth factors and morphogens (McKay, 1997; Stemple and Mahanthappa, 1997; Weiss et al., 1996b). FGF2 has been shown to be a critical mitogen for cortical neural precursor cells in vitro (Ghosh and Greenberg, 1995; Vicario-Abajón et al., 1995).

Heparan sulfate proteoglycans (HSPGs) are important regulators of FGF action (Givol and Yayon, 1992; Rapraeger et al., 1991; Yayon et al., 1991). HSPGs interact with FGFs through their heparan sulfate chains and the association modulates the activity of FGFs. HSPGs have been suggested to be involved in various aspects of neural development by regulating FGF actions, including neurogenesis. Nurcombe et al. (Nurcombe et al., 1993) reported that HSPGs secreted by the E9 mouse neuroepithelial cells preferentially bind FGF2 than FGF1, but concomitant with the onset of FGF1 expression at E11, the HSPGs change their binding preference from FGF2 to FGF1. HSPGs isolated from E11 enhance the mitogenic activity of FGF1 on neuroepithelial cells more highly than that of FGF-2, whereas HSPGs from E9 enhance FGF-2 activity more highly than FGF1 activity.

The molecular identity of HSPG(s) expressed in neural precursor cells has not been fully characterized. We previously reported that glypican-4 (K-glypican), a glycosyl phosphatidylinositol (GPI)-anchored cell surface HSPG, is expressed in the embryonic brain (Watanabe et al., 1995). Glypican-4 is a member of the glypican family of HSPGs characterized by the GPI-anchorage to cell surfaces and strictly conserved cysteine residues in their core proteins. This family includes glypican [glypican-1 (David et al., 1990)], cerebroglycan [glypican-2 (Stipp et al., 1993)], OCI-5 [glypican-3 (Filmus et al., 1988)], glypican-5 (Saunders et al., 1997), glypican-6 (Veugelers et al., 1999), and the Drosophila *dally* gene (Nakato et al., 1995). Genetic evidence suggests that glypicans are involved in embryonic development by regulating the proliferation of precursor cells. For instance, glypican-3 has been shown to be the causative gene for Simpson-Golabi-Behmel syndrome (SGBS), a human genetic disorder characterized by pre- and postnatal overgrowth (Pilia et al., 1996). *Dally*

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has been shown to be crucial for ordered cell cycle progression of lamina precursor cells, specialized precursor cells that generate lamina neurons connecting Drosophila photoreceptor cells to the central nervous system (Nakato et al., 1995).

In this article, we show that expression of glypican-4 in the developing brain is correlated with several loci of proliferating neural precursor cells, most notably the telencephalic ventricular zone. We also show that glypican-4 binds FGF2 through its heparan sulfate chains and suppresses the mitogenic activity of FGF2 on cortical precursor cells. These results suggest that glypican-4 plays a role in the regulation of FGF2 activity in locations associated with neurogenesis.

RESULTS

Expression of Glypican-4 mRNA in the Embryonic Mouse Brain

We previously showed that in the mouse embryo glypican-4 is predominantly expressed in kidney and brain (Watanabe et al., 1995). To further define its spatiotemporal expression pattern in the nervous system, we mapped glypican-4 mRNA expression in the developing mouse brain by *in situ* hybridization.

Transverse sections of mouse embryos from E10 to E17 were analyzed using a mouse K-glypican RNA probe (Fig. 1). The specificity of the probe has previously been demonstrated (Watanabe et al., 1995). Temporally, glypican-4 expression was already detected at E10 in the wall of the telencephalic vesicle. This expression increases as the brain develops, and peaks around E11-13. After E15, the expression is downregulated and is undetectable at E17 (see Fig. 2). The expression of glypican-4 is not ubiquitous, but is restricted to the periventricular areas of the telencephalon (Fig. 1). Outside the telencephalon, only the developing tectum shows moderate levels of glypican-4 expression (arrowheads in Fig. 1D–H).

The periventricular expression of glypican-4 suggests that it is predominantly expressed in proliferating neuroepithelial cells in the ventricular zone. It has been shown that before E11 the ventricular zone occupies the entire telencephalic wall (Bayer and Altman, 1991). As the brain develops, the ventricular zone becomes restricted to the innermost layer, and the intermediate zone and cortical plate, which contain postmitotic neurons, occupy the outer layers. In mice after E15, the ventricular zone is restricted to narrow areas facing the ventricles (Jacobson, 1991). Our analysis revealed that expression of glypican-4 is restricted to the ventricular zone. At E10-11, the expression of glypican-4 was observed throughout the telencephalic wall (Fig. 1A–C). In later stages, the expression of glypican-4 was restricted to the inner layers of the telencephalic wall and the outer layers were devoid of glypican-4 expression (Fig. 1D–K). This distribution pattern is consistent with the localization of the ventricular zone.

To further confirm that glypican-4 is expressed in the ventricular zone, sections of E13, 15, and E17 mouse brain were double-labeled by in situ hybridization and by bisbenzamide staining (Fig. 2). Bisbenzamide labels cellular DNA and allows observation of layers in the telencephalic wall (Emerling and Lander, 1996). At E13, glypican-4 mRNA was detected throughout ~70% of the thickness of the telencephalic wall (right panel), which corresponded to the ventricular zone as shown by bisbenzamide staining (left panel). At E15, the ventricular zone occupies about one third of the thickness of the telencephalic wall, with the subventricular zone, intermediate zone, and cortical plate forming outer layers. Here the expression of glypican-4 was restricted to the ventricular zone, with deeper layers of the ventricular zone showing stronger signals. The more superficial layer of the ventricular zone adjacent to the intermediate layer showed weaker signals. No expression was observed in the outer layers of the E15 telencephalic wall. The expression level in the E15 ventricular zone, however, appeared lower than that observed in E13 mice. In E17 brain, little expression of glypican-4 was observed in the telencephalic wall, while the ventricular zone persisted as a narrow layer facing the lateral ventricle (Bayer and Altman, 1991).

The expression of glypican-4 is not ubiquitous in all ventricular zones of the developing CNS. It is essentially restricted to the ventricular zone of the telencephalon (Fig. 1). The ventricular zones of the diencephalon (third ventricle), mesencephalon (aqueduct), and rhombencephalon (fourth ventricle) do not express glypican-4. Such spatially restricted expression in the telencephalic ventricular zone is not due to differences in the timing of neurogenesis in these areas. As early as E10, when neurogenesis is occurring along the entire neuraxis, no expression of glypican-4 was observed in diencephalon, mesencephalon, or rhombencephalon (Fig. 1).

Glypican-4 Expression in Sites of Neuronal Regeneration in the Postnatal Brain

In situ hybridization studies suggest that glypican-4 expression correlates with sites of active neurogenesis. Neurogenesis is not, however, limited to the ventricular zones of the embryonic brain. In the cerebellum, generation of granule neurons in the external granular layer occurs during early postnatal periods (Jacobson, 1991). In rodent brain, neurogenesis continues during adult life in the dentate gyrus of the hippocampal formation (Altman and Das, 1965) and in the anterior part of the subventricular zone, which generates olfactory neurons (Luskin, 1993). Multipotent stem cells have also been isolated from periventricular areas in the brain stem and spinal cord (Weiss et al., 1996a). Therefore, we examined whether precursor cells in these sites express glypican-4. In situ hybridization revealed strong expression of glypican-4 in the dentate gyrus of adult mice (Fig. 3A). A weaker but substantial

