Glypicans are heparan sulfate proteoglycans that are attached to the cell surface by a GPI (glycosylphosphatidylinositol) anchor. Glypicans regulate the activity of Wnts, Hedgehogs, bone morphogenetic proteins and fibroblast growth factors. In the particular case of Wnts, it has been proposed that GPI-anchored glypicans stimulate Wnt signalling by facilitating and/or stabilizing the interaction between Wnts and their cell surface receptors. On the other hand, when glypicans are secreted to the extracellular environment, they can act as competitive inhibitors of Wnt. Genetic screens in Drosophila have recently identified a novel inhibitor of Wnt signalling named Notum. The Wnt-inhibiting activity of Notum was associated with its ability to release Dlp [Dally (Division abnormally delayed)-like protein; a Drosophila glypican] from the cell surface by cleaving the GPI anchor. Because these studies showed that the other Drosophila glypican Dally was not released from the cell surface by Notum, it remains unclear whether this enzyme is able to cleave glypicans from mammalian cells. Furthermore, it is also not known whether Notum cleaves GPI-anchored proteins that are not members of the glypican family. Here, we show that mammalian Notum can cleave several mammalian glypicans. Moreover, we demonstrate that Notum is able to release GPI-anchored proteins other than glypicans. Another important finding of the present study is that, unlike GPI-phospholipase D, the other mammalian enzyme that cleaves GPI-anchored proteins, Notum is active in the extracellular environment. Finally, by using a cellular system in which GPC3 (glypican-3) stimulates Wnt signalling, we show that Notum can act as a negative regulator of this growth factor.

Key words: glycosylphosphatidylinositol (GPI) anchor, glypican family of proteins, heparan sulfate proteoglycan, mammalian Notum, phospholipase Wnt.

INTRODUCTION

Eukaryotic cells produce a large number of proteins that are anchored to the outer surface of the plasma membrane through a covalent GPI (glycosylphosphatidylinositol) linkage [1,2]. It has been estimated that GPI-anchored proteins constitute an average of 0.5% of the cell proteins [3]. Although GPI-anchored proteins do not have common functions, the presence of the anchor itself appears to confer some important behavioural attributes on them, including preferential localization at the lipid rafts, and susceptibility to cleavage by endogenous and exogenous phospholipase enzymes [4].

Glypicans are a family of HS (heparan sulfate) proteoglycans that are linked to the plasma membrane via a GPI anchor [5–7]. Two glypicans have been identified in Drosophila: Dally (Division abnormally delayed) [8] and Dlp (Dally-like protein) [9]. In mammals, the glypican family has six members [GPC1 (glypican-1)–GPC6] [7,10]. In general, glypicans are expressed predominantly during development, and their expression levels change in a stage- and tissue-specific manner, suggesting that they are involved in morphogenesis [11]. Genetic and functional studies performed in Drosophila, Xenopus, zebrafish and mammals demonstrated that glypicans could regulate the signalling activity of Wnts, Hedgehogs, BMPs (bone morphogenetic proteins) and fibroblast growth factors in a tissue-specific manner [9,12–21]. In the particular case of Wnts, a direct interaction between various Wnt family members and glypicans has been demonstrated [19,20,22–24], and it has been proposed that GPI-anchored glypicans can stimulate signalling by facilitating and/or stabilizing the interaction between Wnts and their cell surface receptors [23]. On the other hand, when glypicans are secreted to the extracellular environment, they can act as competitive inhibitors of Wnt signalling [23]. In addition, secreted glypicans are known to play a role in the transport of Wnts in the wing imaginal discs during Drosophila development for the purpose of morphogen gradient formation [25–27].

