Glypican-3 (GPC3) is one of the six members of the mammalian glypican family. We have previously reported that GPC3 inhibits Hedgehog (Hh) signaling by competing with Patched (Ptc) for Hh binding. We also showed that GPC3 binds with high affinity to Hh through its core protein, but that it does not interact with Ptc. Several members of the glypican family, including GPC3, are subjected to an endoproteolytic cleavage by the furin-like convertase family of endoproteases. Surprisingly, however, we have found that a mutant GPC3 that cannot be processed by convertases is as potent as wild-type GPC3 in stimulating Wnt activity in hepatocellular carcinoma cell lines and 293T cells and in promoting hepatocellular carcinoma growth. In this study, we show that processing by convertases is essential for GPC3-induced inhibition of Hh signaling. Moreover, we show that a convertase-resistant GPC3 stimulates Hh signaling by increasing the binding of this growth factor to Ptc. Consistent with this, we show that the convertase-resistant mutant binds to both Hh and Ptc through its heparan sulfate (HS) chains. Unexpectedly, we found that the mutant core protein does not bind to Hh. We also report that the convertase-resistant mutant GPC3 carries HS chains with a significantly higher degree of sulfation than those of wild-type GPC3. We propose that the structural changes generated by the lack of cleavage determine a change in the sulfation of the HS chains and that these hypersulfated chains mediate the interaction of the mutant GPC3 with Ptc.

**Background:** Glypican-3 (GPC3) is a proteoglycan that is cleaved by convertases and that inhibits Hedgehog signaling.

**Results:** Convertase-resistant GPC3 stimulates Hedgehog signaling, and unlike wild-type GPC3, binds to Patched through its heparan sulfate chains.

**Conclusion:** Convertase cleavage is required for GPC3-induced inhibition of Hedgehog signaling.

**Significance:** The structure of the GPC3 core-protein determines the binding properties of heparan sulfate chains.

Glypicans are a family of proteoglycans that are bound to the plasma membrane by a glycosylphosphatidylinositol anchor (1, 2). Six members of this family have been identified in mammals (GPC1 to GPC6) (1). In general, glypicans display heparan sulfate (HS)-type glycosaminoglycan (GAG) chains (3). The insertion sites for these GAG chains are located close to the C terminus, placing them close to the cell surface, and suggesting that these chains could mediate the interaction of glypicans with other cell membrane proteins (4). Notably, glypicans do not have domains with obvious homology to characterized domains found in other proteins, suggesting that they have unique functions.

Genetic and biochemical studies have demonstrated that glypicans can regulate several signaling pathways, including those triggered by Hedgehogs (Hhs) (5–7), Wnts (8, 9), bone morphogenetic proteins (10–12), and fibroblast growth factors (13). Glypicans can either stimulate or inhibit the interaction of these growth factors with their signaling receptors. The function of a glypican in a specific cellular context depends on its structural features and on the set of growth factors and growth factor receptors present in that cellular context.

GPC3 is widely expressed during development (4). Loss-of-function mutations of GPC3 cause the Simpson-Golabi-Behmel overgrowth syndrome (14), and Gpc3-null mice display developmental overgrowth (15). GPC3 regulates embryonic growth by inhibiting the Hh signaling pathway (5, 16). This inhibition results from the ability of GPC3 to compete with Patched (Ptc), the Hh signaling receptor, for Hh binding. The ability of GPC3 to act as a competitive inhibitor of Hh signaling is due to the fact that this glypican binds to Hh, but not to Ptc (5). Although it is well known that Hh binds with low affinity to the core protein of GPC3 displays high affinity binding to HS (5). Moreover, the interaction of Hh with GPC3 triggers the endocytosis and degradation of the GPC3-Hh complex, reducing the amount of Hh available to bind to Ptc (5, 17).

