Loss of Gab1 adaptor protein in hepatocytes aggravates experimental liver fibrosis in mice

Takashi Kizu,¹* Yuichi Yoshida,¹* Kunimaro Furuta,¹ Satoshi Ogawa,¹ Norihiro Chatani,¹ Mina Hamano,¹ Takayo Takenura,¹ Hisa Ezaki,¹ Yoshihiro Kamada,¹ Keigo Nishida,² Yoshikazu Nakaoka,³ Shinichi Kiso,¹ and Tetsuo Takehara¹

¹Department of Gastroenterology and Hepatology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan; ²Laboratory for Homeostatic Network, RCI, RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa, Japan; and ³Department of Cardiovascular Medicine, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

Submitted 4 August 2014; accepted in final form 18 January 2015

LIVER FIBROSIS IS A MAJOR medical issue worldwide because it affects up to 3% of the world’s population and represents a serious medical burden due to its association with chronic liver disease, cirrhosis, and mortality. Although various growth factors are involved in liver fibrogenesis, the molecular mechanisms by which they affect liver fibrosis remain poorly understood. Gab1, a Grb2-associated binder 1, is a scaffolding adaptor protein that contains a PH domain and potential binding sites for SH2 and SH3 domains. Despite its wide expression in many cell types, the role of Gab1 in liver fibrosis remains unclear. Here, we investigated the role of hepatocyte Gab1 during liver fibrosis using a genetic approach and identified the molecular mechanisms by which Gab1 affects liver fibrosis using mouse models.

The effects of epidermal growth factor (EGF) family members on liver fibrosis have been shown to differ from one another in a mouse model of liver fibrosis (14, 28, 40). We and others recently showed that deletion of heparin-binding EGF enhances liver fibrosis (14, 40). In contrast, deletion of amphiregulin, another member of the EGF family, has been reported to improve hepatic fibrogenesis (28). Although these growth factors are implicated in liver fibrosis, the downstream signaling in hepatocytes remains poorly understood.

Grb2-associated binder 1 (Gab1) is a scaffolding adaptor protein that contains a PH domain and potential binding sites for SH2 and SH3 domains. It could be hypothesized that, because these various growth factors/RTKs are implicated in liver fibrosis, hepatocyte Gab1 might have a possible role in regulating liver fibrosis. However, until now, the role of hepatocyte Gab1 during liver fibrosis has remained unclear.

Here, we investigated the role of hepatocyte Gab1 during liver fibrosis using a genetic approach and identified the molecular mechanisms by which Gab1 affects liver fibrosis using mouse models.

MATERIALS AND METHODS

Mice. We previously described a generation of mice carrying a Gab1 gene with two loxP sequences flanking exon 2 (Gab1floxfloxflox) C57BL/6 mice (25). Hapten-specific Gab1 conditional knockout (Gab1Cko) mice were generated by crossing Gab1floxfloxflox C57BL/6 mice with albumin promoter-driven Cre recombinase (Alb-Cre) transgenic C57BL/6 mice (Jackson Laboratories). All of the animal experiments were approved by the Animal Care and Use Committee of Osaka University Medical School and were conducted according to institutional guidelines.

Polymerase chain reaction analysis for genotyping. Genomic DNA was extracted from mouse tails and subjected to polymerase chain reaction (PCR) to genotype the Gab1floxflox and Cre transgene. The primers used for the PCR of genomic DNA from mouse tails were as follows: Gab1floxflox: 5′-GGGCACTTCTTCTGCTCCTCACTATCTC-3′ and 5′-GTTGAAGGCGCATGCTGACGACGTC-3′; Cre:
**Bile duct ligation.** Control and Gab1CKO mice were subjected to bile duct ligation (BDL) or sham laparotomy (Sham) as described previously (10). Briefly, after midline laparotomy, the common bile duct was mobilized and ligated two times with 5-0 silk sutures and dissected between the ligatures in the BDL group. The operation was performed in the Sham group as in the BDL group, but without the ligation. At 5 or 10 days after BDL, the animals were killed for further analysis.

**Carbon tetrachloride-induced liver fibrosis model.** For the toxin-induced liver fibrosis model, we used chronic administration of carbon tetrachloride (CCL4) (34). Control and Gab1CKO mice were injected intraperitoneally with CCL4 (0.5 ml/kg body wt) or oil two times a week for 6 wk and killed at 24 h after final injection for further analysis.

