Role of the E3 ubiquitin ligase gene related to anergy in lymphocytes in glucose and lipid metabolism in the liver

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Abstract

Gene related to anergy in lymphocytes (GRAIL) is an E3 ubiquitin ligase that regulates energy in T-lymphocytes. Whereas, the relevance of GRAIL to T lymphocyte function is well established, the role of this protein in other cell types remains unknown. Given that GRAIL is abundant in the liver, we investigated the potential function of GRAIL in nutrient metabolism by generating mice in which the expression of GRAIL is reduced specifically in the liver. Adenovirus-mediated transfer of a short hairpin RNA specific for GRAIL mRNA markedly reduced the amounts of GRAIL mRNA and protein in the liver. Blood glucose levels of the mice with hepatic GRAIL deficiency did not differ from those of control animals in the fasted or fed states. However, these mice manifested glucose intolerance in association with a normal increase in plasma insulin levels during glucose challenge. The mice also manifested an increase in the serum concentration of free fatty acids, whereas the serum levels of cholesterol and triglyceride were unchanged. The hepatic abundance of mRNAs for glucose-6-phosphatase, catalytic (a key enzyme in hepatic glucose production) and for sterol regulatory element-binding transcription factor 1 (an important transcriptional regulator of lipogenesis) was increased in the mice with hepatic GRAIL deficiency, possibly contributing to the metabolic abnormalities of these animals. Our results thus demonstrate that GRAIL in the liver is essential for maintenance of normal glucose and lipid metabolism in living animals.

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Introduction

The liver plays a central role in nutrient metabolism. It stores carbohydrate as glycogen in the fed state, and it releases glucose, produced either through the breakdown of glycogen (glycogenolysis) or through de novo synthesis (gluconeogenesis), into the circulation in the fasted state. The liver is also the major site for the synthesis and oxidation of fatty acids as well as a source of secreted triglyceride and cholesterol. Dysregulation of nutrient metabolism in the liver thus results in various disorders including type 2 diabetes mellitus, dyslipidemia, atherosclerosis, and fatty liver disease, all of which have become global health problems.

Gene related to anergy in lymphocytes (GRAIL, also known as the product of the gene Rnf128) is a transmembrane RING finger-type E3 ubiquitin ligase. The abundance of GRAIL mRNA is increased in T-lymphocytes in response to the induction of anergy (Anandasabapathy et al. 2003), and the encoded protein plays an important role in the regulation of anergy in these cells (Serogy et al. 2004, Soares et al. 2004, Lineberry et al. 2008a) by catalyzing the ubiquitination of target proteins including CD40 (Lineberry et al. 2008a) as well as CD81 and CD151 (Lineberry et al. 2008b). Although the expression of GRAIL in T-lymphocytes correlates well with the function of these cells (Heissmeyer et al. 2004), GRAIL is expressed in various other cell types and organs (Anandasabapathy et al. 2003). The physiological relevance of this protein in cells other than T-lymphocytes has remained unknown, however.

Given that, among major organs, the abundance of GRAIL mRNA is greatest in the liver (Anandasabapathy et al. 2003), we have investigated the function of GRAIL in this organ. With the use of adenovirus-mediated transfer of short hairpin RNA (shRNA), a technique that has been widely used to investigate the function of specific proteins in the liver (Koo et al. 2004, 2005, Taniguchi et al. 2005), we have generated mice in which
the abundance of GRAIL is reduced specifically in the liver. We now present evidence that GRAIL in the liver is required for maintenance of normal glucose and lipid metabolism in living animals.

Materials and methods

Antibodies to GRAIL

A cDNA encoding the RING-finger domain of mouse GRAIL (amino acids 231–421) was synthesized with the use of PCR and cloned into the pGEX4T-1 vector (GE Healthcare, Amersham, UK) for expression of a glutathione S-transferase (GST) fusion protein. Polyclonal antibodies to GRAIL were generated by injection of rabbits with the GST–GRAIL fusion protein. For the analysis of GRAIL expression in various tissues, tissue extracts (100 μg protein) prepared from 15-week-old male C57BL/6 mice were subjected to immunoblot analysis.