Genetic screens in Drosophila performed by two laboratories identified a new negative regulator of Wnt signalling called Notum [28,29]. Initially, it was proposed that Notum regulates Wnt activity by modifying the HS chains of Drosophila glypicans [28]. However, more recent studies showed that Notum acts as a lipase, releasing Dlp from the cell surface by cleaving the GPI anchor [30]. Because these studies showed that the other Drosophila glypican (Dally) was not released from the cell surface by Notum, it remains unclear whether Notum is able to cleave glypicans from mammalian cells. Furthermore, it is also not known whether Notum can cleave GPI-anchored proteins that are not members of the glypican family. In the present study, we show that mammalian Notum can cleave several mammalian glypicans. Moreover, we demonstrate that Notum is able to release, from the cell surface, GPI-anchored proteins other than glypicans, such as uPAR (urokinase-type plasminogen activator receptor) and T-cadherin. Another significant discovery is our finding that unlike GPI-PLD...
(phospholipase D), to date the only characterized mammalian lipase that can cleave GPI-anchored proteins, Notum is active in the extracellular environment. We also showed that Notum’s cleavage site in the GPI anchor is similar to that of GPI-PLC (phospholipase C). In addition, by using a cellular system in which GPC3 stimulates Wnt signalling, we show that Notum can act as a negative regulator of this growth factor. Finally, we determined the expression profile of Notum during different stages of embryonic development and in various tissues of adult mice.

**EXPERIMENTAL**

**Cell lines and plasmids**

The HEK-293T [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)] cell line was cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum). L-cells permanently transfected with Wnt3a-pLNCx or empty vector (pLNCx) were obtained from the A.T.C.C. and cultured in DMEM supplemented with 10% FBS.

Expression vectors containing GPC3, the mutant GPC3 that cannot be glycanated (GPC3ΔAGAG), His-tagged GPC4 and CEA (carcinoembryonic antigen) were previously described [31–33]. The expression vectors for GPC5 and GPC6 were generated by inserting their cDNAs into the pEF (where EF is elongation factor) and pcDNA3 expression vectors respectively. The mouse wtNotum (wild-type Notum) cDNA was obtained from Riken and was cloned into the pTracer vector (Invitrogen), which contains a His tag at the C-terminus of the cloning site. To place the His tag in frame with the inserted Notum cDNA, we mutated the last residue (asparagine) to serine. To inactivate the proteolytic activity of Notum, we generated a mutant form of Notum [mutNotum (mutant Notum)] by replacing Ser-237 with alanine by site-directed mutagenesis. The mutation was confirmed by DNA sequencing. The expression vector containing uPAR [34] was provided by Dr J. Aguirre-Ghiso (University of Albany), and the GFP (green fluorescent protein)-tagged T-cadherin expression vector was obtained from Dr J. Yang (Columbia University) [35].

**Notum activity assay**

HEK-293T cells were transiently co-transfected with expression vectors for His-tagged wtNotum or mutNotum, the indicated GPI-anchored protein and GFP as a control for transfection efficiency. The day after transfection, the medium was changed to serum-free DMEM. The conditioned medium was removed 24 h later, and concentrated 10-fold using Centricon, and the concentrate was incubated with Wnt3a-pLNCx or empty vector (pLNCx) cells were plated in a medium with 10% FBS, and transiently transfected with expression vectors for His-tagged wtNotum or mutNotum. The day after transfection, the medium was changed to serum-free DMEM. On the following day, the conditioned medium was removed and concentrated 10-fold by using Centricon. The Notum-containing concentrated medium was then added to the HEK-293T cells transfected with GPC3 and GFP. The conditioned medium was removed 6 h later, and concentrated 5-fold by using Centricon. The amount of GPC3, wtNotum, mutNotum and GFP in the cell lysates and conditioned media was assessed by Western blotting as described above.

**Radiolabelling analysis**

HEK-293T cells were transiently co-transfected with expression vectors for His-tagged wtNotum or mutNotum and for uPAR. The day after transfection, the medium was changed to serum-free DMEM or phosphate-free and serum-free DMEM for 2 h. After incubation, the cells were metabolically labelled with 0.1 μCi/ml [3H]P, (Amersham Bioscience) or 0.1 μCi/ml [3H]ethanolamine (Amersham Bioscience) in the presence or absence of 0.5 unit/ml GPI-PLC (phospholipase C) for 18 h. The conditioned medium was removed, 17 h later, and the released uPAR was immunoprecipitated with uPAR antibody overnight at 4°C, followed by a 1 h incubation at 4°C with Protein G. Immune complexes were then collected and washed three times with PBS. The radioactivity of the proteins from immunoprecipitated samples was determined by using a liquid-scintillation counter (Beckman Coulter). Each experiment was performed in triplicate and the results represent the means ± S.D.