**Determination of liver hydroxyproline content.** Wet liver samples (200 mg) were lyophilized at −40°C for 48 h. Freeze-dried liver samples were hydrolyzed in 6 N HCl at 100°C for 6 h. After filtration using a 0.22-μm membrane, 300 μl of the filtered solution were mixed with 5 μl of 1% phenolphthalein and 290–310 μl of 6 N NaOH until the indicator turned brown in color (pH 7–8). Forty milliliters of this solution were mixed with 25 ml of chloramine-T solution containing 0.56 mM chloramin-T (Sigma-Aldrich, St. Louis, MO) and 10% 2-propanol in chloramin-T buffer (50 g/l citric acid monohydrate, 12 g/l acetic acid, 120 g/l sodium acetate trihydrate, and 34 g/l sodium hydroxide). After this solution was allowed to stand for 20 min at room temperature, 150 μl of Ehrlich’s solution (2 g p-dimethylaminobenzaldehyde, 3 ml of 70% perchloric acid, and 9 ml of 2-propanol) were added, and the solution was incubated for 20 min at 65°C and another 5 min at room temperature. Absorbance was measured at 570 nm. The results are expressed as micrograms of hydroxyproline per gram of wet liver.

**Histological analysis.** Liver tissues were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned (4 μm thick), and stained with hematoxylin and eosin. The number of areas of oncogenic necrosis was counted per view field at ×100 magnification. Hepatocyte apoptosis was analyzed using a transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining kit (Milipore, Molsheim, France) according to the manufacturer’s protocol. The number of TUNEL-positive hepatocytes per view field at ×200 magnification was counted. To determine the proliferation rates of hepatocytes, liver sections were stained with anti-Ki-67 antibody (1:1,000; DAKO). The number of Ki-67-positive hepatocytes per view field at ×200 magnification was counted. To assess intrahepatic macrophage accumulation, liver sections were stained with anti-CD68 antibody (1:100; Serotec, Oxford, UK). Liver fibrosis was evaluated by measuring hepatic collagen deposition with picrosirius red staining. The fibrosis area stained by picrosirius red per view field at 200 magnification was quantified using image analysis software (Photo- shop; Adobe Systems, San Jose, CA). Activation of hepatic stellate cells was assessed by immunohistochemical staining with anti-smooth muscle actin (SMA) antibody (1:200; Abcam, Cambridge, MA). To evaluate the expression of CCL5 protein, liver sections were stained with anti-CCL5 antibody (1:500; BD, Heidelberg, Germany). For immunohistochemical staining, antibody-antigen complexes were detected with a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. A minimum of three view field views per tissue per mouse was counted for each analysis.

**Isolation and culture of mouse primary hepatocytes and nonparenchymal cells.** Hepatocytes and nonparenchymal cells (NPCs) were isolated from control and Gab1CKO mice by two-step pronase-collagenase perfusion of mouse liver as previously described (20). Briefly, after a cannula was inserted in the portal vein and the inferior vena cava was cut, the mouse liver was perfused with preperfusion solution for 5 min to remove mouse blood and protect hepatocytes. For digestion of mouse liver, the tissue was perfused with 53% pronase solution and 0.27% collagenase solution for 1 and 4 min, respectively. All solutions were warmed to 37°C before use and perfused at a flow rate of 4 ml/min. The perfused mouse liver was transferred into Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and minced to release the hepatocytes. The suspension was filtered through a 100-μm membrane and washed three times with centrifugation at 50 g for 1 min at 4°C. After aspiration of the supernatant, the cell pellet was suspended in William’s medium E with 10% fetal calf serum. Hepatocytes with viability of 90% or higher, as determined by trypan blue exclusion, were used for experiments. Hepatocytes (25,000 cells/cm²) were seeded on collagen-coated culture plates in William’s medium E with 10% fetal calf serum, 2 mM l-glutamine, 10-7 M insulin, 10-7 M dexamethasone, 100 μM penicillin, and 100 μM streptomycin. Hepatocytes were allowed to attach to plates overnight, and before treatment the cell culture medium was changed to an insulin-free medium. Isolated hepatocytes were stimulated with HGF (Sigma-Aldrich) for 15 min or with 50 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich) for 24 h, and then hepatocytes or the supernatant was harvested for further analysis.

**Western blot analysis.** Mouse liver samples or cells were homogenized or scraped off in RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, phosphatase inhibitor mixture, and complete protease inhibitor mixture (Roche Applied Science, Basel, Switzerland). Western blot analysis was performed as described previously (40). The following antibodies were used for immunodetection: anti-Phos- pho-Met (Tyr1234), anti-Met, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-Gab1 (Tyr627), and anti-Gab, purchased from Cell Signaling Technology (Danvers, MA), and anti-glycerol-3-phosphate dehydrogenase (GAPDH), purchased from Trevi- gen (Gaithersburg, MD).

**Real-time reverse-transcription polymerase chain reaction.** Total RNA was extracted using a QIAshredder and an RNAeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was carried out using the QuantiFast SYBRGreen RT-PCR kit (Qiagen) according to the manufacturer’s protocol. The Quantitect gene assay kit (Qiagen) was used for analysis of mouse Ccl2, Col1a1, Acta2, TGF-B1, IL-6, Ccl5, Ccl2, CD68, Cc15, and GAPDH. The primers 5′-AACGATCATGGCAAGGTGTAAT-3′ and 5′-TCGCTGTTGCGACATTGTTT-3′ were used to amplify and quantify Gab1 mRNA. The level of gene expression for each sample was normalized to GAPDH mRNA expression using the comparative cycle threshold method.

