Previous studies from our laboratory have found glypican 3 (GPC3) as a negative regulator of growth. CD81 was found to be a binding partner for GPC3, and its expression and co-localization with GPC3 increased at the end of hepatocyte proliferation. However, the mechanisms through which these two molecules might regulate liver regeneration are not known. We tested the hypothesis that GPC3 down-regulates the hedgehog (HH) signaling pathway by competing with patched-1 for HH binding. We found decreased GPC3-Indian HH binding at peak proliferation in mice followed by increase in glioblastoma 1 protein (effector of HH signaling). We performed a yeast two-hybrid assay and identified hematopoietically expressed homeobox (Hhex, a known transcriptional repressor) as a binding partner for CD81. We tested the hypothesis that Hhex binding to CD81 keeps it outside the nucleus. However, when GPC3 binds to CD81, CD81-Hhex binding decreases, resulting in nuclear translocation of Hhex and transcriptional repression. In support of this, we found decreased GPC3-CD81 binding at hepatocyte proliferation peak, increased CD81-Hhex binding, and decreased nuclear Hhex. GPC3 transgenic mice were used as an additional tool to test our hypothesis. Overall, our data suggest that GPC3 down-regulates cell proliferation by binding to HH and down-regulating the HH signaling pathway and binding with CD81, thus making it unavailable to bind to Hhex and causing its nuclear translocation. (Am J Pathol 2013, 183: 153–159; http://dx.doi.org/10.1016/j.ajpath.2013.03.013)

Glypican 3 (GPC3) is a heparan sulfate proteoglycan that is bound to cell surface by glycosylphosphatidylinositol.1 Loss-of-function mutations of GPC3 result in the Simpson-Golabi-Behmel syndrome, an X-linked disorder characterized by prenatal and postnatal organ overgrowth, including liver.2,3 CD81, also known as target of antiproliferative antibody, is a cell surface protein that belongs to tetraspanin 4 superfamily.1 It is involved in mediating signal transduction events that play a role in regulation of cell development, activation, growth, and motility.5,6 Our laboratory has previously demonstrated that GPC3 acts as a negative regulator of liver regeneration.7,8 This observation was accompanied by an increase in expression and co-localization of CD81 (a binding partner for GPC3) in tandem with GPC3 at the end of hepatocyte proliferation.7,8 However, the mechanisms and pathways through which these two molecules might regulate liver regeneration are not known.

In the present study, we sought to identify the mechanisms and potential associated partners through which these two proteins might mediate regulation of liver growth. Previous literature suggests that GPC3 can bind to hedgehog (HH) ligands and inhibit HH signaling during development.9 The HH signaling pathway has been previously reported to play an important role in liver regeneration after partial hepatectomy (PHx) in mice.10 We tested the hypothesis that GPC3 down-regulates the HH signaling pathway by competing with patched-1 for HH binding during liver regeneration (Figure 1). On the other hand, GPC3-HH binding decreases to free up HH signaling at the time of proliferation after PHx (Figure 1). To investigate the mechanisms for CD81’s role in regulating liver growth, we performed a yeast two-hybrid assay to identify potential binding partners for CD81. Hematopoietically expressed homeobox protein (Hhex) was a CD81 binding partner under high-stringency binding conditions. Most of the studies describing Hhex’s role are in liver development.11,12

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Hhex is expressed in some adult tissues, including liver, lung, spleen, thymus, and pancreas. Hhex mRNA has been previously detected in adult mouse liver and has been shown to be a transcriptional repressor. Its role in adult liver remains unexplored for the most part. Previous studies suggest that the role of Hhex is dependent on its shuttling between cytoplasm and nucleus. However, the mechanisms governing its translocation into the nucleus are not known. We hypothesized that Hhex binding to CD81 during hepatocyte proliferation keeps it outside the nucleus (Figure 1). When GPC3 binds to CD81, less CD81 is available for binding to Hhex, resulting in nuclear translocation of Hhex. Hence, GPC3 brings about increased nuclear GLI and decreased nuclear Hhex during cell proliferation. Smo, smoothened; TEM, tetraspanin enhanced microdomain.

Materials and Methods

**GPC3 TG Mice**

Generation of male GPC3 TG mice is described previously. These mice show significant over expression of GPC3 protein and mRNA compared with their WT littermates (friend leukemia virus B strain mice).