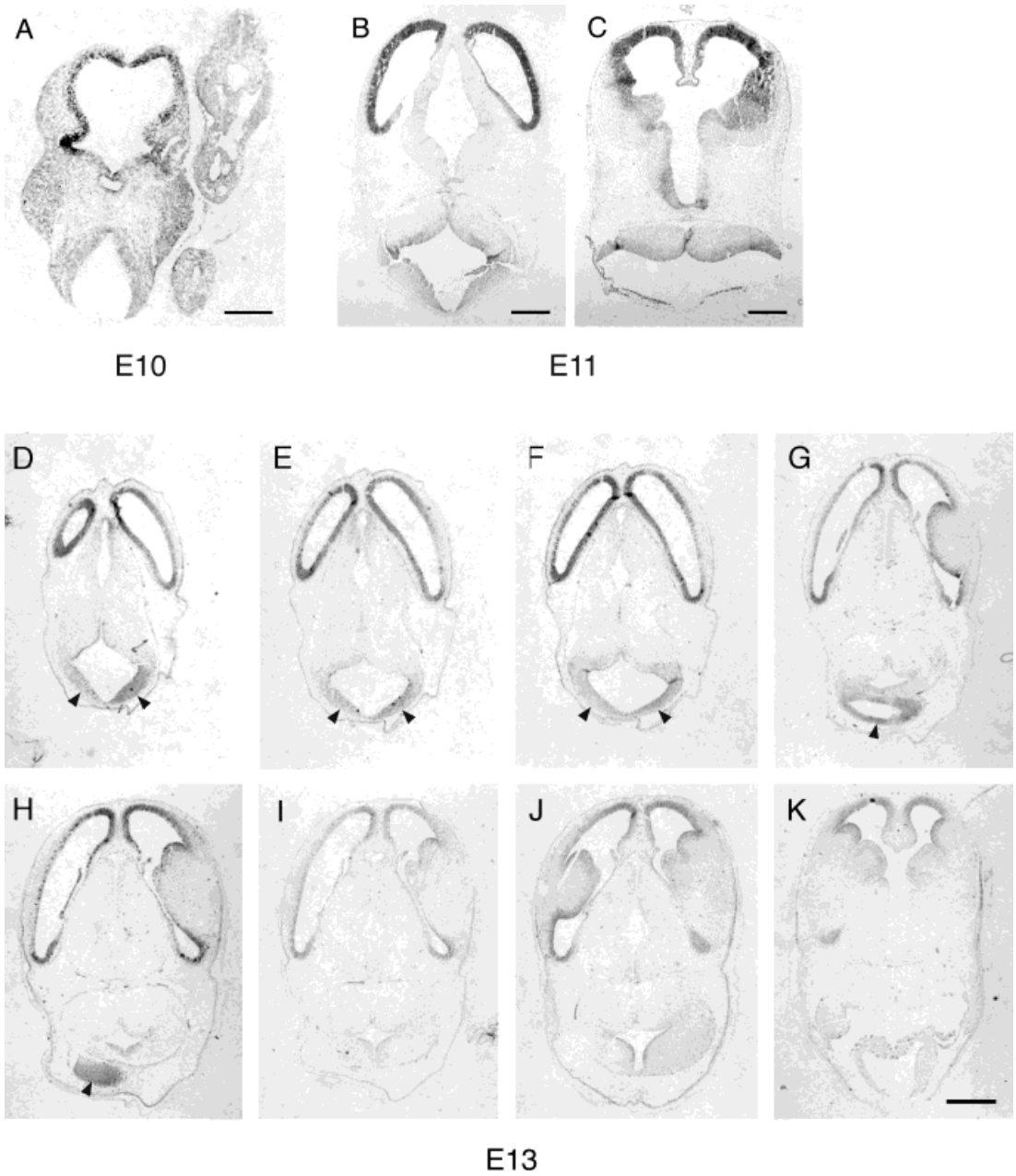

Fig. 1. Expression of glypican-4 mRNA in embryonic mouse brain. In situ hybridization was performed on transverse sections of E10 (**A**), E11 (**B** and **C**), and E13 (**D–K**) mouse brain with a digoxigenin-conjugated mouse glypican-4 RNA probe, followed by visualization of signals with phosphatase-conjugated anti-digoxigenin antibodies. In E11 and E13 brains, transverse sections at different levels (from crown toward rump) were analyzed. Note that both in E10 and E11 brain, the expression of glypican-4 mRNA is observed mainly in the periventricular areas of the telencephalon. At E13, glypican-4 expression is decreased compared with E11. The ventricular zone of the tectum (arrowheads) is positive for glypican-4 expression, while the ventricular zones of the diencephalon, mesencephalon, and rhombencephalon do not express glypican-4. Scale bars = 100 μm (A), 500 μm (B and C), 1 mm (D-K).

expression was detected in the external granular layer of P7 cerebellum (not shown). On the other hand, little glypican-4 mRNA was detected in the subventricular zone of the anterior lateral ventricle (Fig. 3B) or the periventricular areas of the brain stem and the spinal cord (not shown).

Glypican-4 is Expressed as a HSPG in the Embryonic Brain

To demonstrate that glypican-4 is indeed expressed as a HSPG in the embryonic brain, we performed immunoblotting and immunohistochemistry analyses. By

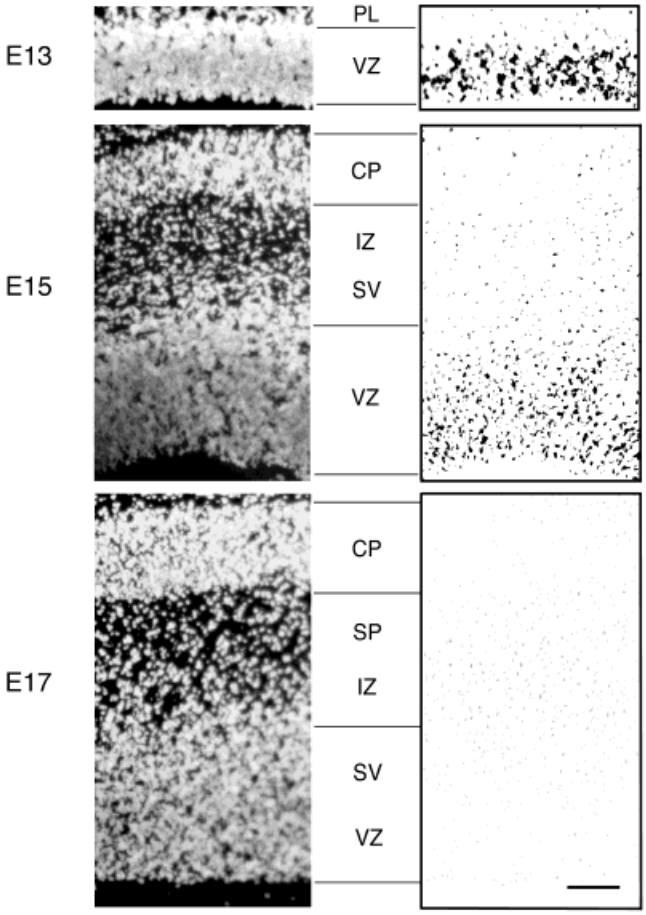

Fig. 2. Expression of glypican-4 mRNA in the developing cerebral wall. Transverse sections of E13, E15, and E17 mouse telencephalon were hybridized with digoxigenin-labeled RNA probes for glypican-4. After visualization of the hybridization signals with phosphatase-conjugated anti-digoxigenin antibodies, sections were stained with bisbenzamide to identify layers of the cerebral wall. Note that the signals of glypican-4 mRNA correlate with the ventricular zone in E13 and E15 brain. CP, cortical plate; IZ, intermediate zone; PL, plexiform layer; SP, subplate; SV, subventricular zone; VZ, ventricular zone. Scale bar = 150 μm.

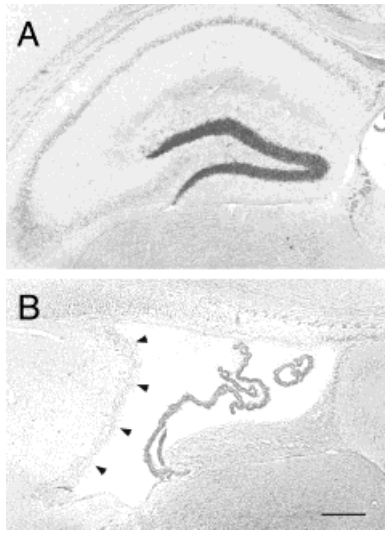

Fig. 3. Expression of glypican-4 mRNA at sites of adult neurogenesis. In situ hybridization was performed on a coronal section of the dentate gyrus (**A**) and on a sagittal section of the anterior part of the lateral ventricle (**B**) of adult mice with a digoxigenin-conjugated mouse glypican-4 RNA probe. Note that strong glypican-4 expression is observed in the dentate gyrus (A). On the other hand, the subventricular zone of the anterior part of the lateral ventricle, which generates olfactory neurons during adult life (arrowheads), does not express significant levels of glypican-4 (B). Scale bar = 300 μm.

immunohistochemistry, strong immunoreactivity of glypican-4 was observed corresponding to the ventricular zone of E16 rat telencephalon, whereas little immunoreactivities were detected in more superficial layers, including the subventricular zone, intermediate zone, and cortical plate (Fig. 4B). This result is consistent with the expression pattern glypican-4 mRNA demonstrated by in situ hybridization.