**Purification of Notum and generation of antibodies against Notum**

HEK-293T cells were plated in a medium with 10% FBS and transiently transfected with an expression vector for His-tagged wtNotum. The day after transfection, the medium was changed to serum-free DMEM. Cells were incubated for 24 h and the conditioned medium was removed and concentrated 10-fold using Centricon, and the concentrate was incubated overnight with Ni-NTA (Ni2+-nitriotriacetate)–agarose beads (Qiagen) in a buffer containing 50 mM NaH2PO4, 300 mM NaCl and 100 mM imidazole (pH 8.0). The beads were then washed in a buffer containing 50 mM NaH2PO4, 300 mM NaCl and 20 mM imidazole (pH 8.0), and bound Notum was eluted with a buffer containing 50 mM NaH2PO4, 300 mM NaCl and 250 mM imidazole. After elution, the sample was dialysed in PBS.

To generate polyclonal antibodies against Notum, rabbits were injected with purified Notum by using standard protocols.

**Immunohistochemistry**

Paraffin-embedded tissue sections were treated with blocking solution before being sequentially incubated with the anti-Notum antibody (Notum, 1:200) overnight at 4°C, followed by biotin-labelled secondary antibody and HRP-conjugated avidin [each for 30 min at room temperature (20°C)]. Detection was achieved with a substrate/chromagen mixture (Zymed Laboratories) and haematoxylin counterstaining. For negative controls, incubation with the primary antibody was omitted.

**Luciferase assay for TOPFLASH reporter activity**

HEK-293T cells were plated on to 24-well plates at a density of 200 000 cells/well and co-transfected with a luciferase reporter vector driven by the TOPFLASH promoter [37], and expression vectors for β-galactosidase, wtNotum or mutNotum and GPC3 or vector control (EF) by using Lipofectamine™ 2000 (Invitrogen). At 1 day after transfection, the cells were incubated for 6 h with conditioned media from Wnt3a-transfected L-cells or...
Mammalian Notum: a novel enzyme with glycosylphosphatidylinositol-anchor-releasing activity

L-cells transfected with vector control. Cells were then lysed, and luciferase activity (Luciferase Assay System; Promega) and β-galactosidase activity were determined. Each luciferase value was normalized for transfection efficiency using the β-galactosidase activity.

Semi-quantitative RT–PCR (reverse transcription)–PCR

Total RNA from each tissue was prepared by using TRIzol® (Invitrogen) according to the manufacturer’s instructions. Total RNA (1.5 µg) was reverse-transcribed with the SuperScript First-strand Synthesis System (Invitrogen). The Notum, GPC3, GPC4, GPC5 and GPC6 cDNA was then amplified by using the following primers: Notum, 5′-TTCCCGCTGACTTTCAGC-3′ and 5′-GGTACCTGTGTTGTCAGC-3′; GPC3, 5′-CAAGAAAGATGGAGGAGG-3′ and 5′-CAAAACTCAAAGCCTATTGGGAGTCAGGCT-3′; GPC4, 5′-CTGCTATTCATGTATAGCATTCA-3′ and 5′-TATACAGAGCTAAGGC-3′; GPC5, 5′-CGAGAGTCCTTATCCAGTG-3′ and 5′-GTTATTGTTCCTCACTGCAG-3′; GPC6, 5′-GTACAGATCTCCATGCAGA-3′ and 5′-ATGCTGATCACTGCAGAT-3′. A total of 30 amplification cycles were performed. To control for amount of intact RNA, β-actin was amplified in parallel with the following primers: 5′-CGCACCACTGGCATTGTCAT-3′ and 5′-TTCTCTTCTTCGTTAAGGC-3′.

RESULTS

Notum induces the release of glypicans and other GPI-anchored proteins into the extracellular environment

First, we investigated whether mammalian Notum can release glypicans from the cell surface. To this end, we cloned the full-length cDNA of His-tagged mouse Notum into an expression vector. We also generated an expression vector containing mutNotum, a His-tagged Notum cDNA that is inactive due to a mutation in the predicted active site (Ser-237 to alanine) [30]. Next, HEK-293T cells were co-transfected with a GPC3 expression vector and vectors containing wtNotum or mutNotum. A GFP expression vector was also included as a control for transfection efficiency. The regular medium was replaced by serum-free medium 24 h after transfection. After an additional 18 h incubation period, the conditioned medium was collected and concentrated, and the amount of GPC3 was assessed by Western-blot analysis. The presence of GPC3 in the corresponding cell lysates was also investigated. As shown in Figure 1(A), we found that, when compared with the conditioned medium from cells