**Microarray analysis.** Gene expression in the mouse livers following BDL was analyzed using an Affymetrix Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA). Control and Gab1CKO mice were subjected to BDL or sham laparotomy and killed. Total RNA was extracted from the livers of control and Gab1CKO mice using a QIAshredder and an RNAeasy Mini kit (Qiagen) according to the manufacturer’s protocol. Total RNA samples from livers of three mice per experimental group were pooled and used for microarray analysis. Samples were hybridized on Affymetrix GeneChip Mouse Genome 430 2.0 arrays in the RIKEN Research Center for Allergy and Immunology. To identify the genes involved in liver fibrosis in Gab1CKO mice, we used a heatmap indicating the top 20 of the 787 genes and selected the genes that had greater than twofold induction in livers of Gab1CKO mice after BDL compared with control mice after BDL.

**Transcript profiling.** The microarray data can be accessed at RCGI REdDIC (URL: http://reddic.rcai.riken.jp/welcome.cgi) under the following accession numbers: RSM10654, RSM10655, RSM10656, and RSM10657.

**Enzyme-linked immunosorbent assay.** The expression levels of CCL5 in liver tissue and cultured supernatant were measured using a Mouse CCL5/RANTES Quantikine enzyme-linked immunosorbent assay.
Detection of NF-κB activity. Murine normal hepatocyte BNL CL.2 cells (ATCC, Manassas, VA) were transfected with 10 nM of either siGab1 or siControl using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions for reverse transfection. After 24 h of incubation, these cells were stimulated with 50 ng/ml of LPS (Sigma-Aldrich) for 60 min, and then nuclear protein was extracted to measure NF-κB activity. NF-κB activity was assessed by the p65 DNA-binding activity using a TransAM NF-κB Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol.

CCL5 antagonist treatment. Control and Gab1CKO mice were subjected to BDL and treated daily until death with intraperitoneal injections of either phosphate-buffered saline (PBS) or CCL5 antagonist (10 μg/mouse; Bachem, Philadelphia, PA), a selective antagonist for CCL5 receptors (29). The mice were divided into groups of eight for each treatment (PBS-treated control, CCL5-antagonist-treated control, PBS-treated Gab1CKO, and CCL5-antagonist-treated Gab1CKO). Animals were killed 10 days after the BDL procedure.

RESULTS

Generation of hepatocyte-specific Gab1 conditional knockout mice. Because the targeted disruption of mouse Gab1 results in embryonic death (15), we generated hepatocyte-specific Gab1CKO mice by crossing Gab1fl/fl control mice with hepatocyte-specific albumin promoter-driven Cre recombinase transgenic C57BL/6 mice (Fig. 1A). Western blot analysis showed that the expression level of Gab1 was dramatically decreased in the liver of Gab1CKO mice but not in other organs (Fig. 1B). We also demonstrated that the deficiency of Gab1 was specific to hepatocytes and was not observed in NPCs (Fig. 1C). Furthermore, we showed that the HGF-induced tyrosine phosphorylation of Gab1 was lost in cultured hepatocytes from Gab1CKO mice (Fig. 1D). The functional effect of this deletion was confirmed by demonstrating that HGF-induced activation of ERK, a downstream target of Gab1 (39), was exclusively attenuated in Gab1CKO hepatocytes (Fig. 1D).

Involvement of Gab1 during liver fibrosis after BDL. To investigate the role of Gab1 during liver fibrosis, we first examined the phosphorylation of Gab1 in the livers after BDL. Western blot analysis showed that Gab1 was tyrosine phosphorylated in the livers of control mice but not that of Gab1CKO mice after BDL (Fig. 2). These results indicated the possible involvement of Gab1 during liver fibrosis.

Hepatocyte Gab1 is required for the protection against liver fibrosis progression after BDL. To elucidate the role of Gab1 in liver fibrosis, we performed BDL or a sham operation (Sham) in control and Gab1CKO mice. Ten days after BDL, Gab1CKO mice displayed a significant increase in the areas of liver fibrosis between portal tracts compared with control mice.
Gab1CKO mice also showed a significant increase in liver fibrosis compared with control mice 14 days after BDL (data not shown). The enhanced liver fibrosis in the Gab1CKO mice was confirmed by significantly increased levels of hepatic hydroxyproline in the livers of Gab1CKO mice compared with control mice (Fig. 3C). Furthermore, the mRNA expression levels of profibrogenic markers, such as Col1α1, Col1α2, TGF-β1, and Acta2, were significantly increased in the livers of Gab1CKO mice compared with control mice (Fig. 3D). Consistent with the Acta2 mRNA result, immunohistochemical analysis confirmed that Gab1CKO mice exhibited a clear increase in α-SMA-positive areas. This result indicates enhanced activation of hepatic myofibroblasts, which are major producers of fibrotic extracellular matrix (ECM; Fig. 3E). These results indicate that hepatocyte Gab1 has a protective role against liver fibrosis progression.