To further characterize glypican-4 in the embryonic brain, detergent extracts of the membrane fraction from E14 mouse brain were probed with the R364 antibody, with or without heparitinase digestion. Without heparitinase digestion, the antibody did not react with any specific band (Fig. 4A, lane 1). After heparitinase digestion, a major 60 kDa band and a weaker ~30 kDa band were detected (lane 2). The size of the 60 kDa band is consistent with that of full-length glypican-4 core protein (Watanabe et al., 1995). The weaker 30 kDa band probably represents a proteolytic fragment of glypican-4 core protein (Watanabe et al., 1995). The lack of reactivity without heparitinase digestion (lane 1) is probably due to poor transfer of undigested glypican-4 to nitrocellulose filters, rather than to the lack of reactivity to the HSPG-form of glypican-4. Poor transfer of proteoglycans to nitrocellulose filters has been reported in several instances (Rapraeger et al., 1985; Saunders et al., 1997). This result indicates that essentially all glypican-4 molecules in E14 mouse brain are in the form of HSPG. Taken together, these immunohistochemical and immunoblotting experiments demonstrate that glypican-4 is indeed expressed as a HSPG in the embryonic brain.

Neural Precursor Cells in Culture Express Glypican-4

To further establish the correlation of glypican-4 expression with neural precursor cells in the embryonic brain, we examined the expression of glypican-4 in cultures of rat neural precursor cells. Cultures of precursor cells were prepared from E13 rat telencephalon (E13 cultures) according to the method by Ghosh and Greenberg (Ghosh and Greenberg, 1995). As shown in Fig. 5A, the majority of cell clusters at one day in vitro (DIV) were immunoreactive for nestin, an intermediate filament protein specific for neural precursor cells (Hockfield and McKay, 1985; Lendahl et al., 1990).

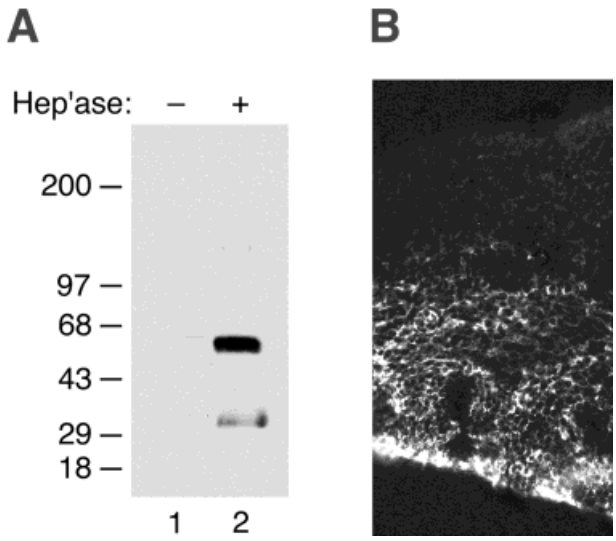

Fig. 4. Glypican-4 HSPG is expressed in the embryonic brain. **A**: Detergent extracts of the membrane fraction from E14 mouse forebrain were immunoblotted with R364 affinity-purified anti-glypican-4 antibodies with (**lane 2**) or without (**lane 1**) heparitinase treatment. A 60 kDa band detected after heparitinase digestion is the full-length glypican-4 core protein (lane 2). The weaker 30 kDa band is a proteolytic fragment of the glypican-4 core protein, which was also observed in cells transfected with glypican-4 cDNA (Watanabe et al., 1995). The absence of reactivity without heparitinase digestion (lane 1) is probably due to poor transfer of undigested HSPGs to nitrocellulose filters (Rapraeger et al., 1985; Saunders et al., 1997; see also text). **B**: Immunohistochemical identification of glypican-4 in the ventricular zone. E16 rat forebrain was stained with R364 antibodies as describe in Materials and Methods. Note that strong immunoreactivity of glypican-4 is detected in the deeper layer of the cerebral wall corresponding to the ventricular zone.

These cells were also immunoreactive for the D1.1 monoclonal antibody, another marker of neural precursor cells (Fig. 5D). The D1.1 antigen, a cell surface ganglioside, is specifically expressed by germinal cells in the ventricular zone and the external granular layer of the cerebellum (Levine et al., 1984; Stallcup et al., 1983). In addition to the expression of these markers, many of the cells in E13 cultures were shown to be replicating as demonstrated by the incorporation of BrdU (Fig. 5G), satisfying another criterion for precursor cells. These results indicate that most of the cells in these cultures retained the phenotype of undifferentiated neural precursor cells.

Expression of glypican-4 was examined in these cultures by double immunostaining with various markers. Strong glypican-4 expression was detected in E13 cultures (Fig. 5B and E). In glypican-4/nestin double staining, immunoreactivity of glypican-4 was detected in a punctate pattern on the surface of nestin-positive cells (Fig. 5A-C). We also examined the co-expression of glypican-4 and the D1.1 antigen (Fig. 5D-F). Because the D1.1 antigen is expressed on the cell surface, colocalization with glypican-4 was observed more clearly than was that of glypican-4 and nestin. Double staining revealed that glypican-4 and the D1.1 antigen are co-expressed on the surface of the same cells (Fig. 5F). These results demonstrate that glypican-4 is expressed in neural precursor cells.

Lack of glypican-4 mRNA in the outer layers of the developing telencephalic wall (see Fig. 2) suggests that expression of glypican-4 is rapidly down-regulated soon after neurons become postmitotic. To define the end point of glypican-4 expression, we analyzed its expression relative to that of neuronal markers. Cultures were prepared from E17 forebrain by the same procedure as for E13 cultures and double stained for glypican-4 and MAP2, a marker of differentiated neurons. In E17 cultures, most cells showed clear neuronal morphology with long processes and were MAP2-positive (Fig. 5I). No glypican-4 expression was detected in cells with neuronal morphology. There were occasionally small clusters of cells (the cluster on the right in Fig. 5I), in which the round cells in the center showed a weak glypican-4 immunoreactivity and the cells with neuronal morphology in the periphery were MAP2-positive. These glypican-4 expressing cells may be small population of E17 cerebral cells that retain the precursor phenotype. It is noteworthy that even in such mixed clusters, immunoreactivities of glypican-4 and MAP2 never overlapped (Fig. 5I). These results suggest that glypican-4 expression is lost before neurons become MAP2-positive.

Because the expression of MAP2 begins relatively late in neuronal differentiation, we examined the relationship between the expression of glypican-4 and another neuronal marker, TuJ1. The TuJ1 monoclonal antibody recognizes neuron-specific type III β-tubulin, which is expressed in neurons as early as the terminal mitosis (Menezes and Luskin, 1994). Thus expression of TuJ1 antigen occurs earlier than that of MAP2. To study this, we employed E13 cultures at 1 DIV. While most of the clusters in these cultures are TuJ1-negative, a small population of clusters contains cells immunoreactive for TuJ1. We examined these TuJ1-positive clusters by double staining with anti-glypican-4 and the TuJ1 antibodies to see if the expression of TuJ1 and glypican-4 may overlap. Double staining revealed that TuJ1-positive cells surround glypican-4-positive cells, which are located in the center of the clusters (Fig. 5H). As is the case with MAP2/glypican-4 double staining, immunoreactivities of glypican-4 and TuJ1 did not overlap. These results suggest that expression of glypican-4 is lost before cells become TuJ1-positive. Taken together, these observations demonstrate that expression of K-glypican is restricted to cells that retain the phenotype of neural precursor cells and that expression is down-regulated in postmitotic neurons.