Contrary to GPC3, glypican-5 (GPC5) stimulates Hh signaling (18). This stimulatory activity is based on the ability of this glypican to increase the binding of Hh to Ptc (18). Consistent with this, GPC5 interacts with both Hh and Ptc. Notably, both interactions are mediated by the GAG chains. The HS chains of GPC5 display a significantly higher degree of sulfation than those of GPC3 (18). Because the negative charge provided by the sulfate groups is responsible for most of the interactions involving HS chains, this variation in the degree of sulfation of...
GPC3 Requires Convertase Cleavage to Inhibit Hedgehog

the GAG chains may explain the differential interaction of GPC3 and GPC5 with Ptc. However, it should be noted that GPC3 displays two GAG chains, and GPC5 displays four GAG chains. This difference in the number of GAG chains could also have an impact in the binding properties of GPC3 and GPC5.

In contrast to the inhibitory effect of GPC3 on Hh signaling, it is now well established that this glypican can stimulate both canonical and non-canonical Wnt activity (19, 20). GPC3 is expressed by most hepatocellular carcinomas (HCC) (21), and it promotes HCC growth by stimulating canonical Wnt signaling (8). Studies in the Gpc3-null mice have also demonstrated that this glypican regulates Wnt signaling in normal embryonic tissues (19).

Several members of the glypican family, including GPC3, are subjected to an endoproteolytic cleavage by the furin-like convertase family of endoproteases (22). The internal cleavage site generates an ~40-kDa N-terminal subunit, as well as an ~30-kDa C-terminal subunit that carries the GAG chains. These subunits remain linked to each other by one or more disulfide bonds. Surprisingly, however, a mutant GPC3 that cannot be processed by convertases is as potent as wild-type GPC3 in stimulating Wnt activity in HCC cell lines and 293T cells and in promoting HCC growth (23).

To better understand the structural requirements for the regulatory function of GPC3 in Hh signaling, we have investigated whether GPC3 needs to be processed by convertases to inhibit the Hh signaling pathway. We provide here experimental evidence showing that the cleavage by convertases is essential for GPC3-induced inhibition of Hh signaling. Moreover, we show that a convertase-resistant GPC3 mutant stimulates Hh signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmids—**293T and NIH 3T3 cells (obtained from the ATCC) were cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere with 5% CO2. 293T cells were transfected with Lipofectamine 2000, and NIH 3T3 cells were transfected with Lipofectamine LTX-Plus (Invitrogen). All conditioned media were prepared in 293T cells transfected with the indicated expression vectors and collected 48 h after transfection in serum-free conditions, with the exception of ShhN conditioned medium, which was collected 6 days after transfection in the presence of 2% FBS. Expression vectors for GPC3 and GPC3ΔGAG in E. coli, GPC3-AP and GPC3ΔGAG-AP in APTag-2, ShhN in pcDNA, and Sonic Hh-alkaline phosphatase (Shh-AP) in APTag-4, and HA-tagged Ptc in 293T and NIH 3T3 cells were transfected with Lipofectamine 2000, and NIH 3T3 cells were transfected with Lipofectamine LTX-Plus (Invitrogen). All conditioned media were prepared in 293T cells transfected with the indicated expression vectors and collected 48 h after transfection in serum-free conditions, with the exception of ShhN conditioned medium, which was collected 6 days after transfection in the presence of 2% FBS. Expression vectors for GPC3 and GPC3ΔGAG in E. coli, GPC3-AP and GPC3ΔGAG-AP in APTag-2, ShhN in pcDNA, and Sonic Hh-alkaline phosphatase (Shh-AP) in APTag-4, and HA-tagged Ptc in murine stem cell virus (5); GPC3 RR-AA in EF (23); and GPC5 in pcMV (18) were previously described. To generate the GPC3 RR-AAΔGAG in EF, the GAG attachment sites (Ser508 and Ser508) were mutated to alanine by site-directed mutagenesis, and the mutations were verified by sequencing. This non-glycanated GPC3 RR-AA was still processed by convertases, and additional mutations were required to generate a non-glycanated convertase-resistant GPC3. These mutations involved changing three additional arginine residues (Arg387, Arg388, and Arg389) and four lysine residues (Lys371, Lys374, Lys394, and Lys396) to alanine, generating GPC3 5R4K-9AΔGAG, named GPC39AΔGAG. These additional seven mutations were also introduced in the GPC3 RR-AA expression vector to generate GPC3 5R4K-9A, named GPC3 9A. The GPC3 RR-AA-AP, GPC3 9AΔGAG-AP, and GPC3 9A-AP vectors were prepared by inserting these cDNAs into the BspE1 site of the pAP-tag2 vector (Gene Hunter Corp.).