![Figure 1](image_url)
transfected with an inactive mutNotum, the conditioned medium from cells transfected with wtNotum contained significantly more GPC3. Conditioned media from cells transfected with mammalian mutNotum contained the same amount of GPC3 as conditioned media from cells transfected with vector control (results not shown). We also observed that cells treated with wtNotum display a reduction in the levels of GPC3 compared with cells treated with mutNotum (Figure 1A). Taken together, these results demonstrate that mammalian Notum can cleave GPC3. We [36] and others [38] have previously reported that many GPC3-expressing cell lines constitutively secrete detectable amounts of GPC3 to the conditioned media. We speculate that this secretion could be the result of the activity of endogenous GPI-PLD (phospholipase D) or endogenous Notum. In agreement with these previous observations, we found that cells treated with mutNotum secrete detectable amounts of GPC3 in the conditioned medium. Figure 1(A) also shows that significant amounts of GPC3 can still be detected in lysates from cells transfected with wtNotum. Most likely, a significant proportion of the GPC3 retained in the cell lysates represents recently synthesized GPC3 that has not yet reached the cell surface. Another possible explanation for the incomplete release of GPC3 is that its GPI anchor displays modifications that do not allow efficient cleavage by Notum. In the case of bacterial GPI-PLC, for example, it has been shown that acylation of the inositol ring strongly inhibits its activity [39]. In addition, the incomplete cleavage could also be due to the fact that the amount of wtNotum produced by the transfected cells was not enough to cleave all the available GPC3.

The results shown in Figure 1(A) do not provide a definitive proof that Notum is acting extracellularly, since it is also possible that GPC3 is cleaved in the endoplasmic reticulum or in the secretory pathway before reaching the cell surface. To investigate whether Notum is able to cleave GPC3 at the cell surface, we decided to incubate GPC3-transfected cells with conditioned medium from HEK-293T cells transiently transfected with Notum (see the Experimental section). After incubation with Notum-containing medium, the levels of GPC3 in the medium and in the cell lysate were assessed by Western-blot analysis. As shown in Figure 1(B), we found that treatment with wtNotum significantly increased the amount of GPC3 detected in the conditioned medium, although the increase appears to be smaller than that generated by direct transfection of Notum into the GPC3-expressing cells. A decrease in GPC3 levels in the cell lysates of the wtNotum-treated cells was also observed (Figure 1B).

Next, we investigated whether Notum can cleave other members of the glypican family. To this end, we co-transfected HEK-293T cells with expression vectors for GPC4, GPC5 or GPC6 and wtNotum or mutNotum, and the effect of Notum on glypican levels in the conditioned medium and cell lysates was investigated. As shown in Figures 1(C)–1(E), we found that transfection with wtNotum significantly increased the amount of glypicans present in the conditioned medium. In the case of GPC5 and GPC6, similarly to GPC3, a significant decrease in glypican levels in the cell lysates of the wtNotum-treated cells was observed. However, no obvious difference in the amount of GPC4 in the cell lysates could be detected.

Mammalian glypicans can also be cleaved at the level of the GPI anchor by endogenous GPI-PLD [40]. Because this enzyme can cleave several kinds of GPI-anchored proteins [3,41,42], we decided to investigate whether, in addition to glypicans, Notum can release other classes of GPI-anchored proteins from the cell surface. To this end, we co-transfected Notum with expression vectors for uPAR, GFP-tagged T-cadherin and CEA, and the effect of Notum on the release of the GPI-anchored proteins into the conditioned media was analysed by Western blotting. As shown in Figures 2(A) and 2(B), wtNotum significantly increased the amount of secreted uPAR and T-cadherin respectively. The cleaving activity of Notum on uPAR is similar to that observed for GPC4 and GPC5. In the case of CEA, however, no increase in the amount of this protein in the conditioned medium from cells transfected with wtNotum was observed (Figures 3A). On the other hand, CEA was released into the conditioned medium when the cells were treated with GPI-PLC (Figure 3B).