Expression vectors for mouse GRAIL

A cDNA encoding full-length mouse GRAIL was isolated by PCR and ligated into pcDNA3.1 (+; Invitrogen). A mutant GRAIL cDNA in which C residues at nucleotide positions 888 and 897 of the open reading frame were replaced by A was generated with the use of a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

Constructs encoding GRAIL shRNAs

The nucleotide sequence of mouse GRAIL mRNA was analyzed for the generation of shRNAs according to the rational design rules (Reynolds et al. 2004). Three complementary antiparallel oligonucleotides corresponding to nucleotides 325–343, 873–891, and 1069–1087 of the open reading frame were synthesized together with a loop sequence (acgtgctgcgtcgt). The respective oligonucleotides synthesized were thus as follows: GSX1, gtttGGGGCATGGTGTAAGTATAacgtgtgctgtccgtGATGTAATTGCAGGCTAGAAT (reverse); GSX2, gtttATTCTAGCCTGCAAT TATAacgtgtgctgtccgtTATGATTGCAAGTGTAGATTTtttt (forward) and atgcaaaaaATC-CTAACCTGCAATCATAacggacagcacacgtTA-GATTGCAGGTTAGGATttttt (forward); and GSX3, gtttGGGGCATGGTGTAAGTATAacgtgtgctgtccgtGATGTAATTGCAGGCTAGAAT (reverse). The forward and reverse oligonucleotides were annealed and then ligated into the pcPURmU6icassette vector (Takara Bio, Ohtsu, Japan), which contains the mouse U6 promoter. For screening of the efficacy and specificity of the shRNA constructs, COS7 cells were transfected both with the pcPURmU6icassette vectors encoding GSX1, -2, or -3 shRNAs and with pcDNA3.1 (+) encoding wild-type or mutant GRAIL with the use of the lipofectamine reagent (Invitrogen). Lysates of the transfected cells were then subjected to immunoblot analysis with antibodies to GRAIL. For production of adenovirus vectors encoding the GSX2 shRNA or containing the U6 promoter alone, the GSX2 sequence together with the U6 promoter or the U6 promoter alone was excised from the corresponding pcPURmU6icassette vector and ligated into the pAxwit cosmid cassette (Takara Bio). The adenoviruses encoding GRAIL shRNA (AxshGRAIL) or containing the U6 promoter alone (AxU6) were then generated with the use of an Adenovirus Expression Vector Kit (Takara Bio) as described previously (Kitamura et al. 1999).

Generation and metabolic analysis of mice with liver-specific deficiency of GRAIL

All animal procedures were performed in accordance with the guidelines of the animal ethics committee of Kobe University Graduate School of Medicine. Eight-week-old male C57BL/6 mice were injected through the tail vein with AxshGRAIL or AxU6 at a dose of 1 × 10⁹ plaque-forming units per 30 g body mass. Mice were subjected to various assays 4 days after adenoviral injection unless indicated otherwise. For a glucose tolerance test (GTT), mice deprived of food for 16 h were loaded intraperitoneally with glucose (2 g/kg). Blood glucose and plasma insulin concentrations were measured as described (Miyake et al. 2002). Serum levels of triglyceride, cholesterol, free fatty acids, and alanine aminotransferase were determined with the use of a Triglyceride E-test, cholesterol E-Test, NEFA C-test, and transaminase CII-test (Wako, Osaka, Japan) respectively in animals deprived of food for 16 h. The amounts of mRNAs derived from various genes in the liver were also determined in mice that had been deprived of food for 16 h.

Gene expression analysis

Reverse transcription (RT) and real-time PCR analysis with 36B4 mRNA as the invariant control was performed as described (Miyake et al. 2002). The primers for glucose-6-phosphatase, catalytic (G6pc), peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A; Inoue et al. 2006), phosphoenolpyruvate carboxykinase 1, cytosolic (PKC1; Teshigawara et al. 2005), fatty acid synthase (FAS; Okamoto et al. 2007), and sterol regulatory element-binding transcription factor 1 (SREBF1; Matsumoto et al. 2002) were designed and synthesized.
were as described. The primers for mouse GRAIL were 5'-GGGAGCACGGTGCAAGTATC-3' (forward) and 5'-TTCCTTTCAGATTGCCAATCAT-3' (reverse). The primers for mouse CREB regulated transcription coactivator 2 (CRTC2) were obtained from QIAGEN (QuantiTect Primer Assay, Valencia, CA, USA). For northern blot analysis, a membrane loaded with 20 μg total RNA extracted from various mouse organs (Mouse Tissue Total RNA Northern Blot; BioChain, Hayward, CA, USA) was probed with a 32P-labeled fragment of mouse GRAIL cDNA (nucleotides 138–482) isolated by PCR. For assay of hepatic gene expression during refeeding, C57BL/6 mice were deprived of food for 24 h and then fed with a high-carbohydrate chow containing 70% sucrose.