**Enhanced liver fibrosis in Gab1CKO mice after the chronic administration of CCl4.** To further confirm the role of Gab1 in liver fibrosis using another well-established mouse model of liver fibrosis, we administered CCl4 to control and Gab1CKO mice. The results are shown in Fig. 2 and Table 1. As expected, the administration of CCl4 significantly increased the levels of hepatic hydroxyproline in the livers of Gab1CKO mice compared with control mice (Fig. 2C). Moreover, the mRNA expression levels of profibrogenic markers, such as Col1α1, Col1α2, TGF-β1, and Acta2, were significantly increased in the livers of Gab1CKO mice compared with control mice (Fig. 2D). Consistent with the Acta2 mRNA result, immunohistochemical analysis confirmed that Gab1CKO mice exhibited a clear increase in α-SMA-positive areas. This result indicates enhanced activation of hepatic myofibroblasts, which are major producers of fibrotic extracellular matrix (ECM; Fig. 2E). These results indicate that hepatocyte Gab1 has a protective role against liver fibrosis progression.

Fig. 2. Involvement of Gab1 during liver fibrosis after bile duct ligation (BDL). Tyrosine phosphorylation of Gab1 in the livers of control and Gab1CKO mice 5 and 10 days after BDL.

Fig. 3. Enhanced progression of liver fibrosis in hepatocyte-specific Gab1CKO mice after BDL. A: representative views of Sirius red staining in liver sections 10 days after BDL (n = 9 control, n = 10 Gab1CKO) or Sham (n = 5 control, n = 5 Gab1CKO). Top: Sham; middle, BDL; bottom, BDL (enlarged view of boxed region in middle). Scale bars: 400 μm. B: quantification of Sirius red-positive areas in liver sections. C: determination of hydroxyproline content in livers. D: gene expression of Col1α1, Col1α2, TGF-β1, and Acta2 in livers. E: representative views of α-smooth muscle actin (SMA) staining in liver sections. Top: Sham; middle, BDL; bottom, BDL (enlarged view of boxed region in middle). Scale bars: 200 μm. Data are means ± SE. *P < 0.05 and **P < 0.01.
liver fibrosis, we chronically administered CCl₄ to control and Gab1CKO mice. After 6 wk of CCl₄ administration, Gab1CKO mice showed significant increases in Sirius red-positive areas and hepatic hydroxyproline content compared with wild-type (WT) mice (Fig. 4, A–C). Quantitative real-time RT-PCR analysis also demonstrated a significant increase in the mRNA expression level of profibrogenic markers, such as Col1α1, Col1α2, TGF-β, and Acta2, in KO mice compared with WT mice (Fig. 4D). Furthermore, KO mice exhibited enhanced activation of hepatic myofibroblasts, as demonstrated by an increase in α-SMA-positive areas (Fig. 4E). These data confirmed the antifibrotic role of hepatocyte Gab1 in liver fibrosis. Gab1CKO mice showed exacerbation of liver injury, impaired hepatocyte proliferation, and enhanced liver inflammation after BDL. Because oncotic necrosis and apoptosis are characteristic features of hepatocellular injury following BDL (10), we evaluated liver injury by quantifying oncotic necrotic areas and hepatocyte apoptosis in liver sections. Five days after BDL, Gab1CKO mice showed a significant increase in the number of oncotic necrotic areas and apoptotic hepatocytes compared with control mice (Figs. 5, A–C). The enhanced liver injury observed in Gab1CKO mice was confirmed by increased levels of serum alanine transaminase (ALT; Fig. 5D). Furthermore, we demonstrated that cholestasis was unchanged be-

---

![Image](http://example.com/fig4.jpg)

**Fig. 4.** Enhanced progression of liver fibrosis in hepatocyte-specific Gab1CKO mice after the chronic administration of carbon tetrachloride (CCl₄). A: representative views of Sirius red staining in liver sections of control and Gab1CKO mice after the chronic administration of CCl₄ and oil. Control and Gab1CKO mice were subjected to intraperitoneal injections of CCl₄ (0.5 ml/kg body wt, n = 10 control, n = 11 Gab1CKO) or oil (n = 5 for control, n = 5 for Gab1CKO) two times per week for 6 wk and were killed 24 h after the final injection. Top, oil; middle, CCl₄; bottom, CCl₄ (enlarged view of boxed region in middle). B: quantification of Sirius red-positive areas in liver sections. C: determination of hydroxyproline content in the liver. D: gene expression of Col1α1, Col1α2, TGF-β1, and Acta2 in livers. E: representative views of α-SMA staining in liver sections. Top, oil; middle, CCl₄; bottom, CCl₄ (enlarged view of boxed region in middle). Scale bars: 200 μm (A and E). Data are means ± SE. *P < 0.05 and **P < 0.01.
These data indicate that loss of Gab1 in hepatocytes affected sensitivity of liver injury induced by cholestasis. We also assessed hepatocyte proliferation in control and Gab1CKO mice following BDL because surviving hepatocytes undergo a complementary proliferative response during cholestasis (10). Staining of liver sections with Ki-67 indicated a decreased number of proliferating hepatocytes in Gab1CKO mice compared with control mice (Fig. 5E). These data suggested that hepatic loss of Gab1 resulted in enhanced liver injury with decreased complementary hepatocyte proliferation during cholestasis.