It is generally believed that the HS chains of proteoglycans mediate their interaction with other proteins. In the case of glypicans, however, we have demonstrated that the HS chains are not required for the interaction of GPC3 with Wnts [23,24]. We decided therefore to investigate whether the HS chains are required for the interaction of Notum with GPC3. To this end, we co-transfected the HEK-293T cells with Notum and a mutant GPC3 cDNA (GPC3ΔGAG) in which the two insertion sites for the HS chains have been mutated. We found that the effect of Notum on the release of non-glycanated GPC3 is similar to its effect on wild-type GPC3 (Figure 4).

Activity of purified Notum

Next, we investigated whether Notum can be purified from conditioned medium in an active form. For the purification, we incubated His-tagged Notum-containing conditioned medium with Ni beads as described in the Experimental section. As shown in the stained SDS/PAGE gel in Figure 5, we obtained Notum that was at least 95% pure. In addition to the 59 kDa band corresponding to the full-length Notum, two smaller bands that correspond to degradation products were also detected by Western-blot analysis. After purification, we tested the activity of Notum by assessing its ability to release GPC3ΔGAG and uPAR from the cell surface. Different concentrations of Notum (from
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Figure 3 The effect of Notum and GPI-PLC on the release of CEA

(A) HEK-293T cells were transiently co-transfected with expression vectors for CEA and His-tagged wtNotum or vector control, and GFP. The expression levels of the transfected proteins in cell lysates (Lys) and conditioned media (Med) were assessed by Western-blot analysis. (B) HEK-293T cells were transfected with an expression vector for CEA and then left untreated or treated with 0.5 unit/ml GPI-PLC for 20 h. The levels of CEA were assessed by Western blotting. Films were scanned, and the fold increase in the released protein after wtNotum treatment compared with mutNotum treatment is indicated at the bottom of each blot. Each experiment was performed at least three times, and one representative blot is shown.

1 to 5 µg/ml) were added for 6 h to HEK-293T cells transfected with GPC3ΔGAG or uPAR, and the effect of the purified enzyme on the release of GPC3ΔGAG and uPAR was analysed by Western blotting. As shown in Figures 5(C) and 5(D), we found that purified Notum is able to release non-glycanated GPC3 and uPAR from the cell surface in a concentration-dependent manner.

Localization of the cleavage site

Although Kreuger et al. [30] clearly demonstrated that Notum-induced cleavage occurs at the level of the GPI anchor, the exact localization of the cleavage site is not known. Each GPI anchor contains one ethanolamine and two phosphates [1], where one of the phosphates is attached to ethanolamine, and the other to inositol. GPI-PLD-released proteins retain one ethanolamine and one phosphate, whereas GPI-PLC-released proteins retain one ethanolamine and two phosphates. In an effort to provide more information with regard to the Notum cleavage site, we transfected HEK-293T cells with uPAR and wtNotum or mutNotum. Transfected cells were metabolically labelled with [32P]Pi and [3H]ethanolamine for 18 h. In parallel, cells were transfected with uPAR and treated with GPI-PLC during the labelling period. The conditioned medium was then collected, and uPAR was immunoprecipitated. The radioactivity of the precipitated uPAR was quantified (Table 1A), and the background radioactivity (represented by the radioactivity released by cells transfected with mutNotum) was subtracted. The ratio of the incorporated [32P]Pi to [3H]ethanolamine was then calculated (Table 1B). The results show that the ratio in the uPAR released by Notum is similar to the ratio in the uPAR released by GPI-PLC. Our results suggest, therefore, that Notum cleaves GPI-anchored proteins at a site similar to that cleaved by GPI-PLC.

Notum regulates Wnt signalling through GPC3

As discussed above, Notum was originally identified as a negative regulator of canonical Wnt signalling in Drosophila. In our laboratory, we have shown that GPC3 stimulates Wnt activity in cultured mammalian cells and that this stimulation requires the anchoring of GPC3 to the cell membrane [23]. Based on this, we speculated that mammalian Notum could act as an inhibitor of the GPC3-induced potentiation of canonical Wnt signalling. To test this hypothesis, HEK-293T cells were transiently transfected with a β-catenin-responsive luciferase vector, GPC3 and wtNotum or mutNotum. The day after transfection, conditioned media from Wnt-3a-producing or vector control L-cells were added to the transfected HEK-293T cells for 6 h, and luciferase activity was measured. As shown in Figure 6, we found that wtNotum significantly inhibits the GPC3-induced stimulation of canonical Wnt signalling.

