LRP1 mediates Hedgehog-induced endocytosis of the GPC3–Hedgehog complex

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Summary

Glypican-3 (GPC3) is a heparan sulfate (HS) proteoglycan that is bound to the cell membrane through a glycosylphosphatidylinositol link. This glypican regulates embryonic growth by inhibiting the hedgehog (Hh) signaling pathway. GPC3 binds Hh and competes with Patched (Ptc), the Hh receptor, for Hh binding. The interaction of Hh with GPC3 triggers the endocytosis and degradation of the GPC3–Hh complex with the consequent reduction of Hh available for binding to Ptc. Currently, the molecular mechanisms by which the GPC3–Hh complex is internalized remains unknown. Here we show that the low-density-lipoprotein receptor-related protein-1 (LRP1) mediates the Hh-induced endocytosis of the GPC3–Hh complex, and that this endocytosis is necessary for the Hh-inhibitory activity of GPC3. Furthermore, we demonstrate that GPC3 binds through its HS chains to LRP1, and that this interaction causes the removal of GPC3 from the lipid rafts domains.

Keywords

Glypican-3; Hedgehog; LRP1; Endocytosis

Introduction

Glypican-3 (GPC3) is a member of the glypican family. Glypicans are cell surface proteoglycans that are linked to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Filmus et al., 2008). GPC3 is highly expressed in many tissues during development and plays an important role in the regulation of embryonic growth (Filmus and Capurro, 2008). Loss-of-function mutations of GPC3 cause the Simpson–Golabi–Behmel overgrowth syndrome (SGBS) (Pilia et al., 1996), and *Gpc3*-deficient mice display developmental overgrowth (Cano-Gauci et al., 1999; Chiao et al., 2002). In addition, it has been reported that *gpc3* polymorphisms have a significant impact in the body size of mice (Oliver et al., 2005).

In a recent study from our laboratory we demonstrated that GPC3 regulates embryonic growth by inhibiting the Hedgehog (Hh) signaling pathway (Capurro et al., 2008). We showed that GPC3 binds Hh at the cell membrane and competes with Patched (Ptc), the Hh

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receptor, for Hh binding. Furthermore, we found that the binding of Hh to GPC3 triggers the endocytosis and degradation of the GPC3–Hh complex, reducing in this way the amount of Hh available for binding to Ptc (Capurro et al., 2008). Consistent with this finding, we showed that *Gpc3*-null mice display increased Sonic Hh (Shh) and Indian Hh protein levels, but similar amounts of the corresponding transcripts, compared with the normal littermates (Capurro et al., 2008; Capurro et al., 2009). In addition, studies performed in GPC3-expressing mouse embryonic fibroblasts showed that soon after internalization the GPC3–Hh complex can be localized in early endosomes and, later on, in the late endosome/lysosome compartment. Additional support for our model was provided by the finding that incubation of mouse embryonic fibroblasts with Shh-containing medium significantly reduces the cellular levels of GPC3 (Capurro et al., 2008). Our studies have provided therefore strong genetic and biochemical evidence indicating that GPC3 negatively regulates Hh signaling by inducing its endocytosis and degradation. However, the mechanism by which the GPC3–Hh complex is internalized remains unknown.

GPI-anchored proteins can be internalized by at least three different mechanisms, and it is currently thought that the localization of a given GPI-anchored protein in a specific cell membrane domain plays a critical role in determining the endocytic mechanism (Mayor and Riezman, 2004). Most GPI-linked proteins are localized in cholesterol-sphingolipid-rich membrane domains or lipid rafts, and much of the experimental evidence published to date indicates that lipid raft-residing GPI-anchored proteins are internalized via clathrin-independent processes. When clustered by extracellular agents, GPI-linked proteins are endocytosed by caveolar-coated vesicles. Alternatively, in the absence of clustering agents, GPI-anchored proteins are internalized through the clathrin-independent carriers (CLIC) of the GPI-enriched early endosomal compartment (GEEC) pathway (Bhagatji et al., 2009). Whether internalization of lipid-bound membrane proteins via the GEEC–CLIC pathway requires specific sequence/structural/topological information, or whether it rests purely on the absence of signals for endocytosis by other mechanisms is currently unknown (Bhagatji et al., 2009). Significantly, some GPI-anchored proteins have been shown to endocytose through a clathrin-dependent mechanism upon binding of ligand. The best-studied examples are the urokinase receptor (uPAR) and the prion protein (PrP) (Lakhan et al., 2009). Unoccupied uPAR is randomly distributed along the plasma membrane. However, the binding of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) to uPAR triggers the rapid association of the ternary complex with the low-density lipoprotein receptor-related protein 1 (LRP1), and its sequestration in clathrin-coated pits (Czekay et al., 2001). The PrP, on the other hand, is localized in lipid rafts, but the binding of cupric ions (Cu^{2+}) causes rapid dissociation from the lipid rafts, and clathrin-mediated, LRP1-dependent internalization (Parkyn et al., 2008; Taylor and Hooper, 2007). Thus, the GPI-anchored proteins uPAR and PrP are endocytosed by piggybacking on LRP1, a well-characterized transmembrane endocytic receptor that interacts with clathrin.

The LDL (low-density lipoprotein) receptor family is a group of cell surface type 1 transmembrane proteins that harbor the NPxY cytoplasmic motif required for clustering into clathrin-coated pits. LRP1, along with LRP2 (megalin), the two largest members of the family, interact and mediate the endocytosis of more than 40 ligands. Their interaction with these ligands is mediated by a large number of ligand-binding repeats that are displayed in

their extracellular domains (May et al., 2007). LRP1 is ubiquitously expressed, but its levels are particularly high in smooth muscle cells, hepatocytes and neurons. Deletion of the *Lrp1* gene leads to lethality in mice, revealing a critical, but still undefined, role in development (Lillis et al., 2008). LRP2/megalin is expressed by the yolk sac and anterior neuroepithelium in the embryo, and in the proximal renal tube and intestinal epithelium in the adult (May et al., 2007). Interestingly, LRP2/megalin has already been shown to be involved in the endocytosis of Shh in the neural tube (McCarthy et al., 2002).

In this study we identified the mechanism of endocytosis of the Hh–GPC3 complex. We provide experimental evidence demonstrating that GPC3 interacts with LRP1, and that this endocytic receptor mediates the Hh-induced internalization of the Hh–GPC3 complex.