Finally, we examined expression of glypican-4 in E13 cultures that were allowed to differentiate for four days. Without FGF2, the cells in E13 cultures differentiate into TuJ1/MAP2-positive neurons over three to four days (Ghosh and Greenberg, 1995). We examined these cultures by double staining of glypican-4 and markers of neural precursor cells (i.e., nestin, D1.1)

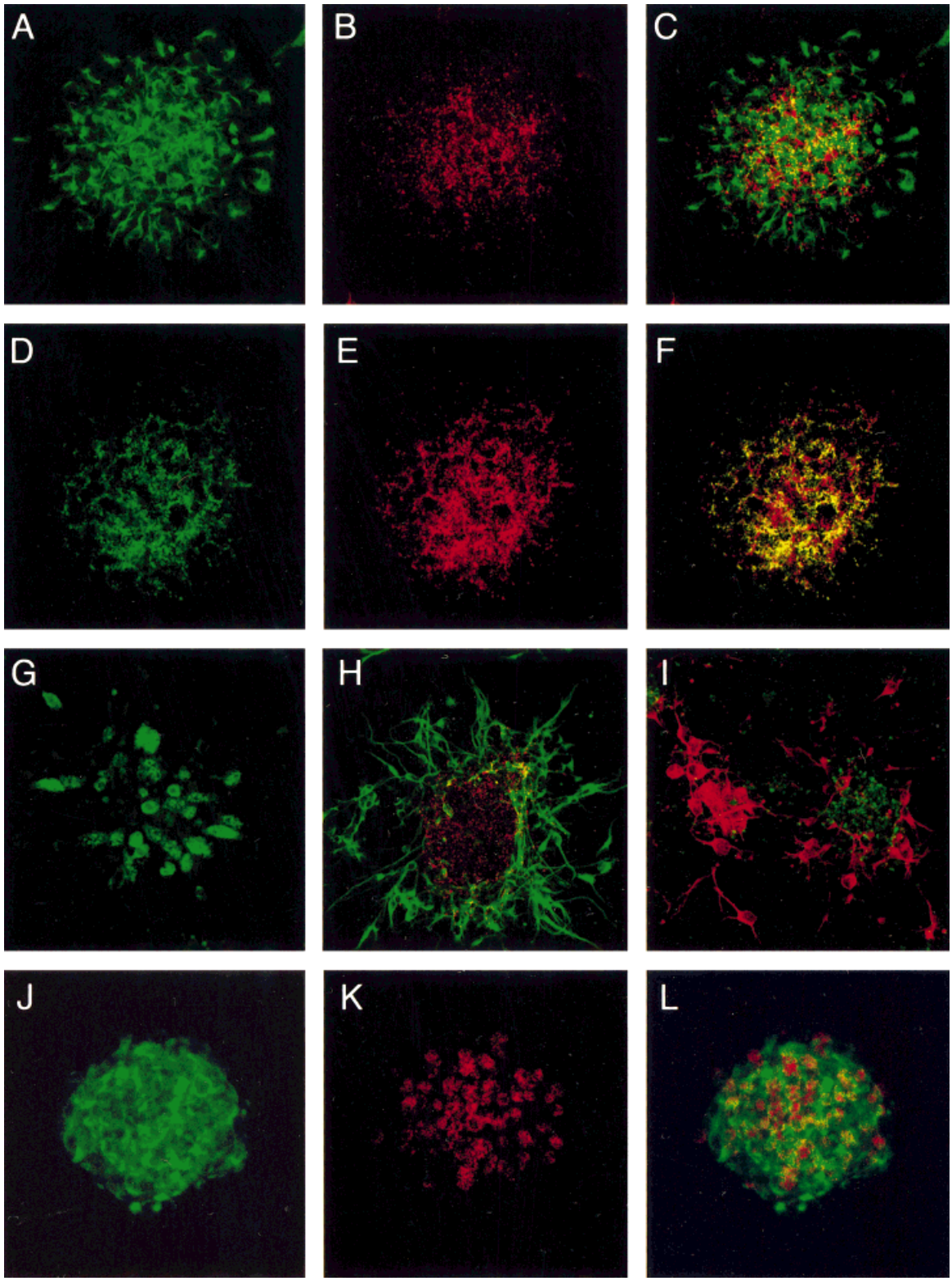

Fig. 5. Expression of glypican-4 in neural precursor cells in culture. (**A–C**) E13 cultures were double-labeled with monoclonal anti-nestin (A) and polyclonal anti-glypican-4 (B) antibodies. Panel C shows a superimposed view. (**D–F**) E13 cultures were double-labeled with the D1.1 monoclonal (D) and polyclonal anti-glypican-4 (E) antibodies. Panel F shows a superimposed view. (**G**) Incorporation of BrdU by the cells in E13 cultures. Cultures were incubated with 100 nM BrdU for 18 h. After fixation and permeabilization, incorporated BrdU was detected with a monoclonal anti-BrdU antibody and FITC-conjugated anti-mouse IgG. (**H**) E13 cultures were double-labeled with the TuJ1 monoclonal (green) and polyclonal anti-glypican-4 (red) antibodies. (**I**) Cultures prepared from E17 rat brain (E17 cultures) were double-labeled with monoclonal anti-MAP2 (red) and polyclonal anti-glypican-4 (green) antibodies. Two types of clusters are shown to illustrate that expression of glypican-4 and MAP2 does not overlap. The cluster on the left consists solely of cells with extended morphology, while the cluster on the right contains glypican-4-positive cells in the center surrounded by MAP2-positive cells with extended morphology. Even in such mixed clusters (which are relatively rare in E17 culture), immunoreactivities of glypican-4 and MAP2 never overlap. (**J–L**) EGF-responsive neurospheres isolated from E15 mouse striata were double-labeled with monoclonal anti-nestin (green) and polyclonal anti-glypican-4 (red) antibodies. Panel L shows a superimposed view.

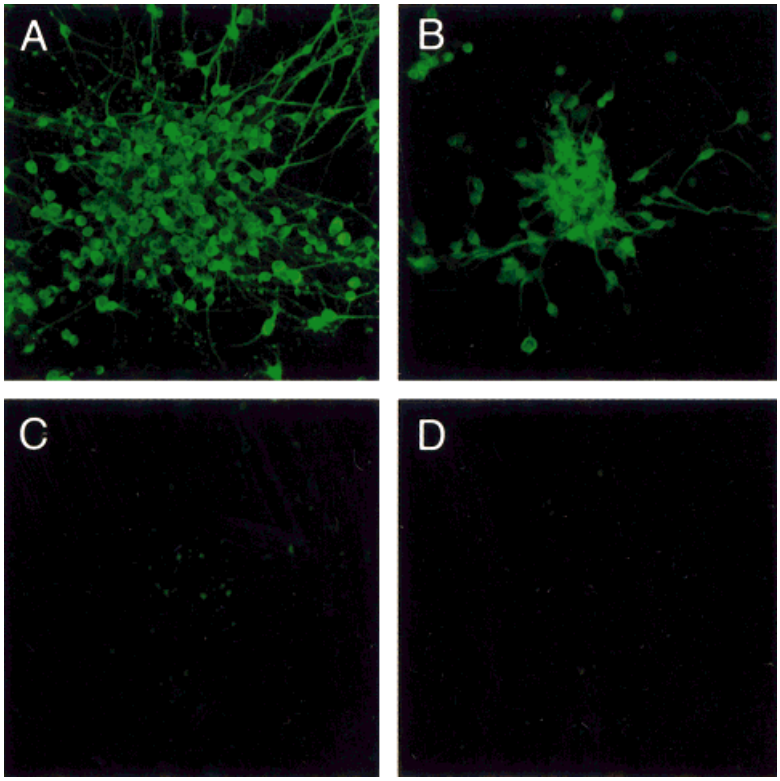

Fig. 6. Glypican-4 is not expressed in cultures of neural precursor cells that have been maintained for four days without FGF2. Cultures of neural precursor cells were prepared from E13 rat brain as described and maintained without FGF2 for four days. Cultures were then double-labeled with following antibodies: (**A**) anti-MAP2 (green) and anti-glypican-4 (red); (**B**) TuJ1 (green) and anti-glypican-4 (red); (**C**) anti-nestin (green) and anti-glypican-4 (red); (**D**) D1.1 (green) and anti-glypican-4 (red). Note that by this treatment cells differentiated into TuJ1/MAP2-positive neurons. Neither nestin (C) nor D1.1 antigen (D) was expressed in these cells. Glypican-4 was not detected in these cells (A–D).

and that of glypican-4 and markers of postmitotic and differentiated neurons (i.e., TuJ1, MAP2). Essentially, all cells were immunoreactive for MAP2 and TuJ1 in these cultures (Fig. 6A and B); little nestin or D1.1 immunoreactivity was detected (Fig. 6C and D), indicating that after four days without FGF2, the cells are no longer neural precursor cell. Double staining of glypican-4 with these markers showed that expression of glypican-4 was also lost from these cells (Fig. 6A–D). These results further confirm that expression of glypican-4 is restricted to cells with precursor properties.

There are culture systems for other types of neural precursor cells. It has been shown that precursor cells that generate both neuronal and glial progenies can be isolated from mouse embryonic striatum and expanded as floating spheres in the presence of EGF (Reynolds et al., 1992; Reynolds and Weiss, 1996). We prepared these EGF-dependent striatal precursor cells and examined whether they express glypican-4. As shown in Fig. 5J-L, these cells were immunoreactive for glypican-4 and for nestin, indicating that striatal precursor cells express glypican-4. We also examined glypican-4 expression in O2A progenitor cells. Unlike cortical and striatal precursor cells, O2A cells exhibit a limited proliferative ability toward oligodendrocyte and type II astrocyte lineages (Raff et al., 1990). Glypican-4 was not detected in O2A cells (not shown).