**Yeast Two-Hybrid Assay**

The Matchmaker GAL4 yeast two-hybrid system (Clontech, Mountain View, CA) was used for identification of CD81-binding partners according to standard protocol. As bait for screening, the vector pGBK7 expressing a fusion protein composed of full-length rat CD81 cDNA linked to the DNA-binding domain was constructed. Positive clones were selected on SD/-Ade/-His/-Leu/-Trp high-stringency YPDA (yeast, peptone, dextrose, adenine) plates with X-Gal. Plasmids from positive clones were subsequently isolated from the yeast, transferred to Escherichia coli Top10 competent cells (Invitrogen, Grand Island, NY), and sequenced. The GenBank/National Center for Biotechnology Information databases were screened for similar sequences using BLAST search.

**Protein Analysis by Western Blot**

Nuclear and cytoplasmic extracts pooled from at least four mice per time point were prepared using NE-PER nuclear and cytoplasmic extraction kit according to the manufacturer’s protocol (catalog number 78833; Pierce Biotechnology, Rockford, IL). Solubilized protein was subjected to Western blot analysis as described previously. Membranes were stained with Ponceau stain and β-actin (for nuclear and cytoplasmic extracts, respectively) to verify equal loading of total protein and transfer efficiency. Horseradish peroxidase–conjugated secondary antibodies were used at a 1:50,000 dilution (Jackson Immunoresearch Laboratories, West Grove, PA). Primary antibodies used with their concentrations were as follows: GPC3 (ARP37665 Aviva Systems Biology Corp., San Diego, CA, 1:1000; SC10456, Santa Cruz Biotechnology, Dallas, TX, 1:200), CD81 (GTX75432 GeneTex, Irvine, CA, 1:1000; SC70803, Santa Cruz, 1:500), Indian HH (IHH) (ab52919, Abcam, Cambridge, MA, 1:5000), Smoothened (ab72130, Abcam, 1:2000), GLI1 (PAB10214, Abnova, Taipei City, Taiwan, 1:2000), patched-1 (ab39266, 1:250; sc6147, 1:250), and Hhex (CA1326, Cell Applications, San Diego, CA).

**70% PHx in Mice**

Isoflurane inhalation (Baxter, Deerfield, IL) was used to anesthetize mice. PHx was performed on at least four mice per group per time point as described previously. Liver tissue samples from GPC3 TG and WT (littermates) were collected on day 0 (baseline), day 2 (proliferation peak), and day 8 (past end of hepatocyte proliferation) after PHx for further analysis.

**IHC for GLI1**

Paraffin-embedded liver sections (4 μm thick) were used for immunohistochemical (IHC) staining. Antigen retrieval was achieved by steaming the slides in target retrieval buffer (Dako, Carpinteria, CA) for 20 minutes. The tissue sections were blocked in protein block for 20 minutes followed by incubation with primary antibody (1:250) for 1 hour at room temperature. The primary antibody was then linked to biotinylated secondary antibody (1:250) followed by routine avidin-biotin complex method. Diaminobenzidine was used as the chromogen, which resulted in a brown reaction product.
Co-Immunoprecipitation

Mouse liver tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer with inhibitors. For immunoprecipitation studies, 500 μg of protein lysates pooled from at least four mice per group per time point were diluted to a final volume of 500 μL and precleared by incubating with IgG from the source of primary antibody and 20 μL of agarose A/G plus beads (Santa Cruz) for 1 hour at room temperature. Protein complexes were immunoprecipitated from cleared lysates with primary antibodies overnight at 4°C, followed by 2-hour incubation at 4°C with agarose A/G plus beads. Immune complexes were then collected and washed three times with RIPA buffer containing protease and phosphatase inhibitors (Sigma, St. Louis, MO) before resuspension in 4× SDS-PAGE sample buffer and 10× reducing reagent (Invitrogen). Proteins from immunoprecipitated samples or 30 μg of crude liver protein were separated by SDS-PAGE and subjected to Western blotting. Note that the Western blots of proteins that are pulled down using this technique are not reflective of their absolute expression levels (that require equal loading). Our analyses in this article rely on the ratios of bound protein to pulled down protein, which reflect changes in protein interactions.