### Statistical analysis

Unless indicated otherwise, quantitative data are presented as means ± S.E.M. Differences between groups were evaluated by Student’s t-test. A P<0.05 was considered statistically significant.

### Results

#### Tissue distribution of GRAIL

Northern blot analysis revealed that GRAIL mRNA was most abundant in heart, kidney, and liver, and was present in smaller amounts in brain, skeletal muscle, and white adipose tissue (Fig. 1A), essentially consistent with the results of a previous study (Anandasabapathy et al. 2003). Although GRAIL mRNA was not detected in lung and spleen by northern analysis (Fig. 1A), it was found to be present in small amounts in these two organs as well as in testis by RT and real-time PCR analysis (Fig. 1B). GRAIL mRNA was also detected in epididymal and brown adipose tissue, but not in s.c. adipose tissue, by RT and real-time PCR analysis. Immunoblot analysis with antibodies to GRAIL revealed that, among major mouse organs, GRAIL protein was most abundant in the liver (Fig. 1C). GRAIL protein was glycosylated in cells and thus observed as multiple bands (Anandasabapathy et al. 2003). The difference in apparent molecular weight of GRAIL protein in multiple tissues might be attributable to the difference in the extent of glycosylation. The abundance of GRAIL mRNA was significantly suppressed after refeeding whereas that of SREBF1 was increased in the liver of mice (Fig. 1D).

#### Generation and specificity of GRAIL shRNAs

Systemic infusion of adenovirus vectors results in the liver-specific expression of exogenous genes (Miyake et al. 2002), and adenovirus-mediated introduction of shRNAs induces a specific reduction in the amount of the target protein in the liver (Koo et al. 2004, 2005, Taniguchi et al. 2005). To explore the function of GRAIL in the liver, we therefore attempted to down-regulate the expression of GRAIL in this organ by adenovirus-mediated introduction of shRNA in mice. We generated three shRNA constructs (GSX1, GSX2, and GSX3) targeted to mouse GRAIL mRNA (Fig. 2A).
Vectors encoding each of these constructs were introduced individually into COS7 cells by transfection together with an expression vector encoding wild-type mouse GRAIL. Although all three constructs inhibited the expression of GRAIL protein, the effect of GSX2 was most pronounced (Fig. 2B). To verify the specificity of the shRNA constructs, we next tested their effects on the expression of a mutant form of GRAIL in which a single nucleotide in the target sequence of GSX2 was altered (Fig. 2A). GSX1 and GSX3 exhibited relatively small inhibitory effects on the expression of the GRAIL mutant (Fig. 2C) as they did on that of the wild-type protein (Fig. 2B). However, the inhibitory effect of GSX2 was almost completely abolished (Fig. 2C), indicating the high specificity of GSX2 for its target sequence in GRAIL mRNA. For subsequent experiments, we therefore focused on the effects of an adenovirus vector encoding GSX2 (AxshGRAIL).

To examine whether GSX2 inhibits the expression of endogenous GRAIL, we infected cultured AML12 mouse hepatocytes with AxshGRAIL or a control adenovirus containing the U6 promoter alone.