Glypican-4 Binds FGF2

Previous reports have shown that FGF2 is expressed in the ventricular zone (Dono et al., 1998; Weiss et al., 1993). The presence of the HSPG form of glypican-4 in the ventricular zone suggests that FGF2 and glypican-4 may associate in vivo. However, because the interaction of heparan sulfate with FGF2 requires specific carbohydrate structures within heparan sulfate chains, it must be demonstrated that glypican-4 produced by neural precursor cells actually binds FGF2. Thus, we examined if glypican-4 indeed has affinity for FGF2. Because it has not been possible for us to purify glypican-4 from embryonic brain tissues in biochemical quantities or to detect non-radiolabeled FGF2 in immunoprecipitates of glypican-4, we used recombinant glypican-4 expressed in the immortalized neural precursor cell line TSM (Chun and Jaenisch, 1996) to address this question. A neural precursor cell line, rather than generic cell lines such as COS and CHO, was used because the ligand binding properties of heparan sulfates can differ depending on the cell types in which glypican-4 is synthesized. Immortalized neu-

ral precursor cells are thought to provide a biosynthetic background equivalent to that of the ventricular zone of embryonic cerebral cortex (Hecht et al., 1996; Weiner et al., 1998).

First, we examined binding of [^{125}I]FGF2 to recombinant $RGSH_6$-tagged glypican-4 captured by Ni-NTA resin. In this assay, recombinant $RGSH_6$-tagged glypican-4 was captured on Ni-NTA resin by incubating the resin with extracts from glypican-4 transfected TSM cells (clone QHK17). Controls were incubated with extracts from control transfected cells (clone QHC1). As shown in Fig. 7A, [^{125}I]FGF2 bound to the resin capturing recombinant glypican-4. Resins incubated with extracts from control transfected cells did not bind [^{125}I]FGF2. The binding of [^{125}I]FGF2 to glypican-4-captured resin was abolished by heparitinase treatment. These results demonstrate that glypican-4 binds FGF2 and that binding is mediated by heparan sulfate chains of glypican-4.

Second, we examined whether anti-glypican-4 antibody coimmunoprecipitates [^{125}I]FGF2. Unlike the first assay, this assay examined soluble phase interaction. Membrane fractions from glypican-4 transfected cells were incubated with [^{125}I]FGF2 and the [^{125}I]FGF2-glypican-4 complex was immunoprecipitated using the R364 anti-glypican-4 antibody. Precipitated materials were resolved on SDS-PAGE and co-precipitated [^{125}I]FGF2 was detected by fluorography. As shown in Fig. 7B, the R364 antibody precipitated [^{125}I]FGF2, whereas the control antibody did not. Again, heparitinase treatment abolished the co-precipitation of [^{125}I]FGF2 with glypican-4. These two experiments demonstrate that glypican-4 synthesized in neural precursor cells binds FGF2 through its heparan sulfate chains.

Effect of Exogenously Added Glypican-4 on the Proliferation of Neural Precursor Cells

It has been shown that different types of HSPGs exert distinct effects on FGF2 activities. While the basement membrane HSPG perlecan strongly potentiates FGF2, glypican-1, syndecan-1, and syndecan-2 show little potentiating activities (Aviezer et al., 1994a, 1994b). To determine the effect of glypican-4 on neural precursor cells, we added recombinant glypican-4 to cultured E13 precursor cells and examined cell proliferation in response to FGF2. For this experiment, we used recombinant glypican-4 produced in immortalized neural precursor cells. As discussed above, we expect that this sample mimics biochemical properties of endogenous HSPGs in the developing cerebral cortex more than such samples as heparin or HSPGs derived from non-neuronal cell types. As shown in Figure 8, addition of recombinant glypican-4 did not enhance the mitogenic activity of FGF2. With higher concentrations, proliferation of precursor cells was suppressed modestly (~30%), presumably due to sequestration of glypican-4-bound FGF2 from cell surface receptors. This effect was not observed with heparitinase-treated

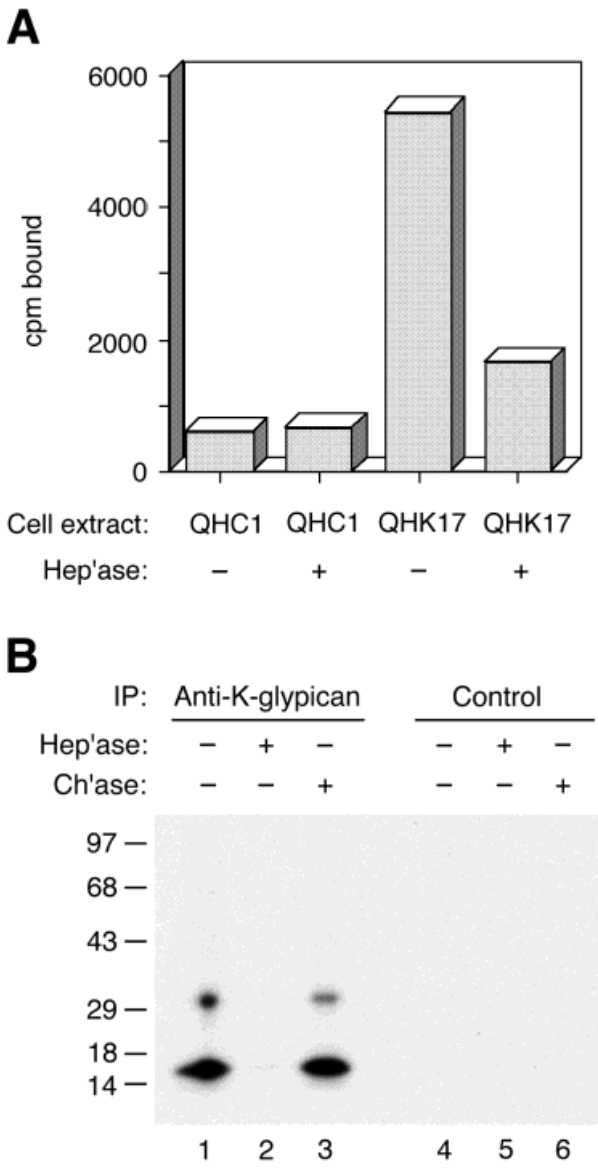

Fig. 7. Glypican-4 binds FGF2. **A**: Solid phase binding assay. The $RGSH_6$-tagged glypican-4 expressed in an immortalized mouse neural precursor cell line TSM (clone QHK17) was captured on Ni-NTA resin. Control resins were incubated with extracts from control transfected cells (QHC1 cells). These resins with or without treatment with heparitinase were then incubated with [^{125}I]FGF2 for 1.5 h. After washing, [^{125}I]FGF2 bound to the resin was eluted with 0.75 M NaCl in 50 mM Tris/HCl (pH 7.0) and counted in a gamma counter. QHC1, control transfected TSM cells; QHK17, K-glypican transfected TSM cells. Hep'ase, heparitinase. Data represent means of duplicates. This experiment was repeated twice with similar results. **B**: Co-immunoprecipitation assay. Membrane fractions prepared from QHK17 cells were incubated with [^{125}I]FGF2 and then immunoprecipitated with affinity purified R364 antibody (**lanes 1–3**) or control rabbit IgG (**lanes 4–6**). Immune complexes were precipitated by incubation with protein G agarose. Precipitated materials were left untreated (lanes 1 and 4) or treated with heparitinase (Hep'ase; lanes 2 and 5) or chondroitinase ABC (Ch'ase; lanes 3 and 6), and resolved by SDS-PAGE in a 8–16% gradient gel. [^{125}I]FGF2 in immunoprecipitates were detected by fluorography.