Results

GPC3 is mainly localized outside lipid rafts

It is well established that the specific mechanism by which cell membrane proteins are internalized depends on their structural features and on their localization on particular cell membrane domains (Wieffer et al., 2009). Thus, as a first approach to identify the mechanism of endocytosis of the GPC3–Hh complex, we investigated whether GPC3 resides in lipid rafts. To this end, we used GPC3-transfected NIH 3T3 mouse embryonic fibroblasts, which are the cells that we previously employed to describe the Hh-induced endocytosis of the GPC3–Hh complex. Cells were lysed with cold 1% Triton-X100, and cell lysates were subjected to a discontinuous sucrose density gradient centrifugation. The gradient was separated in 12 equal-volume fractions, and the presence of GPC3 in each fraction was assessed by western blot analysis. As shown in Fig. 1, GPC3 was predominantly detected in the Triton-X100 soluble fractions at the bottom of the gradient. Only small amounts of GPC3 were present in the low-density fractions, where caveolin-1, a lipid raft marker, was also found. Incubation of the cells with Shh-containing conditioned medium did not alter GPC3 distribution (data not shown). Considering that in NIH 3T3 cells GPC3 massively endocytoses upon binding of Hh (Capurro et al., 2008) (Fig. 2), the fact that only a small proportion of GPC3 resides in the lipid raft domain does not support, in principle, a caveolar or GEEC/CLIC-mediated endocytic process.

GPC3–Shh endocytosis is mediated by clathrin

Next, we investigated whether upon binding to Shh, the GPC3–Shh complex endocytoses via a clathrin-mediated mechanism. To this end, we studied the effect of several treatments that selectively block clathrin-mediated endocytosis on the internalization of this complex. GPC3-transfected NIH 3T3 cells were incubated with Shh-conditioned medium at 8°C. As previously reported (Capurro et al., 2008), Shh strongly binds to the cell membrane of GPC3-expressing cells (Fig. 2B). Transferring these cells to 37°C allows endocytosis to proceed, and consequently the GPC3–Shh complexes disappeared from the cell membrane and were detected in the cytoplasmic/perinuclear region (Fig. 2C). This intracellular dotted pattern of staining was not observed when the GPC3-expressing cells were incubated in similar conditions with control-conditioned medium, indicating that endocytosis is triggered by the binding of GPC3 to Shh (Fig. 2C, inset). We then repeated the endocytosis

experiments after pre-treating the cells with hypertonic sucrose (Fig. 2D), monodansylcadaverine (MDC) (Fig. 2E) and with K⁺ depletion (Fig. 2F) (Peng et al., 2010). The three treatments significantly inhibited GPC3–Shh endocytosis. From these results we conclude that the endocytosis of GPC3–Shh complexes is clathrin-mediated.

RAP inhibits GPC3–Shh endocytosis

Because GPC3 lacks a cytoplasmic tail that could mediate the interaction with the clathrin endocytic machinery, we hypothesized that this glypican may rely on the association with a transmembrane endocytic receptor. Based on the knowledge that LRP1 mediates the endocytosis of two other GPI-anchored proteins (uPAR and PrP), we investigated whether LRP1 is also involved in the internalization of the GPC3–Hh complex. To this end, we studied the effect of RAP on this endocytic process. RAP is a chaperone that binds tightly to the LRP family members in the early secretory pathway to assist correct folding, and to block their premature association with ligands (Lillis et al., 2008). Exogenous RAP has been extensively used as an inhibitor of ligand–LRP interactions (May et al., 2007). When GPC3–Shh internalization assays were performed in the presence of RAP, GPC3 and Shh remained at the cell surface (Fig. 3A). The fact that RAP treatment did not affect transferrin internalization (Fig. 3B), which is also clathrin-dependent, discards a more general toxic effect of RAP on clathrin-mediated endocytosis. Thus, the complete inhibition of GPC3–Shh endocytosis by RAP provides strong evidence indicating that a member of the LRP family is required for the endocytosis of the GPC3–Shh complex.

The ability of RAP to inhibit GPC3–Shh endocytosis was confirmed with an internalization experiment that allows the independent visualization of cell surface-versus internalized-GPC3. GPC3-transfected NIH 3T3 cells growing in tissue culture were incubated with an anti-GPC3 antibody at 8°C. After washing, Shh-conditioned medium was added at 8°C to allow binding without internalization. Shh was then removed, cells washed and transferred to 37°C to allow endocytosis to proceed. After fixation, the detection of GPC3 on the cell surface was done by incubation with a green Alexa-Fluor-conjugated secondary antibody. To detect internalized GPC3, cells were permeabilized and stained with a red Alexa-Fluor-conjugated secondary antibody. Fig. 3F shows that most GPC3 is internalized (red) after incubation for 40 minutes at 37°C with Shh, whereas the majority of GPC3 remains at the cell surface (green) when the Shh incubation was performed in the presence of RAP (Fig. 3G).

LRP1 mediates GPC3–Shh endocytosis

The fact that RAP inhibits GPC3–Shh endocytosis indicates that an LRP family member is involved in this process. As discussed above, LRP1 and LRP2/megalin have been shown to mediate the endocytosis of a large number of proteins. Because LRP2/megalin is expressed in a limited number of tissues, and LRP1, like GPC3, is widely expressed in the embryo (Herz et al., 1992; Li et al., 2005) including tissues where we have previously reported a role of GPC3 on Shh signaling, like digits and duodenum (www.genepaint.org) (Capurro et al., 2008), we decided to investigate whether LRP1 is involved in GPC3–Shh endocytosis. To this end, we investigated the effect of LRP1 knockdown in the Shh-induced endocytosis of the GPC3–Shh complex by following the protocol described in Fig. 2. We found that the

treatment of NIH 3T3 cells with a pool of commercially available LRP1 siRNAs blocks endocytosis at 37°C (Fig. 3C) whereas the treatment with non-targeting control siRNA has no blocking effect (Fig. 3C, inset). We also investigated the effect of LRP1 knockdown on endocytosis by performing the internalization experiment that allows the independent visualization of cell surface versus internalized GPC3. We found dramatic inhibition of Shh-induced GPC3 internalization in cells transfected with LRP1 siRNA (Fig. 3H) as compared with non-targeting control siRNA transfection (Fig. 3H, inset). From these experiments we conclude that LRP1 is the endocytic receptor that mediates GPC3–Shh endocytosis.