**Hh Reporter Assay—**NIH 3T3 cells were seeded in 6-well plates (250,000 cells/well) and cotransfected with a luciferase reporter driven by an Hh-responsive promoter (0.4 μg), the indicated amounts of GPC3 variants or GPC5 expression vectors, and β-galactosidase (50 ng). One day after transfection, the cells were transferred to a 24-well plate at 50% confluence, and on the following day, ShhN or control conditioned medium (diluted 1/10 in DMEM 2% FBS) was added for 48 h. A luciferase assay was then performed, and the luciferase activity was normalized based on the β-galactosidase activity. Glypican expression levels in the whole cell lysates were verified by Western blot analysis.

**Cell Binding Assay—**293T cells transfected with the indicated expression vectors were transferred to 8 °C and incubated for 2 h with Shh-AP or AP conditioned media containing the same amount of AP activity. Unbound ligand was removed with four washes of PBS, and the cells were lysed in 10 mM Tris–HCl, pH 8, 1% Nonidet P-40. Lysate aliquots with equal amounts of protein were heated at 65 °C for 10 min to inactive the cellular phosphatases, and the AP activity was then measured with a SIGMAFAST p-nitrophenyl phosphate tablet set. Glypican expression levels in the whole cell lysates were assessed by Western blot analysis using the anti-GPC3 mAb 1G12. When indicated, endogenous Ptc was immunoprecipitated using the rabbit anti-Ptc polyclonal antibody H-267 (Santa Cruz Biotechnology) before measuring the AP activity in the precipitated material.

**Co-immunoprecipitation—**293T cells were transfected with GPC3 RR-AA and Shh expression vectors or vector controls (EF and pcDNA, respectively). Cells were lysed in radioimmunoprecipitation assay buffer, the lysates were precleared with protein G-Sepharose during 1 h at 4 °C, and GPC3 was immunoprecipitated with the 1G12 mAb. The presence of Shh in the precipitated material was assessed by Western blot with the rabbit anti-Shh polyclonal antibody H-267 (Santa Cruz Biotechnology).

**Pulldown Assay—**293T cells were transfected with HA-tagged Ptc or vector control and lysed in radioimmunoprecipitation assay buffer, and the lysates were subsequently incubated with the 12CA5 anti-HA mAb (Roche Applied Science) and protein G-Sepharose (Sigma Aldrich). Beads were then blocked with 5% BSA in PBS containing 0.1% Triton X-100 for 90 min at room temperature, and aliquots containing equal amounts of beads were incubated for 1 h with the indicated AP-glypican or AP conditioned media. After four washes with 20 mM Hepes, pH 7.4, 150 mM NaCl, 0.25% Tween 20, the AP activity bound to the beads was determined as described above.

**Analysis of GAG Chains—**NIH 3T3 cells were transiently transfected with GPC3-AP and GPC3 RR-AA. Two days after transfection, the AP activity of each conditioned medium was measured. This activity, which reflects the expression levels of the transfected glypicans, was similar for GPC3 (1.326 A405/μl of medium) and GPC3 RR-AA (1.182 A405/μl of medium). AP-
tagged glypicans were purified from conditioned media by an anion-exchange chromatography on DEAE-Sepharose followed by affinity chromatography with the anti-AP mAb coupled to agarose (Sigma-Aldrich). Briefly, DEAE-Sepharose gel was added to the conditioned media, and after an overnight incubation at 4 °C, the suspension was collected in an empty column and washed with PBS, and the bound material was eluted with 2 M NaCl in PBS. The eluate was then diluted 4-fold with water and loaded onto the anti-AP-agarose column. After washing the column with 0.5 M NaCl in PBS, the AP-tagged glypicans were eluted with 100 mM triethylamine, pH 11.5, and immediately neutralized with 1 M NaH₂PO₄. Finally, the glypi- 
can preparations were desalted by using UltraceL® 10K (Milli-
pore). Purified glypicans were then digested with a mixture of heparinase (HSase) I and III. The digest was labeled with 2-aminobenzadine (2AB) and subjected to anion-exchange HPLC on an amine-bound silica PA-03 column (Pack PA; YMC Co., Ltd.).