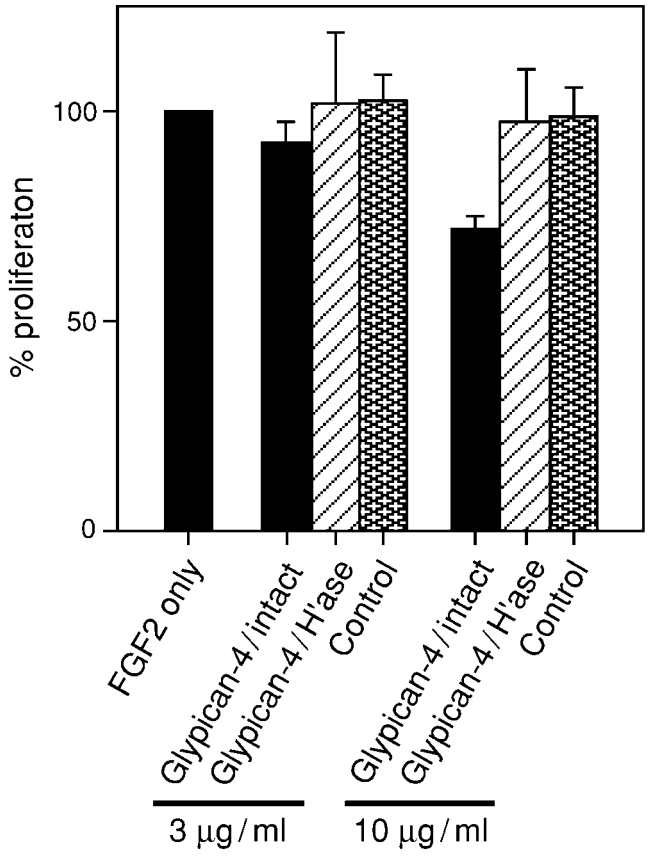

Fig. 8. Effects of exogenously added glypican-4 on the mitogenic activity of FGF2. E13 cortical precursor cells (1×10^4 cells/well) were cultured in the presence of FGF2 (25 ng/ml) and intact glypican-4 (**filled columns**), heparitinase-treated glypican-4 (**hatched columns**), or a negative control sample mock-purified from QHC1 cells (**cross-hatched columns**). Intact and heparitinase-treated glypican-4 were added at 3 or 10 μg/ml. The negative control sample was added to cells in the same volumes as the glypican-4 samples. After a two-day incubation, proliferation of cells were assayed by CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Kit. Data represent means ± 1SD (n = 3) of percent proliferation relative to the proliferation in the presence of FGF2 alone (100%).

glypican-4, indicating that it is mediated by heparan sulfate chains. These results are consistent with previous reports that glypican does not promote receptor binding of FGF2 (Aviezer et al., 1994a, 1994b).

DISCUSSION

There is increasing evidence that FGF2 plays pivotal roles in cortical neurogenesis. FGF2 is a mitogen for stem cells isolated from cerebral cortex and hippocampus (Ghosh and Greenberg, 1995; Vicario-Abajón et al., 1995). FGF2 also promotes survival and proliferation of neuronal progenitor cells isolated from adult brain (Gage et al., 1995; Gritti et al., 1996). Not only does FGF2 act as a mitogen for cortical stem cells, but it also regulates the generation of different neural lineages. Qian et al. (1997) reported that high concentrations of FGF2 promote the generation of glial lineages, while lower concentrations promote continuous generation of neurons. This observation suggests that the regulation of FGF2 concentration in tissue environment may have profound effects on cortical neurogenesis. HSPGs, with their affinity for FGF2, may be the critical component that regulates the effect of FGF2 on neural precursor cells. Identification of HSPG(s) expressed in neural precursor cells would therefore have a considerable implication in our understanding of the regulation of cortical neurogenesis.

In this article, we present several lines of evidence that glypican-4 is expressed by neural precursor cells in the embryonic brain. Histologically, the spatial expression pattern of glypican-4 remarkably correlates with the telencephalic ventricular zone. Moreover, studies with E13 cortical cultures demonstrate that only cells that retain the phenotype of precursor cells express glypican-4. The expression of glypican-4 is lost in cells that have committed differentiation. We found no overlap between glypican-4- and TuJ1-expressing cells in E13 cultures. The TuJ1 antigen is an early marker of neuronal differentiation, expressed as early as the terminal mitosis (Lee et al., 1990; Menezes and Luskin, 1994). This suggests that cessation of glypican-4 expression occurs very early in neurogenesis. Overall, our data indicate that expression of glypican-4 in the brain is highly restricted to undifferentiated precursor cells.

Several HSPGs are known to be expressed in the developing brain. However, no HSPGs other than glypican-4 have been shown to exhibit neuroepithelium-specific expression. For example, cerebroglycan (glypican-2), though widely expressed in the developing nervous system, is not detected in the ventricular zone of any part of the developing CNS (Stipp et al., 1993), but is found in postmitotic neurons and on axon tracts when axons are actively extending (Ivins et al., 1997; Stipp et al., 1993). Glypican-5 is also expressed in postmitotic neurons (Saunders et al., 1997). Glypican-1 has been observed in the ventricular zone (Karthikeyan et al., 1994; Litwack et al., 1998). Its expression, however, is not restricted to the ventricular zone but persists throughout development and increases in the adult brain (Litwack et al., 1994). N-syndecan (syndecan-3) is detected in TuJ1-positive axonal tracts (Kinnunen et al., 1998), and high levels of expression are restricted to the early postnatal brain, mainly in axon tracts (Carey et al., 1997). A 45 kDa perlecan splicing variant (Joseph et al., 1996) has been proposed to be an FGF2-binding HSPG in neuroepithelium (Nurcombe et al., 1993). While this perlecan variant is expressed in neuroepithelium, strong immunoreactivity of this HSPG was mainly seen in the basement membranes of the meninges and blood vessels of the developing CNS (Joseph et al., 1996), an expression pattern consistent with basement membrane molecules such as perlecan.

Not only is expression of glypican-4 in the nervous system specific for the neuroepithelium, but it is essen-

tially restricted to the telencephalic neuroepithelium. This observation relates to a central question of neural development—how regional diversity in the adult brain is generated. Positionally restricted expression within neuroepithelia has been reported for several transcription factors, including MASH1 (Lo et al., 1991), BF-1 (Tao and Lai, 1992), BF-2 (Hatini et al., 1994), Nkx-2.2 (Price et al., 1992), Krox-20 (Wilkinson et al., 1989), Emx1 and Emx2 (Gulisano et al., 1996). It is believed that such positionally restricted expression of transcription factors marks the earliest events in a cascade, which then induces cell surface and/or extracellular molecules that play more direct roles in regulating proliferation, migration, and differentiation of neurons to achieve regional differences. Glypican-4 is one of the several cell surface molecules reported that shows positionally restricted expression in the neuroepithelium. Such cell surface molecules include E- and R-cadherins (Matsunami and Takeichi, 1995; Shimamura and Takeichi, 1992), cadherin-6 (Inoue et al., 1998), and the Le^X carbohydrate (Allendoerfer et al., 1995). Another molecule with extracellular domains that is present in the ventricular zone is the G-protein coupled lysophospholipid receptor lpa1/vzg-1, a receptor for the growth factor-like, signaling lipid lysophosphatidic acid (Fukushima et al., 1998; Hecht et al., 1996). It has a gene expression pattern that is almost identical to that of glypican-4 in the embryonic cortical ventricular zone (Fukushima et al., 1998; Hecht et al., 1996; Weiner et al., 1998). Functional relationships between glypican-4 and lysophosphatidic acid signaling remain to be examined. However, in view of the induction of cadherin expression by lysophosphatidic acid through a homologous lysophospholipid receptor, LPB1/edg-1, an analogous relationship in the ventricular zone may exist (Chun et al., 1999; Contos and Chun, 1998; Lee et al., 1998; Zhang et al., 1999).

HSPGs have been implicated in the regulation of FGF2 activity in neurogenesis (Nurcombe et al., 1993). Like other HSPGs, glypican-4 binds FGF2 through its heparan sulfate chains. Moreover, the distribution of glypican-4 expression correlates with the reported distribution of FGF2 in the developing brain. Dono et al. (Dono et al., 1998) showed that FGF2 is expressed in the ventricular zone of the embryonic mouse brain. Furthermore, FGF2 has been identified on the surfaces of the cells in the ventricular zone (Ford et al., 1994), although the mechanism of its release has not been clarified yet. These observations suggest that FGF2 and glypican-4 interact in vivo.