GST Pull-Down Assay

To confirm Hhex as a true binding partner for CD81 from the yeast two-hybrid assay, we performed a glutathione S-transferase (GST) pull-down assay. Hhex-GST fusion protein or GST protein (control) from Proteintech (Chicago, IL) (catalog number ag13423) was used for the pull-down assay. The fusion protein was first dialyzed using a 10-kDa membrane cup to get rid of residual GSH (approximately 330 Da) that could interfere with the assay. Total cell lysate (500 μg) pooled from four WT mice was diluted in 500 μL of RIPA buffer with protease and phosphatase inhibitors (Sigma). GSH resin (100 μL; Thermo Scientific, Tewksbury, MA) was applied to spin cups, centrifuged, and washed using manufacturer instructions. Hhex-GST fusion protein or GST protein was applied to the spin cups immediately followed by pooled whole cell lysate from WT mice livers. The cup was sealed, vortexed, and incubated at 4°C by gentle rocking for an hour. The spin cups were centrifuged and washed three times. Protein was eluted using 2× sample buffer mixed with β-mercaptoethanol. The elute was used for Western blot analysis to detect Hhex and GST to confirm pull down. The same membrane was stripped (Sigma stripping buffer) and reprobed for CD81.
Statistical Analysis

Data are expressed as means ± SEM. Statistical differences were determined by one-way analysis of variance followed by Tukey’s honestly significant difference test to determine which means were significantly different from each other or from controls using the JMP software version 8 (SAS Institute Inc., Cary, NC). The criterion for significance was $P < 0.05$.

Results

GPC3 Affects the HH Signaling Pathway

Pull down using anti-IHH antibody co-immunoprecipitated GPC3 and patched-1, confirming previous reports that IHH is a binding partner for GPC3 (Figure 2A). The amount of GPC3 and patched-1 that bound to IHH at various time points after PHx normalized to the total amount of IHH pulled down is shown in Figure 2B. WT mice had a significant decrease in GPC3-IHH binding on day 2 after PHx (peak of proliferation) compared with day 0, thus making more IHH available for binding to patched-1 for HH signaling. This finding corroborated the increased patched-IHH binding in WT mice on day 2 (Figure 2, A and B). In contrast to this, GPC3 TG mice (exhibiting lower hepatocyte proliferation) failed to show a decrease in GPC3-IHH binding on day 2 after PHx compared with day 0. A corresponding increase in patched-IHH binding on day 2 was not statistically significant (Figure 2B). Moreover, there was significantly more GPC3 and less patched-1 bound to IHH in TG mice compared with WT mice on day 2. These data suggested a failure in induction of HH signaling pathway in the TG on day 2. Nuclear GLI1, an effector transcription factor of HH signaling pathway, was significantly induced on day 2 in WT mice compared with day 0 (Figure 2, C and D). In contrast, the TG mice had a significant decrease in GLI1 compared with day 0 for TG mice and day 2 for WT mice (Figure 2, C and D). Moreover, GLI1 was also down-regulated in TG mice on day 2 after PHx by IHC (Figure 3). Smoothened (an intracellular mediator of HH signaling) was unchanged in WT mice after PHx, whereas GPC3 TG mice had a mild decrease that was not significant (Figure 2, C and E). Overall, the HH signaling pathway appeared to be up-regulated in WT mice after PHx, whereas it was down-regulated in the GPC3 TG mice.

GPC3-CD81 Binding Changes After PHx

Confirming previous findings from our laboratory in the rat, we found that GPC3 binds to CD81 by co-immunoprecipitation in mouse as well (Figure 4A). The amount of CD81 bound to GPC3 at various time points after PHx normalized to the total amount of GPC3 pulled down is shown in Figure 4B. There was significantly more CD81 bound to GPC3 in TG mice compared with WT mice at
all time points (Figure 4, A and B). Within the group (WT and TG mice), the amount of CD81 bound to GPC3 significantly decreased on day 2 (proliferation peak) and increased by day 8 (past end of hepatocyte proliferation). These data support our previous report in rats\(^7\) that GPC3 and CD81 binding increases toward the end of hepatocyte proliferation. Figure 4A shows lower GPC3 in WT and TG mice on day 8 after PHx. This finding is most likely due to unequal loading (loss of GPC3 pulled down during perfor-
mance of the technique) and not because of less absolute GPC3 expression at this time point. However, because we are analyzing CD81 bound to pulled down GPC3 (ratio), lower GPC3 on day 8 does not affect our interpretation.