GPC3 interacts with LRP1

The fact that LRP1 is required for the endocytosis of the GPC3–Shh complex strongly suggests that this endocytic receptor interacts with GPC3 at the cell surface. As an initial approach to study the interaction of GPC3 with LRP1 we performed a coimmunoprecipitation assay in 293T cells. These cells were transiently transfected with expression vectors for GPC3 and a HA-tagged LRP1 minireceptor that includes the cytoplasmic and transmembrane domains, and the extracellular domain IV (LRP1-IV). This minireceptor has been shown to display similar activity than full-length LRP1 (Zhang et al., 2008). Transfected cells were lysed, GPC3 immunoprecipitated, and the presence of LRP1-IV in the precipitated material was assessed by western blot analysis. Fig. 4A shows that LRP1-IV coimmunoprecipitated with GPC3, suggesting that both proteins are part of the same complex. Next, we analyzed the GPC3–LRP1 interaction in intact cells by performing a cell-binding assay. LRP1-IV- or vector control-transfected cells were incubated with conditioned medium containing equal activities of a GPC3-alkaline phosphatase (AP) fusion protein or AP as control, for 2 hours at 4°C. Cells were then washed with cold PBS, lysed, and the cell-bound AP activity determined. We found a significant specific binding of GPC3–AP to the cells expressing LRP1-IV (Fig. 4B, left).

As an alternative approach to confirm the interaction between GPC3 and LRP1, we performed a pull-down assay. To this end, Protein-G beads covered with LRP1-IV or control beads were incubated with GPC3–AP- or AP-conditioned media. After washing, the amount of AP that remained bound to the beads was measured. As shown in Fig. 4C, there was a significant binding of GPC3–AP to the LRP1-IV-covered beads, suggesting that both molecules directly interact.

To confirm a direct interaction between GPC3 and LRP1, we performed a cell-binding assay after an acid wash treatment of the LRP1-IV-transfected cells to remove endogenous proteins that could be bound to LRP1 and mediate its interaction with GPC3. As shown in Fig. 4B, the acid-treated cells retain most of the GPC3–AP binding capacity of the untreated cells, confirming that GPC3 directly binds to LRP1.

To further characterize the GPC3–LRP1 interaction, we investigated the role of the heparan sulfate (HS) chains of GPC3. For this purpose, we performed an LRP1-IV pull-down experiment with a fusion protein that includes a mutant GPC3 that cannot be glycanated and AP (GPC3^ΔGAG–AP). No significant binding of GPC3^ΔGAG–AP to the LRP1-IV-covered beads was detected (Fig. 5A), indicating that the HS chains of GPC3 mediate the interaction with LRP1. Additional support for this conclusion was provided by performing a pull-down

assay in the presence of increasing concentrations of heparin. We found that heparin inhibits the binding of GPC3 to LRP1-IV in a dose-dependent manner (Fig. 5B). Finally, we investigated the effect of heparin in the Shh-induced endocytosis of the GPC3–Shh complex by following the protocol described in Fig. 2. We found that heparin completely blocks endocytosis at 37°C (Fig. 5C). We also investigated the effect of heparin on endocytosis by performing an internalization experiment that allows the independent visualization of cell surface versus internalized GPC3. As shown in Fig. 5D, we found that no internalized GPC3 can be detected when Shh-triggered endocytosis is performed in the presence of heparin. Altogether, from these experiments we conclude that GPC3 interacts with LRP1, and that the HS chains are essential for this interaction.

It is well established that GPI-anchored proteins are normally localized in lipid rafts. However, as we showed in Fig. 1, in NIH 3T3 cells GPC3 is found mostly outside of these rafts. Based on the knowledge that LRP1 acts as an endocytic receptor in clathrin-coated pits, we hypothesized that this unexpected localization of GPC3 in NIH 3T3 cells is due to its heparan sulfate-mediated association with LRP1. If this hypothesis were correct, a non-glycanated GPC3 should localize to lipid rafts. To test this hypothesis, GPC3 GAG-transfected NIH 3T3 were lysed and subjected to a sucrose density gradient centrifugation. The gradient was separated in 12 fractions, and the presence of GPC3 GAG in each fraction was assessed by western blot analysis. Consistent with our hypothesis, we observed that whereas GPC3 is predominantly found in the soluble fractions (Fig. 5E, right panel, top), GPC3 GAG is mostly detected in the low-density fractions that correspond to the lipid rafts (left panel). As expected, the endogenous LRP1 was found outside of the lipid rafts (Fig. 5E, right panel, bottom).

LRP1 interacts with Shh

It has been reported that LRP2/megalin and Shh interact with high affinity. Therefore, to better understand the nature of the Hh–GPC3–LRP1 complex, we investigated next whether LRP1 also interacts with Shh. First, coimmunoprecipitation experiments were performed in 293T cells transfected with the LRP1-IV minireceptor and Shh or the corresponding control vectors. The coimmunoprecipitation of Shh with HA-tagged LRP1-IV, but not with control HA-tagged DL4, suggests that both molecules are part of the same protein complex (Fig. 6A). We also investigated the interaction between LRP1 and Shh in the context of intact cells. To this end, 293T cells were transiently transfected with LRP1-IV or a control expression vector. Transfected cells were then incubated with Shh–AP- or AP-conditioned medium. After unbound material was washed, cells were lysed and the AP activity determined. We found significant binding of Shh–AP to LRP1-IV-expressing cells (Fig. 6B). Finally, the LRP1–Shh interaction was confirmed by a pull-down assay (Fig. 6C).

Next we investigated whether the binding of Shh to LRP1 is sufficient to induce Shh internalization in the absence of GPC3. Both experimental approaches previously used to study GPC3–Shh endocytosis (Figs 2, 3) failed to detect Shh–LRP1 internalization (data not shown).

LRP1 is required for GPC3-induced inhibition of Hh signaling

We have previously shown that GPC3 inhibits Hh signaling in mouse embryos and in NIH 3T3 mouse embryonic fibroblasts. Because this inhibition was accompanied by the internalization and degradation of the GPC3–Hh complex, we concluded that endocytosis was required for the GPC3-induced inhibition of Hh signaling. Thus, if our finding that LRP1 mediates the GPC3-induced endocytosis of the GPC3–Hh complex is correct, blocking the LRP1-mediated endocytosis should abrogate the ability of GPC3 to inhibit Hh signaling. To test this hypothesis we investigated first the effect of RAP on the GPC3-induced inhibition of Hh signaling by performing an Hh reporter assay in NIH 3T3 cells transfected with a luciferase gene driven by an Hh-responsive promoter. As previously described, the transient expression of GPC3 in these cells inhibits the luciferase activity induced by conditioned medium containing Shh (Capurro et al., 2008) (Fig. 7A). However, when the reporter assay was done in the presence of RAP, the ability of GPC3 to inhibit Hh signaling was abrogated (Fig. 7A).