At present, we do not know the functional role of the glypican-4 binding on FGF2 activity in vivo. It has been shown that HSPGs activate FGF2 and promote its binding to high affinity FGF receptors (Spivak-Kroizman et al., 1994). Thus one possible effect of HSPGs in neural precursor cells is the potentiation of FGF2 activity. However, we found that our recombinant glypican-4 is inefficient in potentiating FGF2. Although we cannot rule out the possibility that our recombinant glypican-4, though produced in immortalized neural precursor cells, lack sulfation patterns of endogenous glypican-4 that is crucial for modulation of FGF2 activity, this result suggests that FGF2 bound by glypican-4 is sequestered from high affinity receptors rather than potentiated. It has been reported that not every HSPG potentiate FGF2: While the basement membrane HSPG perlecan is highly efficient in potentiating FGF2, other HSPGs, including glypicans and syndecans, are inefficient in this regard (Aviezer et al., 1994a, 1994b). It is possible that glypican-4 reduces the effective concentration of FGF2 or fluctuation of FGF2 in the ventricular zone, thereby serving as a reservoir of FGF2 that present stable levels of FGF2 to precursor cells. Such a mechanism may be important for promoting the continuous generation of neuronal progeny, because FGF2 promotes generation of different neural lineages from cortical precursor cells depending of its concentration: High concentrations of FGF2 facilitate the generation of glial progenies whereas low concentrations facilitates generation of neuronal progenies (Qian et al., 1997). On the other hand, it is possible that exogenous addition of soluble HSPGs as employed in this and other studies (Aviezer et al., 1994a, 1994b) does not accurately reflect the function of endogenous glypican-4. Endogenous, membrane-anchored glypican-4 may actually potentiating FGF-2 activity. Thus whether glypican-4 potentiates or suppresses FGF2 activity should be resolved using neural precursor cells entirely lacking glypican-4 expression isolated from glypican-4 mutant mice.

The discovery of a genetic human disorder caused by defective glypican-3 suggests that glypican-4 may be involved in the development of the nervous system in vivo. The Simpson-Golabi-Behmel syndrome (SGBS) is a genetic disorder with overgrowth phenotype (Weksberg et al., 1996), which is caused by the disruption of human glypican-3 gene (Pilia et al., 1996). Recently, we showed that the human glypican-4 gene is located adjacent to the glypican-3 gene, and that in at least one SGBS patient the entire glypican-4 gene is deleted whereas the glypican-3 gene is only partially deleted (Veugelers et al., 1998). This suggests that glypican-4 may also be involved in SGBS. SGBS patients sometimes show nervous system abnormalities, including mental retardation and susceptibility to neuroblastoma. These symptoms cannot be readily explained by deletion of glypican-3, because glypican-3 is not highly expressed in the nervous system (Veugelers et al., 1998). The expression of glypican-4 in the embryonic brain and the adrenal gland is consistent with these phenotypes.

EXPERIMENTAL PROCEDURES

Materials

Heparinase and chondroitinase ABC (protease-free) were purchased from Seikagaku America (Rockville, MD). Digoxigenin RNA labeling mixtures, alkaline phosphatase-conjugated anti-digoxigenin antibodies,

and purified bovine FGF2 were purchased from Boehringer Mannheim (Indianapolis, IN). [^{125}I] FGF2 was purchased from NEN (Boston, MA). Purified mouse laminin, Opti-MEM, N2 supplement, and Lipofectamine were purchased form Life Technologies (Gaithersberg, MD). Poly-L-lysine (Type VIIB), BrdU, and the FITC-conjugated ExtrAvidin reagent were from Sigma (St. Louis, MO). A monoclonal antibody to rat nestin (Rat-401) was from the Developmental Studies Hybridoma Bank, University of Iowa. The D1.1 monoclonal antibody was a gift from Dr. W.B. Stallcup (The Burnham Institute). The TuJ1 monoclonal antibody, anti-rat MAP2 monoclonal antibody (HM-2), and anti-BrdU monoclonal antibody were from BAbCO (Richmond, CA), Sigma, and Amersham (Arlington Heights, IL), respectively. Various FITC- and RITC-conjugated secondary antibodies were purchased from Biosource International (Camarillo, CA). Bisbenzamide (Hoechst 33342) was purchased from Molecular Probes (Eugene, OR). Ni-NTA agarose and the RGS-His monoclonal antibody were from Qiagen (Santa Clarita, CA). A mammalian expression vector pSecTagB and ProBond resin were obtained from Invitrogen (San Diego, CA). CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Kit was purchased from Promega (Madison, WI).

Anti-Glypican-4 Antibodies

Rabbit antisera to mouse glypican-4 were produced with a synthetic peptide RFRPYHPEQRPT (corresponding to residues 364-375 of the mouse glypican-4 amino acid sequence) coupled to keyhole limpet hemocyanin. Antibodies were purified from the antisera on an affinity column of the immunizing peptide. Coupling of the peptide to EAH-Sepharose 4B was performed using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described by Harlow and Lane (Harlow and Lane, 1988). The antisera were applied to the column, and the column was washed first with 10 column volumes of 50 mM Tris (pH 7.4) containing 0.5 M NaCl, and then with 10 column volumes of 50 mM Tris (pH 7.4). Bound antibodies were eluted with 100 mM glycine buffer (pH 2.5). Eluents were immediately neutralized by adding 1/10 volumes of 1 M Tris (pH 8.0). The specificity of the affinity-purified antibody (designated as R364 antibody) was tested by immunocytochemistry with MDCK and Jurkat cells transfected with glypican-4 cDNA. The R364 antibody specifically stained K-glypican-expressing cells but not cells transfected with vector alone (not shown). Preimmune IgG did not stain either glypican-4-transfected or control cells. In immunoblotting, R364 antibody specifically recognizes the 60 kDa full-length core protein and the 30 kDa proteolytic fragment of glypican-4 (see Fig. 4).

Cell Culture

Primary cultures of cortical cells from E13 and E17 rat brain were prepared according to the method by Ghosh and Greenberg (Ghosh and Greenberg, 1995). Briefly, forebrains were dissected from rat embryos and triturated without enzyme treatment. Dissociated cells were plated onto 60 mm culture dishes which had been coated with poly-L-lysine (1 mg/ml) and laminin (7 μg/ml), and cultured in glutamate-free L15 media supplemented with 30 mM glucose, 27.8 mM $NaHCO_3$, 1% N2 supplement, and penicillin-streptomycin. An immortalized mouse neural precursor cell line TSM was established as described previously (Chun and Jaenisch, 1996). Parental and transfected TSM cells were cultured in Opti-MEM supplemented with 2.5% FCS, 14.2 mM β-mercaptoethanol, 20 mM D-glucose, and penicillin-streptomycin. The EGF-dependent neurospheres were prepared from E15 mouse striata according to Reynolds et al. (Reynolds et al., 1992) and maintained for up to four weeks by subculturing every seven days. Cultures of O2A progenitor cells were prepared as described (Nishiyama et al., 1996).

In Situ Hybridization

Mouse embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 18 h at 4°C, followed by dehydration with ethanol and xylene. Embryos were embedded in paraffin and 10 μm sections were cut. Adult mice were anesthetized and perfused transcardially with 0.9% NaCl, followed by 4% paraformaldehyde in PBS. Forebrains were dissected and immersed in 30% sucrose in PBS for 18 h at 4°C. After embedded in OCT compound (Miles, Elkhart, IN), 20 μm sections were cut in cryostat. In situ hybridization was performed with digoxigenin-labeled RNA probes as described previously (Watanabe et al., 1995). For double-labeling with bisbenzamide (Hoechst 33342), sections that had been developed for in situ hybridization were incubated with 1 μg/ml of Hoechst 33342 in PBS for 5 min, followed by washes with PBS.

Immunocytochemistry and Immunohistochemistry

E13 and E17 cortical cell cultures were plated in 60 mm dishes as described above and stained after the period indicated in each experiment. For glypican-4 and the D1.1 antigen, unfixed cells were incubated for 1 h with primary antibodies (R364, 1:25; D1.1, 1:1000) diluted in culture media. After washing with media, cells were incubated with secondary antibodies (RITC- or FITC-conjugated anti-rabbit IgG+IgM, 1:50; FITC-labeled anti-mouse IgG+IgM, 1:50) for 30 min, washed, and fixed for observation. All incubation steps with antibodies were performed under 5% CO_2 at 37°C. For staining with anti-nestin, anti-MAP2, and the TuJ1 antibodies, cells were fixed in 4% paraformaldehide in PBS for 15 min and treated with 0.3% Triton X-100/3% normal goat serum in PBS for 1 h. Cells were incubated for 1 h with primary antibodies (anti-nestin, 1:4; anti-MAP2, 1:500), washed with PBS, and incubated with secondary antibodies (RITC- or FITC-conjugated anti-mouse IgG+IgM, diluted at 1:50) for 30 min. All incubation steps were performed at room temperature. For