**Yeast Two-Hybrid Assay**

Hhex came out as one of the binding partners of CD81 under highest-stringency conditions (YPDA plates with SD/-Adc/-His/-Leu/-Trp). Other proteins identified were secreted phosphoprotein 2, terminal uridyl transferase 1, and HMG-CoA synthase 2 (mitochondrial). We chose to pursue Hhex for its role as a transcriptional repressor. To confirm Hhex as a binding partner for CD81, we performed two additional techniques: HhEX-GST pull-down assay and co-immunoprecipitation with CD81.

**Hhex-GST Pull-Down Assay**

To confirm Hhex as a binding partner for CD81 in mouse liver, we performed a GST pull-down assay. For the yeast two-hybrid assay, CD81 cDNA was our bait protein. In the GST pull-down assay, we used Hhex-GST fusion protein or GST protein (control) as our bait and incubated it with pooled whole liver lysate. CD81 came down in the assay, confirming it as a binding partner (Figure 5, A and B). Lysates incubated with GST protein were unable to pull-down CD81 (Figure 5, C and D), confirming that CD81 is a specific binding partner for Hhex and not GST. Thus, using two completely different techniques and using both proteins as baits, we were able to show that CD81 and Hhex are binding partners for each other.

**CD81-Hhex Binding Changes After PHx**

To study the dynamics of CD81-Hhex binding during liver regeneration, we performed co-immunoprecipitation for the two proteins (Figure 6A). The amount of Hhex bound to CD81 at various time points was normalized to the total

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**Figure 5** Hhex-GST pull-down assay. **A:** Western blot analysis of pull-down of Hhex-GST fusion protein confirmed using anti-Hhex antibody. **B:** CD81 detected on the same membrane using anti-CD81 antibody, indicating it to be a binding partner for Hhex. **C:** GST protein detected after using GST protein instead of HHEX-GST fusion protein for the assay as a negative control. **D:** CD81 could not be detected on the same membrane, confirming the specificity of CD81 binding to Hhex.

**Figure 6** Interaction of CD81 with Hhex. **A:** Western blot of Hhex and CD81 after co-immunoprecipitation using anti-CD81 antibody in WT and GPC3 TG mice at days 0, 2, and 8 after PHx. **B:** Ratios of densitometry of bound Hhex normalized to the amount of CD81 pulled down during a time after PHx in WT and TG mice. **C:** Western blot of nuclear Hhex and Ponceau stain as loading control in WT and GPC3 TG mice during time after PHx. **D:** Densitometric analyses of nuclear Hhex normalized to Ponceau stain. \(^* P < 0.05\) versus the corresponding 0 hour time point. \(^\dagger P < 0.05\) versus WT mice at the same time point.
CD81 pulled down (Figure 6B). In WT mice, less Hhex was bound to CD81 on day 0 (Figure 6, A and B). There was a significant increase in bound Hhex on day 2 after PHx followed by its decrease to normal levels by day 8. In stark contrast to this pattern, in the TG mice, there was significantly higher Hhex bound to CD81 to start with on day 0, which decreased significantly on days 2 through 8. To test whether the amount of Hhex bound to CD81 (near the membrane) inversely correlated with the amount of nuclear Hhex (transcriptional repressor), we looked at nuclear Hhex by Western blot (Figure 6C) normalized to Ponceau stain for equal loading (Figure 6D). As expected, nuclear Hhex was significantly lower in WT mice on day 2 compared with day 0. In contrast, it significantly increased in TG mice on day 2 compared with day 0. There was also significantly higher nuclear Hhex in TG versus WT mice at the peak of proliferation (day 2). These data support our hypothesis that the binding of CD81 to Hhex might keep it outside the nucleus and prevent its transcriptional repressor functions.

Expression of Patched-1 Receptor

Expression of patched-1 by hepatocytes is controversial. To test whether hepatocytes express patched-1, we perfused WT mouse liver and looked at patched-1 protein by Western blot analysis in hepatocyte pellet and nonparenchymal cell pellet (NPC) fraction using two different antibodies. We were able to detect a clear band with both the antibodies in whole liver lysates, hepatocyte pellet lysate, and NPC pellet lysate (Figure 7).