Macrophages are known to play a major role in liver inflammation during the progression of liver fibrosis (35). We therefore examined macrophage infiltration in the livers of control and Gab1CKO mice by immunohistochemical staining for CD68, which is a surface marker for macrophages. After BDL, Gab1CKO mice exhibited a clear increase in the number of CD68-positive cells in the liver compared with control mice (Fig. 5F). Quantitative real-time RT-PCR analysis also confirmed a significant increase in the hepatic mRNA expression levels of CD68 in Gab1CKO mice (Fig. 5G). Furthermore, the mRNA expression levels of the proinflammatory cytokine IL-6 and the proinflammatory chemokine Ccl2 were also significantly higher in the livers of Gab1CKO mice (Fig. 5G). These data indicated that the hepatocyte-specific deletion of Gab1 led to an enhancement of liver inflammation during cholestasis.

Microarray analysis identifies upregulation of CCL5 gene in the livers of Gab1CKO mice during fibrosis after BDL. To investigate Gab1-mediated downstream signaling during the

---

**Fig. 5.** Exacerbation of liver injury, impaired hepatocyte proliferation, and enhancement of liver inflammation in hepatocyte-specific Gab1CKO mice after BDL. A: representative hematoxylin and eosin staining of liver sections 5 days after BDL (n = 9 control, n = 11 Gab1CKO) or Sham (n = 5 control, n = 5 Gab1CKO). Scale bars: 400 μm. B: quantification of oncocytic necrotic areas in liver sections. C: quantification of the no. of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive hepatocytes in liver sections. D: serum levels of alanine transaminase (ALT), total bilirubin, and alkaline phosphatase (ALP). E: quantification of the no. of Ki-67-positive hepatocytes in liver sections. F: representative CD68 staining of liver sections. Scale bars: 100 μm. G: gene expression of CD68, IL-6, and Ccl2 in livers. Data are means ± SE. ns, Not significant. *P < 0.05 and **P < 0.01.
progression of liver fibrosis, we next performed cDNA mi-
croarray analysis using total RNA from the livers of control
and Gab1CKO mice 10 days after BDL. cDNA microarray
analysis revealed the induction of 787 genes in the livers of
Gab1CKO mice compared with control mice after BDL.
Among these genes, Ccl5 (also known as RANTES) was
identified as strongly upregulated only in the livers of
Gab1CKO mice after BDL (Fig. 6A). We focused on CCL5
because it has been shown to have fibrosis-promoting activity
(4). Further validation by quantitative real-time RT-PCR dem-
onstrated an approximate fivefold increase in Ccl5 mRNA levels
in Gab1CKO mice when compared with control mice 10 days after
BDL (Fig. 6B). To identify the hepatic cells in which CCL5 was
upregulated in Gab1CKO mice, we isolated hepatocytes and
NPCs by two-step pronase collagenase perfusion of the livers of
control and Gab1CKO mice 10 days after BDL. CCL5 gene
expression was significantly higher in the hepatocytes of
Gab1CKO mice than in the hepatocytes of control mice (Fig. 6C).
Conversely, Ccl5 gene expression in NPCs did not significantly
differ between control and Gab1CKO mice (Fig. 6D).

Loss of Gab1 upregulates synthesis of CCL5 protein in
hepatocytes during liver fibrosis after BDL. Staining of liver
sections with CCL5 revealed increased CCL5 protein expres-
sion in the hepatocytes of Gab1CKO mice compared with
control mice (Fig. 7A). Quantification of the immunohisto-
chemical staining confirmed that the number of CCL5-positive
hepatocytes in Gab1CKO mice was significantly higher than
that in control mice (Fig. 7B). In contrast, the number of
CCL5-positive cells in NPCs did not differ between the two
groups after BDL (Fig. 7C). To assess the CCL5 protein
expression level in a more quantitative manner, we measured
the concentration of hepatic CCL5 by ELISA and confirmed
that CCL5 expression was significantly higher in the livers of
Gab1CKO mice than in those of control mice 10 days post-
BDL (Fig. 7D). These findings suggest that hepatic deletion
of Gab1 upregulates protein synthesis of CCL5 in hepatocytes at
the fibrosis stage after BDL.