staining with the TuJ1 antibody, cells were fixed and blocked as above, and incubated with the TuJ1 antibody (1:100). After washing, cells were incubated for 1 h with biotinylated anti-mouse IgG (1:200), followed by a 30 min incubation with FITC-conjugated ExtrAvidin. In the case of double-labeling, cells were first stained for cell surface molecules (glypican-4 and the D1.1 antigen) without fixation, then fixed and permeabilized, and stained for intracellular molecules (nestin, MAP2, the TuJ1 antigen). BrdU labeling of the E13 cortical cultures were performed according to Ghosh and Greenberg (Ghosh and Greenberg, 1995). Briefly, 4 h after plating, BrdU was added to the culture at a final concentration of 100 nM and cells were incubated for 18 h. After fixation and permeabilization, cells were incubated with anti-BrdU antibody for 18 h at 4°C. After washing, cells were stained with FITC-conjugated anti-mouse IgG (1:50) for 30 min at room temperature. For staining of fixed cells, all antibodies and avidin reagents were diluted in 0.3% Triton X-100/3% normal goat serum in PBS. For immunostaining of EGF-dependent striatal precursors, spheres were collected from supernatants of four week cultures and transferred into dishes coated with laminin (7 μg/ml) and poly-L-lysine (1 mg/ml). Four h after plating, cells were stained alive with the R364 antibody as described for E13 cortical precursors. After washing, cells were fixed with 4% paraformaldehyde, treated with 0.3% Triton X-100/3% goal serum in PBS, and then stained with anti-nestin antibodies as described above. O2A cells were stained with R364 antibodies without fixation. After staining with R364 antibodies, O2A cells were fixed with 4% paraformaldehyde, and stained with A2B5 antibodies (1:1000), followed by FITC-labeled anti-mouse IgG+IgM. For immunohistochemical detection of glypican-4, tissues were stained alive to preserve antigenicity of glypican-4. Perpendicular slices of cerebral cortex were dissected from E16 rat forebrain in ice-cold PBS containing 0.1% BSA and 0.45% glucose. The tissue slices were transferred into microcentrifuge tubes and incubated for 1 h with R364 antibodies diluted in 3% goat serum/PBS, followed by incubation with RITC-conjugated anti-rabbit IgG+IgM for 30 min. After washing, the slices were fixed with ice-cold 4% paraformaldehyde in PBS, mounted on a slide, and observed under a Nikon epifluorescence microscope.

Recombinant Glypican-4

Mouse glypican-4 cDNA was tagged with a RGSHHHHHH peptide sequence ($RGSH_6$ tag) at the N-terminus. To prepare this construct, a 1601 bp fragment corresponding to nucleotides 543-2143 of glypican-4 (Watanabe et al., 1995) was amplified by PCR with the following primers: forward, 5′-AGCTAAGCTTAGAGGATCGCATCACCATCACCATCACCCCGGGCTCAAGTCGAAAAGTTGCTC (this primer contains sequences for the $RGSH_6$ tag); reverse, 5′-GCTCTAGATTATCTCCACTCTCCCTGCAC. The authenticity of the amplified fragment was verified by sequencing. The amplified fragment was digested with HindIII and XbaI and ligated to pSecTagB linearized by HindIII/XbaI digestion. Transfection of TSM cells was performed using Lipofectamine according to the manufacturer's instructions. Control transfectants were generated by transfecting insertless pSecTagB. Seven stable transfectant clones were isolated for both K-glypican and the control construct by selection in 500 μg/ml Zeocin. Among these, clones QHK17 (glypican-4 transfectant) and QHC1 (control transfectant) were used in this study. For preparation of membrane fractions from QHK17 and QHC1 cells, cells were collected by scraping, washed twice with PBS, and resuspended in 10 mM Hepes (pH 7.25) containing 0.3 M sucrose, 1 mM PMSF, and 0.3 μM aprotinin. For extraction of E14 mouse brain tissues, forebrains were dissected, rinsed with PBS, and minced lightly by scissors. Cells or minced tissues were homogenized by 16 strokes in a chilled Dounce homogenizer and nuclei were pelleted at 1,000 x *g* for 5 min. Supernatants were then ultracentrifuged in a Beckman TLS55 swinging bucket rotor at 35,000 rpm for 1 h at 4°C. Pellets were resuspended in 50 mM Tris/HCl (pH 8.0) containing 20 mM CHAPS and 0.15 M NaCl. Insoluble materials were removed by centrifugation at 16,000 x *g* in a microcentrifuge. Supernatants were collected as the membrane fraction. $RGSH_6$-tagged recombinant glypican-4 was purified from the QHK17 membrane fraction by using ProBond resin according to manufacturer's instructions. Eluents were dialyzed against 50 mM Hepes (pH 7.0), divided into two aliquots, and one of the aliquots was digested with heparitinase at 37°C for 1 h. A control sample corresponding to the glypican-4-containing fraction was mock-purified from the same amount of the membrane fraction from control transfected cells (QHC1) by the same procedure and used as a negative control in mitogenesis assay.

FGF2 Binding Assay

In this assay, recombinant $RGSH_6$-tagged glypican-4 was captured onto Ni-NTA agarose and used for a binding assay with [^{125}I]FGF2. For capturing $RGSH_6$-tagged glypican-4, Ni-NTA resins (10 μl/tube) were incubated in an Eppendorf tube with membrane fractions (100 μg protein) of either QHK17 or QHC1 cells in 20 mM phosphate buffer (pH 7.8) containing 0.5 M NaCl and 10 mM CHAPS (binding buffer) for 18 h at 4°C. After incubation, resins were washed first with binding buffer and then with 20 mM phosphate buffer (pH 6.0) containing 0.5 M NaCl. Resins were resuspended in 50 μl/tube of 50 mM Hepes (pH 7.0) containing 1 mM calcium acetate, and incubated with or without 5 mU of heparitinase for 2 h at 37°C. After heparitinase digestion, resins were washed once with 20 mM Tris (pH 7.4) containing 0.15 M NaCl (TBS) and blocked by incubation with 100 μl of TBS containing 1% ovalbumin (blocking buffer) for 30 min. After blocking, resins were centrifuged and resuspended in 100

μl/tube of [^{125}I]FGF2 diluted in blocking buffer (0.0002 μCi/μl). After a 1.5 h incubation with [^{125}I]FGF2 at room temperature, resins were washed four times with TBS containing 0.1% Tween 20 and once with TBS. Finally, bound [^{125}I]FGF2 was eluted with 200 μl/tube of 50 mM Tris/HCl (pH 7.0) containing 0.75 M NaCl and counted in a gamma counter.

Co-Immunoprecipitation of Glypican-4 and FGF2

Membrane fractions from QHK17 cells were incubated with [^{125}I]FGF2 in TBS containing 1% BSA and 0.1% Tween 20 (IP buffer). After a 2 h incubation at room temperature, free [^{125}I]FGF2 was removed by dialysis using Spectra/Por 7 membranes (molecular weight cut off: 50,000 dalton). Dialyzed samples were divided into three Eppendorf tubes and incubated with 10 μl per tube of affinity-purified R364 antibody in IP buffer for 18 h at 4°C. Fifteen μl of a 50% gel suspension of protein G agarose were added to each tube and incubated for 1 h at 4°C to precipitate glypican-4. After the incubation, gels were washed five times with TBS containing 0.1% Tween (TBS/Tween). The washed gels were incubated for 30 min at 37°C with 3 mU of heparitinase, 20 mM of chondroitinase ABC, or buffer alone (50 mM Hepes containing 1 mM calcium acetate, pH 7.0). After the enzyme treatment, bound materials were eluted with 50 mM Tris/HCl (pH 7.5) containing 1.5 M NaCl. Eluents were resolved by SDS-PAGE on a 8–16% gradient gel and coprecipitated [^{125}I]FGF2 was detected by fluorography.

Mitogenesis Assay

Rat E13 cortical cultures were prepared by dissociating cells from E13-14 cortex as described above, except that the tissues were pretreated with 6.67 U/ml papain and 300 U/ml DNase for 20 min before trituration. After washing, cells were counted and plated in wells of a 96-well tissue culture plate at 1×10^4 cells/well in F-12:DME (1:1) containing 5 mM Hepes, 0.6% glucose, 2 mM glutamine, and 1× N2 supplement. Cells were incubated in the presence of FGF2 (25 ng/ml) and varying concentrations of intact or heparitinase-treated glypican-4 (0–10 μg/ml). As a negative control, a sample mock-purified from QHC1 cells (see Recombinant Glypican-4) was added to cells in the same volumes as the glypican-4 samples. After a two-day incubation, proliferation of cells were assayed by CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Kit.

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