As an additional approach to investigate the role of LRP1 on the Hh-inhibitory function of GPC3, we studied the effect of LRP1 knockdown on the Hh reporter assay. To this end, NIH 3T3 cells were incubated with LRP1 siRNA or non-targeting control siRNA for 48 hours prior to adding the Shh-conditioned medium. As shown in Fig. 7B, LRP1 siRNA completely blocked the GPC3-induced inhibition of Shh signaling in the luciferase reporter assay. This result provides additional experimental evidence indicating that LRP1-mediated endocytosis is required for GPC3-induced inhibition of Hh signaling.

LRP1 is required for Shh-induced endocytosis of GPC3 in breast cancer cells

We have previously reported that GPC3 is downregulated in human breast cancers. Moreover, we showed that GPC3 inhibits the *in vitro* growth of several breast cancer cell lines, suggesting that GPC3 has a tumor suppressor activity (Xiang et al., 2001). During those studies we noticed that addition of Shh-conditioned medium to GPC3-transfected SKBR3 breast cancer cells induced a significant reduction in the levels of glycanated GPC3, which unlike the non-glycanated GPC3, is localized at the cell membrane (Fig. 8A). Because GPC3 expression in these cells is driven by a viral promoter, this result suggests that Shh induces the endocytosis and degradation of GPC3. We already reported a similar observation with GPC3-transfected NIH 3T3 cells (Capurro et al., 2008). We decided therefore to investigate whether, like in NIH 3T3 cells, LRP1 is required for the Shh-induced endocytosis of GPC3 in breast cancer cells. To this end, we tried first to perform internalization assays as described in Figs 2, 3. Unfortunately, this approach was not successful. The low levels of GPC3 expression in the SKBR3 cells did not allow a clear visualization of membrane versus intracellular staining patterns. As an alternative approach we decided to investigate the effect of RAP on the Shh-induced degradation of GPC3 by immunoprecipitation and western blot analysis. As shown in Fig. 8B, we found that RAP significantly blocks the Shh-induced degradation of GPC3. This result indicates that the role of LRP1 in Hh-induced endocytosis is not unique to mouse embryo fibroblasts.

Discussion

In this study we show that LRP1 acts as an endocytic receptor for the GPC3–Hh complex. This conclusion is supported by our finding that endocytosis of the complex can be blocked by RAP, and by RNAi-induced downregulation of LRP1.

LRP1 mediates the internalization of numerous ligands through clathrin-coated pits (Nykjaer and Willnow, 2002). Because glypicans are GPI-anchored proteins, it would be expected that they are localized in lipid rafts and are, therefore, internalized through caveolae-mediated or GEEC/CLIL mechanisms. However, we show here that GPC3 is found mostly outside of the lipid rafts. Thus, a clathrin-mediated endocytic mechanism for GPC3 is consistent with its localization.

As discussed in the Introduction, LRP1 also mediates the endocytosis of the uPAR–uPA–PAI complex. Studies on the mechanism of endocytosis of this complex have shown that both uPA and PAI can independently interact with LRP1 with low affinity. However, endocytosis occurs only when PAI binds to the uPAR–uPA complex (Nykjaer et al., 1994). Because the affinity of the uPAR–uPA–PAI complex for LRP1 is higher than that of the individual components uPA and PAI, it has been proposed that this high affinity binding is required to trigger internalization (Nykjaer et al., 1994). In this study, we have shown that GPC3 can directly bind to LRP1. However, endocytosis is only triggered upon the addition of Shh (Figs 2, 3). Similarly, we show that Shh can interact with LRP1 but endocytosis does not occur in the absence of GPC3. Based on these observations, it could be speculated that the simultaneous interaction of GPC3 and Shh with LRP1 generates a complex that is stable enough to allow rapid internalization.

Endogenous cell surface HSPGs have been implicated as partners of LRP1 in the endocytosis of numerous proteins, including amyloid- β peptides, coagulation factor VIII, the cellular prion protein, and lipoproteins. These proteins first bind with low affinity to the glycosaminoglycan (GAG) chains of cell surface HSPGs, and then they are ‘presented’ to LRP1 which mediates their internalization (Kanekiyo et al., 2011; Nykjaer and Willnow, 2002; Sarafanov et al., 2011; Wilsie and Orlando, 2003). In other words, the HSPGs act like docking sites that facilitate the interaction of the ligands with the endocytic receptors. Consistent with this mechanism, the endocytosis of these proteins can be blocked by heparin, and by treating cells with heparitinase. Furthermore, endocytosis does not take place in cells that cannot produce HSPGs (Kanekiyo et al., 2011). It should be noted, however, that the specific HSPGs involved in these endocytic processes have not been identified, and that an interaction between cell surface HSPGs and LRP1 has not been established. Based on the results presented here, it is clear that there are obvious differences between the role of HSPGs in the mechanism described above, and the role of GPC3 in the LRP1-mediated endocytosis of Hh. First, GPC3 binds to Shh with high affinity, and this high affinity interaction is mediated by the core protein (Capurro et al., 2008). Second, GPC3 directly binds to LRP1 via its GAG chains, and is internalized together with Shh and LRP1. Thus, GPC3 is more than just a docking site that facilitates Shh–LRP endocytosis.