Figure 2 Efficiency and specificity of GRAIL shRNAs. (A) Schematic of wild-type (wt) and mutant (mt) GRAIL mRNAs and of the shRNA constructs. (B and C) COS7 cells cultured in 6 cm plates were transfected with 1.5 μg pcDNA3.1 (+) encoding wild-type (B) or mutant (C) GRAIL as well as with 1.5 μg pcPURmU6icassette either encoding GSX1, GSX2, or GSX3 GRAIL shRNAs or containing the U6 promoter alone, as indicated. The cells were then subjected to immunoblot analysis with antibodies to GRAIL. (D) AML12 cells cultured in six-well plates were infected for 48 h with the indicated volumes of AxshGRAIL or AxU6 at a concentration of 1×10^10 PFU/ml. The cells were then lysed and subjected to immunoblot analysis with antibodies to GRAIL. (E–G) C57BL/6 mice were injected or not (–) with AxshGRAIL or AxU6 at a dose of 1×10^9 PFU/30 g. The liver was removed and subjected to immunoblot analysis with antibodies to GRAIL (E and G) or to RT and real-time PCR analysis of GRAIL mRNA (F) either 4 days (E and F) or the indicated times (G) after adenoviral infection. All immunoblots are representative of two or three experiments, and the data in (F) are means ± S.E.M. of 11 experiments; **P < 0.01 versus U6 (Student’s t-test).
(AxU6). Immunoblot analysis of cell lysates showed that infection of the cells with AxshGRAIL reduced the amount of endogenous GRAIL protein in a dose-dependent manner, whereas AxU6 had no such effect (Fig. 2D).

Specific depletion of GRAIL in mouse liver

We infected 8-week-old male C57BL/6 mice with AxshGRAIL or AxU6 through injection into the tail vein, and we subjected the animals to various assays 4 days after the injection. As expected, infection of mice with AxshGRAIL markedly reduced the hepatic abundance of GRAIL protein whereas that with AxU6 did not (Fig. 2E). RT and real-time PCR analysis revealed that infection with AxshGRAIL reduced the hepatic abundance of GRAIL mRNA by ~80% compared with that in mice infected with AxU6 (Fig. 2F). The marked reduction in the amount of GRAIL protein in the liver persisted for at least 14 days after AxshGRAIL infection (Fig. 2G). Serum levels of alanineaminotransferase, a marker of liver damage, were similar in mice injected with AxshGRAIL or AxU6 and were within normal limits in both groups of mice (17.85 ± 0.59 and 17.93 ± 1.15 IU/l, respectively, n=5) indicating that neither adenoviral infection at the selected dose nor the ~80% reduction in the level of GRAIL expression induced liver damage.

Metabolic alterations in mice with liver-specific deficiency of GRAIL

Given that the liver plays a central role in nutrient metabolism, we investigated various parameters of glucose and lipid metabolism in mice with liver-specific depletion of GRAIL. Body mass in the fasted (21.2 ± 0.22 and 21.2 ± 0.41 g, n=5) or randomly fed (23.5 ± 0.24 and 23.4 ± 0.18 g, n=5) states did not differ between mice injected with or AxU6 (respectively). Although blood glucose levels in the fasted (Fig. 3A) or randomly fed (Fig. 3B, AxshGRAIL) states were similar in the two groups of mice, the increase in those during a GTT was exaggerated in mice injected with AxshGRAIL (AxshGRAIL, Fig. 3C). Plasma insulin levels during the GTT did not differ significantly, however, between the two groups of mice (Fig. 3D). Whereas, the serum concentrations of cholesterol and triglyceride were similar in the two groups of mice, the serum concentration of free fatty acids was greater in mice injected with than in those injected with AxU6 (Fig. 3E).

Changes in hepatic gene expression in mice with liver-specific deficiency of GRAIL

Regulation of hepatic gene expression is largely responsible for the control of both glucose and lipid metabolism (O’Brien et al. 2001, Shimano 2001). We therefore investigated hepatic gene expression in mice infected with AxshGRAIL. The abundance of mRNAs for G6pc, an important enzyme in hepatic glucose production (Radziuk & Pye 2001), and for SREBF1, a transcription factor that regulates the expression of various lipogenic genes (Shimano 2001), was increased in mice injected with AxshGRAIL compared with that in those injected with AxU6 (Fig. 4). The amounts of mRNAs for PCK1, a rate-limiting enzyme for gluconeogenesis (Radziuk & Pye 2001), and for FAS, an enzyme that contributes to fatty acid synthesis (Shimano 2001), were both slightly increased by infection with AxshGRAIL, but these changes were not statistically significant. PPARGC1A and CRTC2 are transcriptional coactivators that contribute to regulation of gluconogenic genes (Herzig et al. 2001, Yoon et al. 2001, Koo et al. 2005). The mRNA abundance of PPARGC1A as well as of CRTC2 was not affected by infection with AxshGRAIL.