Localization of GPC3—NIH 3T3 cells were transfected with the indicated HA-tagged glypicans, and on the following day, they were plated on poly-l-lysine-treated coverslips. Cells were then starved in serum-free medium during 5 h to favor cilia formation and fixed with 4% paraformaldehyde. For immuno-
staining, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked for 30 min in 5% nonfat dry milk in PBS. Primary cilia were visualized with the anti-acetylated tubulin mAb (T7451, Sigma-Aldrich), and glypicans were detected with the anti-HA rat mAb 3F10 (Roche Applied Sci-
ence) and the corresponding fluorescein-conjugated secondary antibodies. Confocal images were generated using a scanning laser microscope LSM510 version 3.2 SP2 (Carl Zeiss) and a Zeiss LSM image browser. Image analysis was performed using the program ImageJ, as reported previously (17). Briefly, a mask, which was constructed by manually outlining cilia in the image of acetylated tubulin staining, was applied to GPC3/ 
GPC3 RR-AA-stained images to measure the fluorescence intensity at cilia. The average fluorescence intensity of other regions of the cell was obtained by measuring several representa-
tive regions on the cell by moving the mask. After subtracting background from both fluorescence intensities obtained above, the ratio of fluorescence intensity in the cilium to that outside of the cilium was calculated.

RESULTS

GPC3 Cleavage by Convertases Is Required for GPC3-induced Inhibition of Hh Signaling—We have previously generated a mutant GPC3 (GPC3 RR-AA) that cannot be processed by con-
vertases by replacing Arg₃⁵⁵ and Arg₃⁵⁸ in the intraproteolytic processing site RQYR with alanine residues (23). This mutant was as potent as wild-type GPC3 in stimulating Wnt signaling in HCC cell lines and 293T cells (23).

To determine the role of convertase cleavage in the inhibi-
tory activity of GPC3 on Hh signaling, we investigated the effect of the GPC3 RR-AA mutant in an Hh reporter assay in NIH 3T3 cells. We have previously used these cells to demonstrate that GPC3 inhibits Hh signaling (5).

We first confirmed that, as in 293T and other cell lines (23), GPC3 is cleaved by convertases in NIH 3T3 cells, and this cleav- 
age is blocked in the GPC3 RR-AA mutant (Fig. 1A). Next, NIH 3T3 cells were transfected with an expression vector where the luciferase gene expression is driven by an Hh-responsive pro-
moter. As described previously (5), the transient expression of wild-type GPC3 in these cells inhibits the luciferase activity induced by Shh-containing conditioned medium in a dose-de-
pendent manner (Fig. 1B). However, this inhibitory activity was not observed with the convertase-resistant GPC3 mutant. In fact, a modest but significant stimulatory effect was found (Fig. 1C). It should be noted that the stimulatory effect is more evi-
dent at lower GPC3 RR-AA expression levels, and it tends to disappear at higher expression levels. However, the convertase-
resistant mutant was never able to inhibit Hh signaling, even at very high levels of expression where wild-type GPC3 displays a strong Hh-suppressive activity (see Fig. 5). As an additional control, we also performed a Hh luciferase reporter assay in the presence of increasing amounts of GPC5 (Fig. 1D). As reported previously (18), GPC5 stimulated Hh signaling in a dose-depen-
dent manner. This stimulation was observed even at high expression levels.