The interaction between GPC3 and LRP1 is one of the most important findings of this study. This binding was demonstrated by co-immunoprecipitation, pull-down assays, and cell-binding studies. Furthermore, we were able to inhibit the interaction with heparin, and a mutant GPC3 that is not glycanated was not pulled down by LRP1, indicating that the binding is mediated by the GAG chains. Interestingly, we also showed that the non-glycanated GPC3 is mostly localized in lipid rafts. Based on this, we conclude that the HS-mediated interaction of GPC3 with LRP1 is required to keep GPC3 out of detergent-insoluble lipid rafts domains. It should be noted, however, that we previously reported that this non-glycanated GPC3 mutant has a partial inhibitory effect on Hh signaling (Capurro et al., 2008). Considering that the GPC3 displays a high affinity interaction with Shh (Capurro et al., 2008) and that LRP1 could transiently associate with lipid rafts (Wu and Gonias, 2005), a possible explanation for the partial inhibitory effect of GPC3 GAG is that some GPC3–Shh–LRP1 complexes could be formed within the lipid rafts with Shh bridging the GPC3–LRP1 interaction, and that these complexes could then move out of the lipid rafts to be endocytosed by clathrin coated pits. Alternatively, this partial Hh inhibitory effect may be the consequence of a clathrin- and LRP1-independent low rate GPC3 GAG–Shh internalization by caveolar-coated vesicles. In fact, it has been reported that both, uPAR and PrPc also internalize from lipid rafts through a low rate constitutive mechanism (Cortese et al., 2008; Parkyn et al., 2008).

The finding that HS-mediated lateral interactions of glypicans can determine their localization in specific cell membrane subdomains is not completely novel. It has already been reported that in polarized CaCo-2 and MDCK cells GPC1 is sorted almost exclusively to the basolateral cell membrane, something unexpected for a GPI-anchored protein (Mertens et al., 1996). However, upon removal of the GAG chains GPC1 was localized mostly at the apical side of the cell membrane (Mertens et al., 1996). In addition, in a recent study from our laboratory we reported that unlike GPC3, GPC5 stimulates Shh signaling by increasing the binding of Hh to its signaling receptor Ptc (Li et al., 2011). We found that the HS chains of GPC5 are required to interact with Ptc and to localize GPC5 in the primary cilia. A mutant GPC5 that cannot be glycanated does not interact with Ptc, it is not localized at the cilium, and it does not stimulates Hh signaling (Li et al., 2011). Our results also showed that even in the same cell type, specific glypicans may carry GAG chains with different modifications. Certainly, these glypican-specific modifications could have an impact in determining with which other cell membrane proteins each glypican is able to interact with, and which internalization route is used for its endocytosis. GPC1 has been shown to endocytose via caveolae in human bladder carcinoma cells (Cheng et al., 2002), indicating that most likely this glypican does not interact with LRP1. It will be of interest, therefore, to compare the structural properties of the GAG chains in GPC1 with those of GPC3. Additional studies will be required to determine whether other members of the glypican family can interact with LRP1. It should also be noted that GAG chain modifications are cell-type specific, even for the same protein core. We have shown here that, in addition to NIH 3T3 embryonic fibroblasts, an LRP is involved in the endocytosis of the GPC3–Hh complex in breast cancer cells. However, whether GPC3–Hh complexes are internalized by LRP-mediated processes in other cell types remains to be determined.

It should be noted that a small proportion of GPC3 produced by the NIH 3T3 cells can be detected at the 5%/30% interface of the sucrose gradient that contains the lipid raft-associated proteins. At this point in time we do not know whether this association with lipid rafts is due to the fact that a proportion of GPC3 is binding to a protein(s) that is located in such rafts, or whether this is the result of GPC3 overexpression.

Although all members of the LDL receptor gene family share the common structural cytoplasmic motif required for clathrin-mediated endocytosis (Nykjaer and Willnow, 2002), only two members of the family (LRP1 and LRP2/megalin) have been clearly shown to play a role as endocytic receptors (May et al., 2007). By inhibiting LRP1 expression in NIH 3T3 cells, we have demonstrated that this endocytic receptor mediates the endocytosis of the GPC3–Hh complex. However, it is possible that other members of the LDL receptor family could be involved in this internalization process in specific tissues. As discussed above, LRP2/megalin has also been shown to act as an endocytic receptor for Shh (McCarthy et al., 2002). The fact that *megalin*- and *Shh*-deficient embryos display similar neurodevelopmental abnormalities indicates that LRP2/megalin, contrary to GPC3, is a positive regulator of Shh signaling. Consistent with this observation, Shh internalized by LRP2/megalin is not targeted to lysosomes for degradation, and it has been proposed that this endocytic receptor may play a role in Shh transcytosis that is required for long-range signaling (McCarthy et al., 2002). So far, glypicans have not been implicated in the LRP2/megalin-mediated endocytosis of Shh. However, this endocytic activity of LRP2/megalin can be inhibited by heparin, suggesting that HSPGs could be involved (McCarthy et al., 2002).

Another member of the LDL family, LRP4/MEGF7 is also expressed early in development. *Lrp4*-deficient mice display growth retardation, polydactyly and syndactyly at both the fore and hind limbs, suggesting a role for LRP4 as a modulator of the signaling pathways that control limb development (May et al., 2007). Interestingly, polydactyly and syndactyly are some of the phenotypes reported in SGBS patients, and we have found increased Shh levels in the digits of *Gpc3*-knockout embryos (Capurro et al., 2008). Based on these observations, at this point in time we cannot discard the involvement of LRP4 in GPC3–Shh endocytosis-degradation in specific tissues where these molecules are coexpressed.

Another important finding of this study is that endocytosis of the GPC3–Hh complex is essential for GPC3-induced inhibition of Hh signaling. By using luciferase reporter assays in NIH 3T3 cells we clearly showed that GPC3 does not inhibit Hh activity when GPC3–Shh internalization is impaired by the presence of RAP or LRP1 siRNA. This finding provides additional support for the mechanism of GPC3-induced inhibition of Hh signaling that we have previously proposed (Capurro et al., 2008).

Materials and Methods

Cell lines and transfections

The 293T and NIH 3T3 cell lines were obtained from the ATCC (Manassas, VA, USA), and 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 with Lipofectamine Plus (Invitrogen, Burlington, ON, Canada). All conditioned media were

prepared in 293T cells after transfection with the indicated vectors. Media were collected 48 h after transfection in serum-free conditions, with the exception of the ShhN-conditioned medium. This medium, which was used in the Hh reporter assay, was collected 6 days after transfection in the presence of 2% FBS. The SKBR3 cell line (from the ATCC) was maintained in RPMI 1640 medium supplemented with 10% FBS. Expression vectors for GPC3 and GPC3 GAG in EF, GPC3-AP and GPC3 GAG-AP in APTag-2 (GeneHunter Corporation, Nashville, TN), ShhN in pCDNA and Shh-AP in APTag4 (GeneHunter Corporation) were previously described (Capurro et al., 2008; Gonzales et al., 1998). Hemagglutinin A (HA)-tagged LRP1-IV minireceptor in pCDNA was provided by D. Strickland, University of Maryland. The HA-tagged Delta 4 (DL4) construct was a gift from J. C. Zuniga-Pflucker (Sunnybrook Research Institute).