Notum is expressed during different stages of embryonic development and in various tissues of adult mouse

To determine the expression profile of Notum in the mouse, we performed semi-quantitative RT–PCR in multiple tissues and in...
Figure 5 Analysis of purified Notum and its activity

SDS/PAGE of two aliquots of purified Notum, where one sample was analysed by Western blotting (WB) by using an anti-His antibody (A). The gel containing the other sample was stained with Coomassie Brilliant Blue (CBB) (B). HEK-293T cells were transiently co-transfected with expression vectors for GFP (loading control) and GPC3/D1GAG (C) or uPAR (D). Transfected cells were incubated with the indicated concentrations of purified Notum for 6 h and the expression levels of GPC3/D1GAG, Notum, GFP and uPAR in the conditioned media were assessed by Western-blot analysis. Numbers on the sides represent molecular mass markers expressed as kilodaltons.

Figure 6 The effect of Notum on Wnt3a signalling in GPC3-expressing cells

HEK-293T cells were transiently transfected with expression vectors for GPC3, wtNotum or mutNotum, and β-galactosidase. Transfected cells were then treated with conditioned media from Wnt3a-transfected or vector control-transfected L-cells. Cells were then lysed, and the luciferase and β-galactosidase activities were measured. The β-galactosidase activity was used to normalize the luciferase values. Experiments were repeated three times in triplicate, and one representative experiment is shown. Each bar represents the mean+S.D. and is given in arbitrary units.

Figure 7 Expression of Notum and glypicans at various stages of embryonic development and in different tissues of the adult mouse

RNA was extracted from the indicated tissues and Notum, GPC3, GPC4, GPC5 and GPC6 mRNA levels were assessed by RT–PCR. Electrophoresis of the PCR products revealed bands with the expected sizes. Actin was used as a control for the amount and integrity of the RNA.

Table 1 [3H]Ethanolamine and [32P]Pi radioactivities in the precipitated uPAR

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<th>[3H]Ethanolamine radioactivity (c.p.m.)</th>
<th>[32P]Pi radioactivity (c.p.m.)</th>
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<tr>
<td>mutNotum</td>
<td>951 ± 58</td>
<td>289 ± 36</td>
</tr>
<tr>
<td>wtNotum</td>
<td>1482 ± 180</td>
<td>1017 ± 220</td>
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<tr>
<td>GPI-PLC</td>
<td>2143 ± 25</td>
<td>1830 ± 20</td>
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<td>[3H]Ethanolamine</td>
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whole embryos from different developmental stages. As shown in Figure 7, we found that Notum is expressed in lung, ovary, kidney, liver and brain. On the other hand, Notum was not detected in thymus, heart, spleen, stomach, skeletal muscle and bone marrow. In addition, we found that Notum is expressed in all the tested developmental stages. We also investigated the expression pattern of GPC3, GPC4, GPC5 and GPC6 in the same tissues. As shown in Figure 7, GPC3, GPC4 and GPC6 are expressed in almost all the tissues analysed. On the other hand, the pattern of expression of GPC5 is more restricted. There is no obvious correlation between the expression pattern of Notum and any of the glypicans.

Detection of endogenous Notum protein

By injecting rabbits with purified Notum, we were able to generate an anti-Notum polyclonal antibody. To verify the specificity of the antibody, we used it to perform a Western-blot analysis of HEK-293T cells that were transiently transfected with mouse Notum. Figure 8 shows that the antibody specifically detects a 59 kDa band, which corresponds to the predicted molecular mass of Notum. In addition, a smaller band, which most likely represents a degradation product, was also detected by the antibody. No bands were detected in vector-transfected HEK-293T cells, indicating that the antibody specifically detects Notum. The antibody can detect endogenous Notum in a Western-blot analysis of conditioned medium of Hep3B hepatocellular carcinoma cells (Figure 8A). The specificity of the antibody was also verified.
DISCUSSION

We have shown in the present study that mammalian Notum is able to release glypicans from the cell surface into the extracellular environment. In addition, we showed that mammalian Notum could also cleave other types of GPI-anchored proteins that have no functional or structural similarities to glypicans. It remains to be determined whether Drosophila Notum has a similar activity.