Loss of Gab1 in hepatocytes enhances NF-κB activation and
CCL5 synthesis in hepatocytes in vitro. To confirm the in vivo
data showing CCL5 upregulation in the livers of Gab1CKO mice
after BDL, we performed in vitro experiments using primary
mouse hepatocytes isolated from control and Gab1CKO mice.
Because it has been reported that LPS is involved in the progress-
ion of liver fibrosis (35), we stimulated primary hepatocytes from
control and Gab1CKO mice with LPS for 24 h and analyzed
CCL5 gene and protein expression. Quantitative real-time RT-
PCR demonstrated significantly higher Ccl5 mRNA expression
in Gab1CKO hepatocytes than in control hepatocytes (Fig. 8A).
Likewise, CCL5 protein expression in the culture supernatants
of Gab1CKO hepatocytes was significantly increased com-
pared with control hepatocyte culture supernatants (Fig. 8B).
Because CCL5 gene expression is regulated by transcription
factors, including NF-κB (26), we further examined the role of
Gab1 on LPS-induced NF-κB activation using a normal murine
hepatocyte cell line, BNL CL.2, in vitro. Consistent with the in
vivo data, siRNA-mediated depletion of Gab1 enhanced LPS-
duced NF-κB activation in BNL CL.2 cells (Fig. 8, C and D).
These data indicate that loss of Gab1 stimulates NF-κB signal-
ning and subsequent CCL5 synthesis from hepatocytes in
vitro.

Pharmacological antagonism of CCL5 reduces liver fibrosis
after BDL in Gab1CKO mice. To clarify the functional role of
CCL5 overexpression, control and Gab1CKO mice were sub-
jected to daily intraperitoneal injections of a pharmacological
CCL5 antagonist (29) or PBS after BDL. Ten days after BDL,
the daily administration of the CCL5 antagonist had signifi-

A

Sham BDL

Gene Symbol

Affymetrix

Dmbt1

1418287_a_at

Gb1p1

1420549_at

Ccl5

1418126_at

Camp

1419691_at

Ngp

1418722_at

Ms4a4b

1423467_at

Ch131

1451537_at

Gbp2

1418240_at

Fam26f

1436576_at

Gzma

1417898_a_at

Cilp

1457296_at

Gdpb3

1449526_a_at

Igtp

1417141_at

Gdp2

1435906_x_at

Entpd1

1422326_at

Ly6a

1417165_at

Lair1

1439067_at

Trem2

1421792_s_at

Ccl24

1450488_at

Hk3

1435490_at

B

Control

Gab1 CKO

C

D

Sham BDL

CCL5 mRNA

fold changes

Sham BDL

CCL5 mRNA

fold changes

Sham BDL

CCL5 mRNA

fold changes

Sham BDL

Non-parenchymal cells

* n.s.

Fig. 6. Microarray analysis reveals upregulation of CCL5 gene in the livers of hepatocyte-spe-
cific Gab1CKO mice after BDL. A: heatmap analysis revealed upregulation of Ccl5 mRNA
in the livers of Gab1CKO mice 10 days after BDL. B: gene expression of Ccl5 in the livers
after BDL (n = 9 control, n = 10 Gab1CKO) or Sham (n = 5 control, n = 5 Gab1CKO). C: gene
expression of CCL5 in isolated hepatocytes. D: gene expression of CCL5 in isolated nonparenchymal
cells. Data are means ± SE. *P < 0.05.

Fig. 7. A: Change in CCL5-positive cell number
in the liver sections from control and Gab1CKO
mice 10 days after BDL (n = 5 control, n = 5
Gab1CKO). B: ELISA analysis of the concentration
of hepatic CCL5 in the livers of control and
Gab1CKO mice 10 days after BDL (n = 5 control,
n = 5 Gab1CKO). Data are means ± SE.*P < 0.05.

Fig. 9. Pharmacological antagonism of CCL5
reduces liver fibrosis after BDL in Gab1CKO mice.
To clarify the functional role of CCL5 overexpres-
sion, control and Gab1CKO mice were subjected to
daily intraperitoneal injections of a pharmacological
CCL5 antagonist (29) or PBS after BDL. Ten days after BDL,
the daily administration of the CCL5 antagonist had signifi-

G619ROLE OF HEPATOCYTE Gab1 DURING LIVER FIBROSIS

AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00289.2014 • www.ajpgi.org
cantly improved liver fibrosis in Gab1CKO mice, as demonstrated by measurements of Sirius red-positive areas, compared with the PBS treatment (Fig. 9, A and B). This improvement in liver fibrosis in the Gab1CKO mice upon daily administration of the CCL5 antagonist was confirmed by the measurement of hepatic hydroxyproline content (Fig. 9C). These data indicate that hepatic overexpression of CCL5 in Gab1CKO mice functioned to exacerbate liver fibrosis.