Isolation of lipid rafts

GPC3-transfected NIH 3T3 cells were suspended in 1 ml of ice-cold MBS buffer (25 mM MES, 150 mM NaCl pH 6.5) containing 1% Triton X-100, incubated on ice for 30 minutes, and homogenized with 10 strokes in a Dounce homogenizer. The cell lysates were then gently mixed with an equal volume of 80% sucrose in MBS buffer, and loaded in the bottom of a centrifuge tube. The mixture was sequentially overlaid with 30% (2 ml) and 5% (1 ml) sucrose in the same buffer. The discontinuous sucrose gradient was centrifuged at 180,000 *g* for 20 hours in a swinging rotor, and 0.4 ml fractions were collected from the top of the tube.

Endocytosis assays and siRNA treatment

NIH 3T3 cells were plated on poly-L-lysine-treated coverslips, and transfected with GPC3. For the GPC3–Shh co-localization experiment, 2 days after transfection, cells were starved in serum-free medium for 1.5 hours, and incubated with Shh- or control (pCDNA)-conditioned medium supplemented with 1% BSA for 50 minutes. Unbound ligand was removed by washing, and cells were fixed with 4% paraformaldehyde or transferred at 37°C for 40 minutes prior to fixation. To block clathrin-mediated endocytosis, 0.4 M sucrose or 100 μ M MDC (Sigma) (methanol as control) were added to the serum-free medium during the starvation period, and to the Shh-conditioned medium (Peng et al., 2010). For K⁺ depletion, cells were starved in serum-free medium for 1 hour, rinsed with potassium-free buffer (140 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml D-glucose, pH 7.4) and subsequently incubated in hypotonic medium (50% potassium-free buffer, 50% H₂O) for 5 minutes, and in potassium-free buffer for 20 minutes at 37°C. A control was performed with 10 mM KCl added to the potassium-free buffer (Peng et al., 2010). When indicated, RAP [Innovative Research (Novi, Michigan), 25 μ g/ml] was added to the serum-free medium during the starvation period and during the incubation with the Shh-conditioned medium. For immunostaining, cells were permeabilized with 0.1% Triton X-100 for 15 minutes, and blocked for 30 minutes with 5% non-fat dry milk in PBS (blocking buffer). All incubations with primary and secondary antibodies were performed for 1 hour at room temperature in blocking buffer. Antibodies used were: mouse anti-GPC3 monoclonal antibody 1G12 (Capurro et al., 2003), rabbit anti-Shh polyclonal antibody (H-160, Santa Cruz, Santa Cruz, CA), and the corresponding fluorescein-conjugated secondary antibodies. For transferrin internalization, NIH 3T3 cells were starved as above and incubated with

Alexa-Fluor-555-conjugated transferrin (Invitrogen, 25 µg/ml) in serum-free medium supplemented with 1% BSA for 50 minutes. Unbound ligand was removed by washing, and cells were fixed or transferred at 37°C for 40 minutes prior to fixation. When indicated, RAP treatment was performed as above.

For the internal/external GPC3 detection experiment, GPC3-transfected NIH 3T3 cells were incubated with the 1G12 anti-GPC3 antibody (5 µg/ml) in blocking buffer for 1 hour at 8°C. Cells were then incubated with Shh- or control-conditioned medium as described above. After fixation in 4% paraformaldehyde-4% sucrose on ice for 30 minutes, cell surface GPC3 was detected by incubating with Alexa-Fluor-488-conjugated secondary antibody (Invitrogen, 1:500) for 2 hours at room temperature. Cells were then permeabilized for 30 minutes with ice-cold 0.2% Triton X-100 in blocking buffer, and the internalized GPC3 detected with Texas-Red-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, Maine, 11:200). When indicated, RAP was added during the labeling with the 1G12 antibody and the incubation with Shh-conditioned medium. For the competition experiment, heparin (10 µg/ml) was added during the incubation with the Shh-conditioned medium and during the internalization at 37°C. To silence LRP1, 100 nM of LRP1 siRNA (mouse, sc-40102, Santa Cruz) or non-targeting control siRNA (sc-37007, Santa Cruz) were transfected in the NIH 3T3 cells using DharmaFECT-1 transfection reagent (Dharmacon, Lafayette, CO). siRNA-mediated knockdown of LRP1 was confirmed by western blot analysis using the anti-LRP1 rabbit monoclonal antibody LRP1 RabMAb (Epitomics, Burlingame, CA). Actin detection with an anti-actin antibody (AC-40, Sigma) was performed as a loading control. Confocal images were generated using a scanning laser microscope LSM 510 version 3.2 SP2 (Carl Zeiss Inc., Pickering, ON, Canada), and a Zeiss LSM Image Browser.

Coimmunoprecipitation

Transfected 293T cells were lysed in RIPA buffer, and the cell lysates were precleared with Protein-G Sepharose during 1 hr at 4°C. GPC3 and HA-tagged LRP1-IV or DL4 were then immunoprecipitated by incubating the cell lysates over night with 1G12 or 12CA5 (Roche, Laval, QC, Canada) antibodies and then with Protein-G Sepharose for 90 minutes at 4°C. The presence of HA-tagged LRP1-IV or Shh in the precipitated material was assessed by western blot.

Cell-binding assay

293T cells were transfected with HA-tagged LRP1-IV or pCDNA as control. Two days after transfection, the cells were transferred to 8°C and GPC3-AP-, Shh-AP- or AP-conditioned media containing the same amount of AP activity were added to the cells for 2 hours. After unbound ligands were removed by four washes with PBS, cells were lysed in 10 mM Tris-HCl, pH 8, containing 1% NP40. Lysate aliquots with equal amount of protein were heated at 65°C for 10 minutes to inactivate the cellular phosphatases, and the AP activity was then measured with a Sigma fast p-nitrophenyl phosphate tablet set. For acid treatment, cells were incubated in 50 mM Glycine hydrochloride, 100 mM NaCl, pH 3, for 3 minutes at 0°C (to remove endogenously bound ligands), and quickly neutralized with a half volume of 0.5 M HEPES, 100 mM NaCl, pH 7.4 before the incubation with GPC3-AP- or AP-conditioned

media was performed. Control for HA-tagged LRP1-IV expression in the whole cell lysates was performed by western blot.