Discussion

GPC3 acts as a growth regulator in the absence of a known signaling domain in the cell membrane. GPC3 TG mice have inhibited liver regeneration response on day 2 after PHx compared with WT mice as assessed by Ki-67—positive nuclei. Our data indicate at least two different pathways through which GPC3 regulates growth. First, it binds to HH ligand (IHH) and down-regulates HH signaling in liver. Second, it binds to CD81, leading to decreased CD81-Hhex binding, which in turn causes nuclear translocation of transcriptional repressor Hhex. Whether treatment of TG mice with smoothened agonist can rescue this phenotype at least partly would be an interesting experiment. Inhibition of Hhex might not result in complete reversal of growth inhibitory effects of GPC3 because of GPC3-IHH interactions and functions of Hhex other than transcriptional repression. To our knowledge, this is the first study describing CD81 and Hhex to be binding partners for each other. We confirmed this using three different techniques in the present study.

Although our results suggest down-regulation of HH signaling in the liver as a whole, whether GPC3 can regulate HH signaling specifically in hepatocytes is not clear.

Previous studies have found that hepatocytes are not responsive to HH signaling because they do not express the HH receptor patched-1. Our data suggest that hepatocytes express patched-1 (Figure 7). Moreover, hepatocytes express GLI1 (Figures 2C and 3) and GLI2, the transcriptional effectors of HH signaling. We also saw GLI2 expression in WT and GPC3 TG mice by Western blot and IHC but did not see any change in them after PHx (data not shown).

Despite the fact that the GLI family of transcription factors are recognized as predominantly regulated by HH signaling, a few other studies have reported that GLI may also be activated independent of HH-patched-1—smoothened pathway through TGFβ and KRAS in pancreatic adenocarcinoma cells. Such an event has not been demonstrated in nonneoplastic cells. GPC3 TG mice have less GLI1 on day 2 after PHx compared with WT mice (Figures 2C and 3). This finding could be partially attributed to previous findings from our laboratory indicating more than threefold down-regulation of TGFβ gene in GPC TG mice compared with WT mice.

We chose to include GPC3 TG mice in this study to understand if and how overexpression of GPC3 changed the mechanisms for its growth regulatory function. Our data suggest that there were several differences in WT and GPC3 TG mice at baseline (day 0) that changed further after a regenerative stimulus (PHx). First, along with overexpression of GPC3 in TG mice, these mice exhibited a higher level of preparedness for proliferation as evidenced by increased GLI1 by Western blot and IHC (Figures 2C and 3), suggesting that the pathway is more activated in TG than in WT mice in resting livers. The HH signaling pathway has been reported to be involved in epithelial-to-mesenchymal transition and progenitor cell phenotype. We were not
able to detect any enrichment of progenitor cell population in these livers by microscopy, which looked pretty comparable to WT mice. However, we have not looked at specific markers of progenitor cells in the livers of GPC3 TG mice at day 0 to conclusively exclude the possibility. Second, nuclear Hhex (transcriptional repressor) was significantly less in TG mice resting liver compared with WT mice (Figure 6, C and D), again suggesting more preparedness for cell division in these TG mice. However, this preparedness for proliferation did not result in a higher rate of proliferation at baseline as evidenced by comparable Ki-67 staining in TG and WT mice at day 0 shown previously.8 These changes in protein expression in resting livers of TG mice are suggestive of adaptive changes due to gene modification. However, at day 2 after PHx (proliferation peak), TG mice were unable to induce the HH pathway sufficiently to mount a compensatory regeneration response possibly due to changes in GPC3-IHH, GPC3-CD81, and CD81-Hhex binding (an effect of GPC3 overexpression in these mice). The upstream factors that might influence the observed changes in GPC3 binding to its partners are not known and worthy of further investigation. This study demonstrates that changes in protein interactions (demonstrated by ratios of bound protein normalized to pulled-down protein), independent of their absolute levels, might influence regulation of growth.

Even though our study presents evidence that the HH system is operative in hepatocytes, our studies with whole liver homogenates encompass the entire population of hepatic cells (hepatocytes and nonparenchymal cells). In addition to the potential effects on hepatocytes, binding of GPC3 to HH ligands in the extracellular space can decrease the availability of HH ligands for patched-1 binding in HH responsive cells (stellate or endothelial) other than hepatocytes, also leading to an overall down-regulation of HH signaling pathway in the liver. Because hepatocyte proliferation after PHx is dependent on hepatocyte growth factor made by stellate cells, which are known to be HH responsive, GPC3-IHH binding could also affect hepatocyte proliferation in an indirect (paracrine) mechanism.

References