GPC3 RR-AA Increases the Binding of Hh to Ptc—We have previously shown that wild-type GPC3 inhibits the binding of 
Hh to Ptc (5). Based on the results of the Hh reporter assay described above, we hypothesized that the convertase-resistant mutant would display an opposite effect on the Hh/Ptc interac-
tion. To investigate this hypothesis, NIH 3T3 cells were tran-
siently transfected with GPC3 RR-AA and were then incubated with a Shh-AP fusion protein at 4 °C. After removing the unbound material, cells were lysed, Ptc was immunoprecipi-
tated, and the amount of Shh-AP that coimmunoprecipitated with Ptc was quantified by measuring the AP activity in the precipitated material. We found that at low levels of expression, 
GPC3 RR-AA significantly stimulates in a dose-dependent manner the binding of Hh to Ptc (Fig. 2). As control, we showed that in the same assay, expression of wild-type GPC3 inhibits the binding of Shh-AP to Ptc (Fig. 2). Consistent with the lucif-
erase assay results, the stimulatory effect of GPC3 RR-AA on the binding of Shh-AP to Ptc was not observed at higher levels of expression.

GPC3 RR-AA Interacts with Both Hh and Ptc—Next, we 
investigated the mechanism by which GPC3 RR-AA stimulates the binding of Hh to Ptc. We previously reported that GPC3 binds with high affinity to Hh but that it does not interact with Ptc (5). Based on this, we have proposed that GPC3 inhibits Hh-Ptc binding by acting as a competitive inhibitor. GPC5, on the other hand, stimulates Hh signaling and interacts with both Hh and Ptc (18). We hypothesized therefore that GPC3 RR-AA should also interact with Hh and Ptc. As a first approach to study the interaction of GPC3 RR-AA with Hh, we performed a coimmunoprecipitation assay in 293T cells. These cells were transiently transfected with expression vectors for GPC3 
RR-AA and Shh and lysed, and GPC3 RR-AA was immunoprecipitated. The presence of Shh in the precipitated material was then assessed by Western blot. Fig. 3A shows that Shh coimmu-
noprecipitated with GPC3 RR-AA. We also analyzed the GPC3 RR-AA-Shh interaction in intact cells by performing a cell binding assay. GPC3 RR-AA- or vector control-transfected 
293T cells were incubated with conditioned medium contain-
ing Shh-AP, or AP alone as control, for 2 h at 8 °C. The unbound material was then washed with PBS, cells were lysed, and the cell-bound AP activity was determined. Fig. 3B shows that Shh-AP binds significantly more to the GPC3 RR-AA-transfected cells than to control cells.

To investigate the interaction between GPC3 RR-AA and Ptc, we performed a pulldown assay. To this end, protein-G beads covered with Ptc or control beads were incubated with conditioned media containing equal activities of AP, GPC3 RR-AA-AP, or GPC3-AP fusion proteins. After washing, the amount of AP that remained bound to the beads was measured. As shown in Fig. 3C, there was a significant specific binding of GPC3 RR-AA-AP to the Ptc-containing beads. Notably, a much lower binding of GPC3-AP to Ptc-containing beads was also detected (Fig. 3C). A pulldown assay was then repeated with various dilutions of the corresponding AP conditioned media, revealing that GPC3 RR-AA-AP displays a higher binding capacity to Ptc than GPC3-AP in all the dilutions tested (Fig. 3D). It should be noted that we have previously shown relatively low levels of interaction between GPC3 and Ptc in binding assays (18). Because GPC3 is normally outside of the cilium, where Ptc is activated, we have considered that the GPC3/Ptc interaction detected in pulldown assays was not physiologically relevant (18).