Fig. 7. Loss of Gab1 upregulates protein synthesis of CCL5 in hepatocytes during liver fibrosis after BDL. A: representative CCL5 staining of liver sections 10 days after BDL (n = 9 control, n = 10 Gab1CKO) or Sham (n = 5 control, n = 5 Gab1CKO). Top, Sham; middle, BDL; bottom, BDL (enlarged view of boxed region in middle). Scale bars: 50 μm. B: quantification of CCL5-positive hepatocytes. C: quantification of CCL5-positive nonparenchymal cells. D: quantification of CCL5 protein levels in whole liver extracts. Data are means ± SE. *P < 0.05 and **P < 0.01.

Fig. 8. Loss of Gab1 in hepatocytes enhances NF-κB activation and CCL5 synthesis in hepatocytes in vitro. A: gene expression of Ccl5 in cultured primary hepatocytes from control and Gab1CKO mice in response to lipopolysaccharide (LPS, n = 8/group). B: secretion of CCL5 protein in the supernatant of cultured primary hepatocytes. C: activation of NF-κB activation in Gab1-depleted BNL CL.2 cells in response to LPS (n = 4/group). D: Western blot confirms the depletion of Gab1 in siRNA-treated BNL CL.2 cells. Data are means ± SE. **P < 0.01.
DISCUSSION

To our knowledge, the present study is the first to reveal that hepatocyte Gab1 plays a crucial role in liver fibrosis. In this study, we demonstrated that the hepatocyte-specific deletion of Gab1 resulted in enhanced liver fibrosis in a mouse model of cholestasis. We also observed similarly enhanced liver fibrosis in hepatocyte-specific Gab1-KO mice in a model of liver fibrosis induced using the chronic administration of the toxin CCl4 (Fig. 4, A–E), suggesting that Gab1 plays an antifibrotic role in hepatocytes irrespective of the etiology of the chronic liver disease.

Gab1 belongs to the Gab/Daughter of Sevenless (DOS) family of adaptor molecules (12, 24, 27), and the Drosophila homolog of Gab1, DOS, has been shown to act downstream of several RTKs, such as Sevenless, Torso, and the EGFRs (30). Like DOS, Gab1 has also been shown to be tyrosine phosphorylated in response to growth factors/RTKs and is able to amplify signals downstream (25, 27, 45). In this study, we demonstrated that Gab1 was tyrosine phosphorylated in response to the induction of liver fibrosis (Fig. 2). Indeed, we observed that BDL induced the gene expression of several ligands for RTKs in our model, including HGF and EGF family members (data not shown). These findings indicate that growth factor/RTK/Gab1-mediated signals are involved in the progression of liver fibrosis.

To investigate the potential molecular mechanisms of the Gab1-mediated regulation of liver fibrosis in hepatocytes, we performed cDNA microarray analysis using total RNA obtained from the livers of control and Gab1CKO mice 10 days after BDL. We found that several genes, including Gbp2 (11), Fam26f (8), Igtp (41), Ly6a (13), and CCL5 (1), were upregulated in the livers of Gab1CKO mice. Among these genes, the functions of Gbp2, Fam26f, Igtp, and Ly6a in liver fibrosis have remained unclear to date (6–8, 11, 13, 17, 19, 41). Our validation study revealed no statistically significant difference in the expression of these genes between control and Gab1CKO mouse livers 10 days after BDL (data not shown). In contrast, the gene expression of CCL5 was statistically...
significantly upregulated only in the livers of Gab1CKO mice after BDL (Fig. 6, A and B). CCL5 (also known as RANTES) belongs to the C-C chemokine family, which has powerful chemoattractant effects on a variety of cell types (1). Importantly, in experimental mouse models, CCL5 and its receptors have been shown to promote liver fibrosis by increasing the migration, proliferation, and ECM production of hepatic stellate cells (4, 34). In this study, we demonstrated that Ccl5 gene expression was increased in the hepatocytes of Gab1CKO mice but not in NPCs after BDL (Fig. 6, C and D). Our immunohistochemical analysis and in vitro studies using primary hepatocytes isolated from control and Gab1CKO mice also showed loss of Gab1 in hepatocyte-upregulated CCL5 synthesis after BDL (Fig. 7, A–D). These data suggest that hepatocytes are the main source of CCL5 in our Gab1CKO mice. In contrast, a previous report showed that the main source of CCL5 in the liver was considered to be NPCs (4). Indeed, we detected CCL5-positive NPCs in control mice and Gab1CKO mice, but the CCL5-positive cells in the liver of Gab1CKO mice were primarily hepatocytes (Fig. 7, A–C). Consistent with this finding, recent publications have also demonstrated that CCL5 is produced from hepatocytes in mice and humans (23, 33). In addition, our results are in accordance with another study using hepatocyte-specific c-Met KO mice (23) in which Marquardt et al. performed cDNA microarray analysis of hepatocytes isolated from WT and c-Met-KO livers during the early fibrotic response and identified Ccl5 as an upregulated gene in the hepatocytes of c-Met KO mice (23). Together, these findings support that an increase in hepatocyte CCL5 synthesis is involved in the enhanced liver fibrosis observed in our Gab1CKO mice.