A very important finding of the present study is that Notum can cleave GPI-anchored proteins by acting in the extracellular environment and in the context of the intact cell membrane. Another mammalian enzyme that has been shown to be able to cleave GPI-anchored proteins at the anchor level is GPI-PLD. However, this enzyme seems to be active only in the intracellular environment, because it cannot release GPI-anchored proteins when added to intact cells [4,42]. It has been recently reported that the ACE (angiotensin-converting enzyme) is also able to cleave the lipid tails of GPI-anchored proteins [43]. However, the validity of this report has been questioned and it remains controversial [44]. The availability of an enzyme that can cleave GPI anchors at the level of the cell surface will provide a novel tool for the functional study of GPI-anchored proteins, and it may contribute to the understanding of various physiological events in which these proteins play important roles, including T-cell activation, cell adhesion and the signalling pathways triggered by various growth factors [4].

We have assessed the amount of [32P]Pi and [3H]ethanolamine incorporated into uPAR that was released by Notum and by GPI-PLC. Because the ratio between the two isotopes after each of the treatments was found to be similar (Table 1), we propose that Notum cleaves the GPI anchor between the inositol-bound phosphate and the membrane-bound lipid, as it has been reported for GPI-PLC.

In the study by Kreuger et al. [30], where Drosophila Notum activity was characterized, a significant increase in the SDS/PAGE mobility of Dlp was observed after cleavage of the GPI anchor by Notum. This mobility shift was also observed when Dlp was cleaved by GPI-PLC [30]. The change in mobility was larger than would be expected from the mass of the GPI anchor alone, but, as indicated by the authors, anomalous migration of proteins in SDS/PAGE has been previously observed following removal of GPI anchors. In the present study, we have not observed any significant shift in the mobility of any of the GPI-anchored proteins investigated, indicating that the loss of the GPI anchor in these proteins does not generate cleavage products with anomalous SDS/PAGE migration.

We showed here that Notum is able to cleave uPAR and T-cadherin, but not CEA. This suggests that this enzyme has some kind of specificity. The reasons for the resistance of CEA to Notum-induced cleavage are currently unknown. It has been previously reported that the modification of GPI anchors by acylation can inhibit the ability of GPI-PLC to cleave GPI-anchored proteins [39]. However, it seems unlikely that acylation of the GPI anchor in CEA is responsible for the resistance to Notum cleavage in HEK-293T cells, because we could release CEA from the same cells by using GPI-PLC.

We have demonstrated that the HS chains of GPC3 are not required for the Notum-induced cleavage. This is consistent with our finding that Notum can cleave proteins such as uPAR or T-cadherin, which do not display HS chains.

As discussed in the Introduction section, it is currently believed that glypicans can stimulate Wnt signalling by facilitating and/or stabilizing the interactions between Wnts and their signalling receptors (Frizzleds). This hypothesis is based on the finding that glypicans can interact with both Wnts and Frizzleds [19,20,22,24]. Our laboratory has recently reported that ectopic GPC3 stimulates Wnt signalling in HCC (hepatocellular carcinoma) cells [23]. However, the GPC3-induced stimulation of Wnt signalling was significantly reduced when the same HCC cells were transfected with a mutant form of GPC3 that lacks the GPI anchor domain and is consequently released into the conditioned medium. We have speculated that this effect is due to the fact that, contrary to cell-attached GPC3, secreted GPC3 acts as an inhibitor of Wnt-Frizzled interactions [23]. These observations are consistent with the results shown here demonstrating that ectopic Notum significantly reduces GPC3-induced stimulation of Wnt3a signalling in HEK-293T cells. The reduction in Wnt signalling produced by Notum was only partial. This partial effect is probably due to the fact that under our conditions, Notum is not releasing all the available GPC3 into the extracellular environment.

By using RT–PCR, we showed that Notum is expressed in various tissues in the adult mouse and at various stages of development, and is expressed in various tissues in the adult mouse and at various stages of development.
development. Because Notum has the potential to release several classes of GPI-anchored proteins, it remains to be investigated which are the in vivo substrates of Notum in the various tissues in which it is expressed. In this regard, we showed here that several glypicans are produced in tissues that express Notum. These glypicans should be considered, therefore, potential in vivo substrates of Notum. Finally, we have generated polyclonal antibodies that specifically detect endogenous Notum produced by cultured cells and normal liver.

In summary, our results suggest that mammalian Notum plays an important role in the regulation of signals from the extracellular environment by modulating the localization of glypicans and other GPI-anchored proteins.

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