**GPC3 RR-AA-Shh and -Ptc Interactions Are Mediated by the GAG Chains**—We have previously reported that the wild-type GPC3 core protein binds with high affinity to Shh (5). To investigate whether the core protein of the GPC3 RR-AA mutant also interacts with high affinity with Shh, we generated a convertase resistant mutant GPC3 that cannot be glycanated.
To confirm that the lack of interaction of the GPC3 9AΔGAG mutant with Hh and Ptc is due to the absence of the GAG chains, and not the consequence of the additional mutations, a glycanated GPC3 mutant containing the same mutations as GPC3 9AΔGAG (GPC3 9A) was generated. This mutant was as potent as GPC3 RR-AA in stimulating Hh activity in NIH 3T3 cells, and it had a similar capacity to interact with Hh and Ptc (Fig. 4, E–G). Altogether, from these experiments, we conclude that GPC3 RR-AA interacts with Hh and Ptc and that the GAG chains are essential for these interactions.

**GPC3 9R-AΔGAG Does Not Stimulate Hh-induced Luciferase Activity**—If the binding of the convertase-resistant mutant GPC3 RR-AA to Hh and Ptc is required for the GPC3 RR-AA-induced stimulation of Hh signaling, it would be expected that the GPC3 9AΔGAG cannot stimulate Hh signaling. To test this, we performed an Hh luciferase reporter assay in NIH 3T3 cells. As shown previously (5), the transient expression of wild-type GPC3 or the non-glycanated GPC3 (GPC3ΔGAG) inhibits the Shh-induced luciferase activity in a dose-dependent manner, whereas GPC3 RR-AA displays a stimulatory effect at low levels of expression (Fig. 5). As expected, we found that GPC3 9AΔGAG does not stimulate Hh signaling (Fig. 5).

**The GAG Chains of GPC3 RR-AA Display a Higher Degree of Sulfation than Those of Wild-type GPC3**—It is well established that the structure of a protein core of a glypican can have an impact in the type, size, and modifications of their GAG chains (24, 25). Thus, the conformational changes generated by the lack of cleavage into two glypican subunits could alter the access of one or more HS-synthesizing enzymes to the site of GAG synthesis. It is also known that the binding specificity of HS chains is predominantly determined by the degree and type of sulfation. In fact, we have previously reported that the HS chains of GPC5, which mediate the high affinity interaction of this glypican with Ptc, display a significantly higher degree of sulfation than those of GPC3, which do not interact with Ptc (18). Because the interaction between the convertase-resistant GPC3 and Ptc is mediated by the GAG chains, we hypothesized that the differential interaction of GPC3 RR-AA and GPC3 with Ptc is a consequence of a different sulfation profile in their GAG chains. To investigate this possibility, we decided to compare the sulfation profile of the GPC3 and GPC3 RR-AA GAG chains. To this end, GPC3 RR-AA-AP and GPC3-AP fusion proteins were purified from the conditioned medium of transiently transfected NIH 3T3 cells and digested with a mixture of heparinase I and III. The disaccharides generated by the digestion were then labeled with 2AB, separated by anion-exchange HPLC, and identified by comparing the position of the eluted peaks with 2AB-labeled standard HS disaccharides. We found that that the proportion of non-sulfated disaccharides (0S) in GPC3-AP is significantly higher than in GPC3 RR-AA-AP (Fig. 6). Consistent with this finding, the HS chains purified from GPC3 RR-AA-AP display a higher proportion of sulfated disaccharides (Fig. 6). This result suggests that the higher sulfation of the HS chains of GPC3 RR-AA is responsible for the larger binding capacity of GPC3 RR-AA to Ptc as compared with that of GPC3.

**GPC3 RR-AA-AP Localizes to the Primary Cilium**—It is now well established that in mammalian cells, Hh signaling is trig-
tered at the primary cilium where Hh-Ptc interaction takes place (26). We have previously reported that GPC5, a glypican that stimulates Hh signaling and interacts with Ptc, can be found at the ciliary membrane (18). Conversely, we could not detect GPC3 at this location. We therefore decided to investigate whether the convertase-resistant GPC3 RR-AA also localizes to the primary cilium. To this end, NIH 3T3 cells were transfected with GPC3 RR-AA, fixed, and immunostained for GPC3 and acetylated tubulin as a marker of the primary cilium. We found that in cells expressing GPC3 RR-AA levels that stimulate Hh signaling, this mutated glypican can be clearly detected at the primary cilium (Fig. 7A). As reported previously (18), we could not detect wild-type GPC3 at this location (Fig. 7B).