Pull-down assay

293T cells transfected with HA-tagged LRP1-IV or vector control were lysed in RIPA buffer and the cell lysates were first incubated overnight with an anti-HA antibody and then with Protein-G Sepharose for 90 minutes at 4°C. Beads were washed four times with RIPA buffer, and blocked with 5% BSA in PBS containing 0.1% Triton X-100 for 90 minutes at room temperature. Aliquots containing equal amount of beads were then incubated for 1 hour with GPC3-AP, GPC3 GAG-AP, Shh-AP- or AP-conditioned media. Beads were washed four times with 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% Tween20. The AP activity bound to the beads was determined as described above. When indicated, heparin (0.1 to 1 µg/ml) was added to GPC3-AP- or control-AP-conditioned media. The presence of HA-tagged LRP1-IV in the beads was confirmed by western blot.

Hh reporter assay

NIH 3T3 cells were seeded in 6-well plates (250,000 cells/well) and cotransfected with a luciferase reporter vector driven by an Hh responsive promoter (0.4 µg), the indicated amounts of GPC3 or EF expression vectors, and β-galactosidase (50 ng). One day after transfection, cells were transferred to 24-well plates at 50% confluence, and the following day ShhN- or control-conditioned medium (diluted 1:10 in DMEM 2% FBS) was added for 48 hours. A luciferase assay was then performed. The luciferase activity was normalized based on the β-galactosidase activity. When indicated, RAP (20 µg/ml) was added to the ShhN-conditioned medium. RAP was replenished after 24 hours.

Generation of GPC3-expressing SKBR3 cell line

SKBR3 cells were infected by overnight incubation with medium containing a GPC3 lentivirus (GPC3-LV) or a GFP lentivirus (GFP-LV) as control, and 10 µg/ml of Polybrene (Sigma, St Louis, MO). Two days after infection, cells were harvested and GPC3-LV-infected cells were stained with 1G12 and a FITC-conjugated secondary antibody. GPC3 or GFP-expressing cells were then isolated by FACS and expanded.

Western blot analysis of endocytosis

GPC3-LV or GFP-LV transduced cells were plated at high confluence (500,000 cells/well) in 6-well plates, and incubated over night with Shh- or control-conditioned medium. Cell lysates were then prepared in RIPA buffer, and the levels of GPC3 assessed by western blot with the 1G12 antibody. Actin was used as loading control. When indicated, cells were incubated with RAP (20 µg/ml) for 1 hour before adding Shh-conditioned medium supplemented with the same concentration of RAP. The levels of GPC3 were then assessed by immunoprecipitation of GPC3 from the indicated lysates with the 1G12 antibody followed by western blot analysis.

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Fig. 1. GPC3 is mostly localized outside of lipid rafts

Lipid rafts were isolated from GPC3-transfected NIH 3T3 cells by cold lysis with Triton X-100 followed by centrifugation on a discontinuous sucrose gradient. The gradient was separated into 12 equal-volume fractions (Fr 1–12) and the pellet (P). The presence of GPC3 and caveolin-1 (N-20, sc-894, Santa Cruz) in each fraction was assessed by western blot. Lipid rafts partitioned in the 5% to 30% interface of the sucrose gradient, are identified by the presence of caveolin-1.

Fig. 2. Clathrin mediates GPC3–Shh endocytosis

NIH 3T3 cells transfected with GPC3 were incubated with control-conditioned medium (pCDNA CM) (A, inset in C) or Shh-containing conditioned medium (Shh CM) (B–F); for 50 minutes at 8°C, and fixed (A,B) or transferred to 37°C for 40 minutes before fixation (C–F). GPC3 (red) and Shh (green) were then immunostained. Yellow in merged pictures indicates co-localization. Cells were preincubated for 1.5 hours with 0.4 M sucrose (D), 100 μM MDC (E) or methanol as control (E, inset), before adding Shh CM with the corresponding inhibitors. (F) Cells were subjected to K⁺ depletion before adding Shh CM. Control cells (with K⁺) are shown in F, inset. Dotted white lines mark cell boundaries. Scale bars: 10 μm.

Fig. 3. Knockdown of RAP and LRP1 inhibits Shh-GPC3 endocytosis

NIH 3T3 cells were transfected with GPC3 and incubated with Shh-conditioned medium (Shh CM) (**A,C**) or incubated with Alexa-Fluor-555-transferrin (TF) (**B**) for 50 minutes at 8°C, and fixed (**B**, left) or transferred to 37°C for 40 minutes before fixation. GPC3 (red) and Shh (green) were then immunostained as indicated. Yellow in merged pictures represents co-localization. Cells in right panel of **A,B** were preincubated with RAP (25 µg/ml) (+ RAP) for 1.5 hours before adding Shh CM or TF, which also contained RAP. Controls without RAP (-RAP) are shown in **A**, inset and **B**, middle panel. (**C**) After transfection with GPC3, and before adding the Shh CM, cells were transfected with LRP1 siRNA or non-targeting control siRNA (**C**, inset). Dotted white lines mark cell boundaries. (**D-H**) GPC3-expressing NIH 3T3 cells were incubated with anti-GPC3 antibody 1G12 for 1 hour at 8°C before addition of Shh CM (**E-H**) or control-conditioned medium (pCDNA CM) (**D,F** inset). After incubation for 50 minutes at 8°C, cells were fixed, or transferred to 37°C for 40 minutes before fixation. Cell-surface GPC3 (GPC3 Ext) was labeled with a

green Alexa-Fluor-conjugated secondary antibody, and cells were then permeabilized. Internalized GPC3 (GPC3 Int) was detected with a red Alexa-Fluor-conjugated secondary antibody. (G) RAP (25 $\mu\text{g/ml}$) was added to the cells during the incubations with the 1G12 antibody and Shh CM. (H) Cells were transfected with LRP1 siRNA or non-targeting control siRNA (H, inset) before addition of the Shh CM. Note that, as expected, some red Alexa-Fluor-conjugated secondary antibody also labeled cell-surface GPC3 by displacement of the previously bound green label. Scale bars: 10 μm .