CCL5 expression is known to be regulated by transcription factors, including NF-κB (26). In addition, a previous study provided an important link between hepatocyte NF-κB activation and liver fibrosis development (38). Furthermore, a very recent report demonstrated an important role of hepatocyte Toll-like receptor 4 (TLR4) in obesity-induced inflammation in mice (16). Based on these findings, we performed in vitro experiments using primary hepatocytes isolated from control and Gab1CKO mice to investigate the possibility of a molecular link between the deletion of Gab1 and abnormal CCL5 synthesis in hepatocytes. To activate the TLR4-NF-κB pathway in hepatocytes, we stimulated primary hepatocytes from control and Gab1CKO mice in vitro with LPS, a ligand for TLR4, and analyzed the gene and protein expression levels of CCL5. Consistent with the in vivo data, the gene and protein expression levels of CCL5 in response to LPS were significantly increased in Gab1CKO hepatocytes (Fig. 8, A and B). As expected, siRNA-mediated depletion of Gab1 resulted in enhanced LPS-induced NF-κB activation in mouse hepatocyte cell lines (Fig. 8, C and D). In addition, we confirmed that gene expression of other potential NF-κB targets, including TNF-α and IL-6, was increased in hepatocytes of Gab1CKO mice stimulated with LPS when compared with control mice (data not shown). Considering these findings, our in vitro data indicate that loss of hepatocyte Gab1 enhances NF-κB activation and increases subsequent CCL5 production in hepatocytes during liver fibrosis, leading to the exacerbation of liver fibrosis. Several factors might damage the hepatocytes during liver fibrosis in vivo. For example, serum bile acid levels are reported to be elevated in patients with chronic hepatitis C and positively correlated with the degree of liver fibrosis (36), so factors including bile acid other than LPS might damage hepatocytes and then stimulate expression of CCL5 from Gab1-deficient hepatocytes in vivo. Further study is needed to shed light on this issue.

Finally, to elucidate the functional role of abnormal CCL5 overexpression, we administered a pharmacological CCL5 antagonist during fibrosis progression in Gab1CKO mice after BDL. As expected, administration of the CCL5 antagonist significantly improved liver fibrosis in Gab1CKO mice but not in control mice after BDL (Fig. 9, A–C). This result might be caused by the difference in the induction of CCL5 expression between Gab1CKO mice and control mice after BDL (Fig. 6B). In contrast, the CCL5 antagonist did not affect liver injury as assessed by areas of oncocytic necrosis, apoptotic hepatocytes, and serum ALT levels in Gab1CKO mice (data not shown). These data indicate that overexpression of hepatocyte CCL5 in our Gab1CKO mice functioned to exacerbate liver fibrosis. In addition, our data showed that antagonism of CCL5 did not completely abolish the exacerbation of liver fibrosis observed in Gab1CKO mice, suggesting that there are other possible mechanisms to explain the enhanced liver fibrosis (Fig. 9, A–C). Because Gab1CKO mice showed exacerbated liver injury, inflammation, and decreased hepatocyte proliferation after BDL (Fig. 5, A–G), the genes related with the aforementioned pathways, in addition to CCL5, might contribute to exacerbation of liver fibrosis. Collectively, because CCL5 has been reported to be increased in patients with various chronic liver diseases (4, 18), the novel regulation of CCL5 through hepatocyte Gab1 is clinically relevant.

In conclusion, we provide evidence indicating that hepatocyte Gab1 regulates liver fibrosis in mice. Our findings also suggest that hepatocyte CCL5 could be an important contributor to enhanced liver fibrosis in our hepatocyte-specific Gab1CKO mice. Therefore, our present study provides a novel function of hepatocyte Gab1 in liver fibrosis; that is, in addition to hepatoprotective potentials (2), Gab1 has a protective role against liver fibrosis by regulating hepatic CCL5 production. Because Gab1 amplifies signals downstream of a broad range of growth factors/RTKs, the results observed in our hepatocyte-specific Gab1CKO mice also highlight the important role of the signaling of growth factors/RTKs in hepatocytes during liver fibrosis. Taken together, our current data indicate that Gab1 may be a potential target for the treatment of liver fibrosis.

ACKNOWLEDGMENTS

We thank Dr. Toshio Hirano (Osaka University) for helpful discussions and critical review of this paper.

Present address for K. Nishida: Laboratory of Immune Regulation, Faculty of Pharmaceutical Sciences, Suzuka, University of Medical Science, Suzuka, Mie, Japan.

GRANTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