**DISCUSSION**

In this study, we show not only that the convertase processing of GPC3 is required for GPC3-induced inhibition of Hh signaling, but also that a convertase-resistant GPC3 has an unexpected stimulatory effect on Hh signaling. Notably, unlike wild-type GPC3, the convertase-resistant mutant is able to interact with Ptc. Another important change that we found in this mutant is that its interaction with Shh is mediated by the GAG chains, and not by the core protein as we previously reported for wild-type GPC3 (5). It should be noted that only two amino acids were mutated in the primary sequence of GPC3 to abolish the processing by convertases. Thus, to explain the drastic changes in the ability of the convertase-resistant mutant to interact with Shh and Ptc, we propose that the lack of cleavage into two subunits induces a structural change in GPC3. As shown in Fig. 8, this structural change in the GPC3 mutant core protein could alter or hide the Hh binding site. In addition, this structural change could also induce a significant change in the sulfation pattern of the GAG chains (see below).

Considering the ability of the convertase-resistant GPC3 mutant to interact with both Ptc and Shh, along with the observation that more Hh is bound to Ptc in the presence of this GPC3 mutant, our results strongly suggest that GPC3 RR-AA stimulates the Hh signaling pathway by facilitating or stabilizing the interaction between Hh and Ptc. Interestingly, a similar

**FIGURE 3.** GPC3 RR-AA interacts with both Hh and Ptc. A, GPC3 RR-AA-Shh coimmunoprecipitation. 293T were transfected with the indicated expression vectors, and GPC3 was immunoprecipitated (IP). Top panel: the presence of Shh in the precipitated material was probed with an anti-Shh antibody. Middle and bottom panels: the amount of ectopic Shh or mutant GPC3 in whole cell lysates was assessed by Western blot (WB). Arrowhead: GPC3 core protein; bracket: glycanated GPC3. B, cell binding assay. 293T cells transfected with the indicated expression vectors were incubated with Shh-AP or AP alone for 2 h at 8 °C. Cells were then washed and lysed, and the AP activity of aliquots of cell lysates containing equal amounts of protein was determined. Bars represent the mean ± S.D. of triplicates. The background binding of AP alone to the cells was subtracted from each measurement. OD, optical density. C and D, pulldown assay. Ptc-covered protein-G beads or control beads were incubated with equal activities of GPC3-AP, GPC3 RR-AA-AP, or AP alone (C) or the indicated dilutions (D). After washing, the AP activity retained by the beads was measured. Bars represent the specific AP activity (mean ± S.D. of triplicates) bound to the Ptc beads after subtracting the corresponding AP values obtained for the control beads. All the experiments were repeated at least three times. One representative experiment is shown. Statistical analysis comparing each data set with the correspondent control or between the indicated data sets was performed with the unpaired t test. Significance is indicated as: * = p < 0.05; ** = p < 0.01; and *** = p < 0.001. CM, conditioned medium.
GPC3 Requires Convertase Cleavage to Inhibit Hedgehog

FIGURE 4. The interaction between GPC3 RR-AA-Shh and Ptc is mediated by the GAG chains. A and C, cell binding assay. A, 293T cells were transiently transfected with expression vectors for wild-type GPC3 (GPC3), non-glycanated GPC3 (GPC3ΔGAG), or vector control (EF). Cells were then incubated with Shh or control conditioned medium. A luciferase assay was then performed. Bars represent -fold stimulation induced by Hh (mean or between the indicated data sets was performed with the unpaired t test). Significance is indicated as: *p < 0.05; **p < 0.01; and ***p < 0.001. B, Western blot analysis of the GPC3 variants expression levels in the cell lysates. Arrowhead: GPC3 core protein; bracket: glycanated GPC3. One representative experiment of four is shown.

mechanism has been proposed to explain GPC3-induced stimulation of Wnt signaling (27). We have recently shown that GPC3 plays a direct role in the activation of Wnt signaling by binding to both Wnt and its receptor Frizzled (Fz) and by stimulating the formation of signaling complexes (27). Because the convertase-resistant GPC3 is as potent as wild-type GPC3 in the stimulation of Wnt signaling (23), our data suggest that the structural changes generated by the lack of convertase cleavage do not alter GPC3-Wnt and GPC3-Fz binding interactions. Thus, the processing of GPC3 by convertases allows this glypic to have the opposite effect in two key signaling pathways.