Fig. 4. GPC3 interacts with LRP1

(A) Coimmunoprecipitation. 293T cells were transfected with the indicated expression vectors and GPC3 was immunoprecipitated. Top panel shows the presence of LRP1-IV in the precipitated material as assessed by western blotting (WB). Levels of immunoprecipitated GPC3 (middle) and LRP1-IV expression (bottom) in whole cell lysates were verified by western blot. IP, immunoprecipitation; LC; immunoglobulin light chain. (B) Cell-binding assay. 293T cells transfected with LRP1-IV or vector control were incubated with GPC3-AP or AP alone for 2 hours at 8°C (left) or acid-treated with 50 mM glycine-hydrochloride, 100 mM NaCl, pH 3, before the addition of AP ligands (right). Cells were then washed, lysed, and the AP activity of aliquots of cell lysates containing equal amount of protein was determined. The bars represent the mean + s.d. of triplicates. The background binding to cells transfected with vector control (pCDNA) was subtracted from each measurement. Western blot assessment of LRP1-IV expression in the indicated cell lysates is shown below the graph. (C) Pull-down assay. LRP1-IV-covered Protein-G beads were prepared and incubated with GPC3-AP or AP alone. 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 LRP1-IV beads after subtracting the corresponding AP activity bound to the control beads. Inset shows western blot analysis, confirming the presence of LRP1-IV in the beads.

Fig. 5. The heparan sulfate chains of GPC3 mediate the interaction with LRP1 and direct GPC3 out of lipid rafts

Pull-down assay. (A) LRP1-IV-covered Protein-G beads were prepared and incubated with GPC3-AP, GPC3 Δ GAG-AP or AP alone. 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 LRP1-IV beads after subtracting the corresponding AP activity bound to the control beads. (B) An LRP1-IV–GPC3-AP pull-down assay was performed in the presence of the indicated concentrations of heparin. Each point represents the specific AP activity (mean $\pm$ s.d. of triplicates) bound to the beads after subtracting the corresponding control AP values. Western blot confirming presence of LRP1-IV in the beads is shown on the right. (C,D) Heparin inhibits GPC3–Shh endocytosis. (C) NIH 3T3 cells transfected with GPC3 were incubated with Shh-conditioned medium (Shh CM) in the presence of heparin for 50 minutes at 8°C and transferred to 37°C for 40 minutes before fixation. GPC3 immunostaining is shown in red, and Shh in green. Yellow in merged pictures indicates colocalization. Dotted white line identifies cell boundary. (D) GPC3-expressing NIH 3T3 cells were incubated with the anti-GPC3 antibody 1G12 for 1 hour at 8°C before adding Shh-conditioned medium (Shh CM) in the presence of heparin. After a 40 minute incubation at 37°C, cell surface GPC3 (GPC3 Ext) and internalized GPC3 (GPC3 Int) were detected as described in Fig. 3. Scale bars: 10 μ m. (E) Lipid rafts were isolated from GPC3 Δ GAG- or GPC3-transfected NIH 3T3 cells by cold lysis with Triton X-100 followed by centrifugation on a discontinuous sucrose gradient. The gradient was then separated into 12 equal-volume fractions (Fr 1–12) and the pellet (P). The presence of GPC3 Δ GAG/GPC3 and caveolin-1 in each fraction was assessed by western blot. Western blot analysis of endogenous LRP1 throughout the sucrose gradient is shown at the bottom.

Fig. 6. LRP1 interacts with Shh

(A) Coimmunoprecipitation. 293T cells were transfected with the indicated expression vectors, and LRP1-IV or DL4 was immunoprecipitated with anti-HA antibody. Top panel shows the presence of Shh in the precipitated material as assessed by western blotting (WB). Levels of immunoprecipitated LRP1-IV and DL4 (middle) and Shh expression (bottom) in whole cell lysates were verified by WB. IP, immunoprecipitation. (B) Cell-binding assay. 293T cells transfected with LRP1-IV or vector control were incubated with Shh-AP or AP alone for 2 hours at 8°C. Cells were then washed, lysed and the AP activity of aliquots of cell lysates containing equal amount of protein was determined. The bars represent the mean AP activity + s.d. of triplicates. The background binding to cells transfected with vector control (pCDNA) was subtracted from each measurement. (C) Pull-down assay. LRP1-IV-covered Protein-G beads were prepared and incubated with Shh-AP, or AP alone. After washing, the AP activity retained by the beads was measured. Bars represent the specific AP activity (mean + SD of triplicates) bound to the LRP1-IV beads after subtracting the corresponding AP values obtained for the control beads. Western blot analysis assessing LRP1-IV levels in the transfected cells and in the beads used for the pull-down assay is shown below the graph.

Fig. 7. LRP1 is required for GPC3-induced inhibition of Hh signaling

Hh reporter assay in the presence of RAP (**A**) or LRP1 siRNA (**B**). NIH 3T3 cells were transfected with a GPC3 expression vector or vector control (EF), along with a luciferase reporter vector driven by an Hh responsive promoter and β -galactosidase. Cells were incubated for 48 hours with Shh- or control-conditioned medium, and luciferase and β -galactosidase assays performed. Bars represent luciferase activity (mean + s.d. of triplicates) relative to that induced by Hh in EF-transfected cells (arbitrarily defined as 1). Bottom panels show western blot analysis of GPC3 and LRP1 expression levels in the transfected NIH 3T3 cells. Actin was used as loading control.

Fig. 8. LRP1 is required for Shh-induced endocytosis of GPC3 in breast cancer cells

(A) Incubation with Shh induces GPC3 degradation. SKBR3 breast cancer cells transduced with GPC3-LV (GPC3 +) or GFP-LV (GPC3 -) were incubated in the presence of Shh-containing conditioned medium (Shh +) or control conditioned medium (Shh -) overnight at 37°C. Cell lysates were prepared and the levels of GPC3 were assessed by western blot. Actin was used as loading control. (B) RAP inhibits Shh-induced degradation of GPC3. GPC3-transduced SKBR3 cells were incubated with Shh-containing conditioned medium (Shh) in the absence or presence of RAP (Shh + RAP) or control conditioned medium (pCDNA) overnight at 37°C. Cell lysates were prepared and GPC3 levels assessed by immunoprecipitation followed by western blot. Bottom graph shows scanned densitometry quantification of bands corresponding to glycanated GPC3. Density corresponding to cells incubated with control conditioned medium was arbitrarily defined as 1. Relative mean density + s.d. of three independent experiments is shown.