As mentioned above, like the convertase-resistant GPC3, GPC5 also binds to Hh and Ptc through the GAG chains and stimulates Hh signaling. Interestingly, GPC5 is not processed by convertases. However, the lack of convertase processing cannot be considered a requirement for the Hh stimulatory activity of glypicans because the Drosophila glypican Dally-like (Dlp), which also stimulates Hh signaling, is cleaved by convertases (6). Interestingly, contrary to what we have found with GPC5 and GPC3 RR-AA, the GAG chains are not required for the stimulatory activity of Dlp on Hh signaling because the Dlp core protein interacts with Hh (28). Thus, Hh interacts with the core proteins of two glypicans that are cleaved by convertases, GPC3 and Dlp. Whether the processing by convertases is also required for the exposure of the Hh-interacting domain in Dlp remains to be studied.

Taken together, our results indicate that in the context of Hh signaling, GPC3 RR-AA behaves like GPC5. It should be noted, however, that although GPC5 stimulates Hh-induced luciferase activity in a dose-dependent manner reaching high levels of stimulation at higher levels of expression, GPC3 RR-AA dis-
plays a reduced stimulatory activity at higher levels of expression. The reasons for this differential behavior are currently unknown.

Another interesting finding of this study is that the convertase-resistant GPC3 acquires the ability to interact with Ptc. This interaction is mediated by the HS chains, suggesting that the chains carried by GPC3 RR-AA are different from the ones carried by the wild-type GPC3. It is well established that the binding specificity of HS chains predominantly depends on the degree and type of sulfation. Based on this, we compared the sulfation profile of the GPC3 and GPC3 RR-AA HS chains. We found that indeed the HS chains of GPC3 RR-AA display a significantly higher degree of sulfation than those of GPC3, suggesting therefore that this higher sulfation level allows GPC3 to interact with Ptc. This is consistent with our previous observation that the HS chains of GPC5 also display more sulfation than those of GPC3 (18).

It is well known that the level of sulfation in the HS chains of a given cell type is determined by the levels of sulfotransferases and sulfatases (24). In this study, we have compared the HS chains of GPC3 and GPC3 RR-AA purified from the same cell line. Thus, it could be proposed that the differences that we found in the sulfation of the HS chains are the consequence of the structural changes generated by the cleavage of the GPC3 core protein by convertases. This modification of the structure of the core protein could alter the accessibility of one or more of these HS-modifying enzymes to the site of HS chain synthesis in the GPC3 RR-AA mutant. Alternatively, the conformational change generated by the lack of cleavage of GPC3 could alter its ability to interact with other proteins, and could consequently change the Golgi compartment in which GPC3 becomes glycinated. It has been proposed that different Golgi compartments could harbor different relative amounts of the various enzymes involved in the synthesis of GAG chains (29).

Another important observation of this study is that GPC3 RR-AA is detected in the primary cilium, where Hh-Ptc interaction occurs. This finding is consistent with our results that GPC3 RR-AA interacts with Ptc and Hh and increases the binding of Hh to Ptc. As reported previously (17), we did not detect wild-type GPC3 at this location. It remains to be elucidated whether the ability to bind to Ptc is the determinant factor for the localization of GPC3 in the cilium.

Note Added in Proof—Hiroshi Kitagawa’s contributions to this article fulfill the JBC authorship criteria, but his authorship was inadvertently omitted from the version of the article that was published on February 4, 2015 as a Paper in Press.

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GPC3 Requires Convertase Cleavage to Inhibit Hedgehog