Discussion

With the use of adenovirus-mediated transfer of shRNA, we have generated a mouse model in which the expression of GRAIL is acutely reduced in the liver. These animals manifested an exaggerated increase in blood glucose levels in response to glucose challenge. Given that the associated increase in plasma insulin levels of these mice was similar to that of control mice, the glucose intolerance induced by hepatic GRAIL deficiency was likely attributable not to a defect in insulin secretion but to impairment of insulin sensitivity. The dysregulation of hepatic glucose production is an important pathological feature of type 2 diabetes (DeFronzo 2004), and the control of hepatic glucose production is thought to be largely dependent on the abundance of enzymes that mediate this process (Radziuk & Pye 2001). Indeed, forced expression of G6pc or PCK1 in the liver induces frank glucose intolerance in rodents (Valera et al. 1994, Trinh et al. 1998). It is thus likely that the observed increase in the abundance of G6pc mRNA in the liver contributes to the glucose intolerance of mice with hepatic deficiency of GRAIL.

The expression of genes involved in hepatic glucose production is controlled by an elaborate network of transcriptional regulators. Such regulators include the transcription factors cAMP response element-binding protein (CREB), forkhead box O1 (FoxO1), hepatocyte nuclear factor 4 alpha (HNF4α), signal transducer and activator of transcription 3 (STAT3), and Krüppel-like factor 15 (KLF15; Yoon et al. 2001, Teshigawara et al. 2005, Inoue et al. 2006, Gross et al. 2008) as well as the transcriptional coactivators CREB-binding protein.
Moreover, several nuclear receptors including liver X receptor (Cao et al. 2003), farnesoid X receptor (Zhang et al. 2006), and small heterodimer partner (Kim et al. 2008) also appear to contribute to the regulation of the expression of genes for hepatic glucose production. Evidence suggests that the activity, abundance, or subcellular distribution of certain transcriptional regulators is influenced by ubiquitination (Huang et al. 2001, Yoon et al. 2001, Koo et al. 2005, Gross et al. 2008).

**Figure 3** Metabolic characteristics of mice with liver-specific deficiency of GRAIL. (A and B) Blood glucose concentrations in the fasted (A) or randomly fed (B) states of mice injected with AxshGRAIL or AxU6. (C and D) Blood glucose (C) and plasma insulin (D) concentrations during a GTT in mice injected with AxshGRAIL or AxU6. Values at various times after glucose administration are shown in the left panels, and the area under the curve (AUC) during the test is shown in the right panels. (E) Serum concentrations of cholesterol, triglyceride, and free fatty acids (FFA) in the fasted state of mice injected with AxshGRAIL or AxU6. Data are means ± S.E.M. of values from 5 (A and E), 8 (B), or 13 and 11 (shGRAIL and U6 respectively in (C) and (D)) mice. **P<0.01 versus U6 (Student's t-test).
GRAIL might also affect the expression of genes related to glucose production through the ubiquitination of such transcriptional regulators. Although the mRNA abundance of PPARGC1A and CRTC2 in mouse liver was not affected by shRNA of GRAIL, we cannot exclude the possibility that GRAIL affects the protein abundance of these molecules.

Another metabolic alteration induced by depletion of GRAIL in the liver was an increase in the serum concentration of free fatty acids. Given that an acute increase in the circulating concentration of free fatty acids results in insulin resistance in both humans and rodents (Savage et al. 2007), this defect in lipid metabolism might also be related to the glucose intolerance in these mice. SREBF1 is a transcription factor that controls the expression of several genes that encode enzymes involved in fatty acid synthesis (Shimano 2001). The observed increase in the abundance of SREBF1 mRNA in the liver of mice with hepatic GRAIL deficiency is thus consistent with the
increase in the serum level of free fatty acids. Furthermore, the mRNA abundance of hepatic GRAIL was suppressed after refeeding whereas that of SREBF1 was increased. This observation is consistent with a notion that GRAIL contributes to negative regulation of SREBF1. The abundance of FAS mRNAs which is regulated by SREBF1 (Shimano 2001), was slightly greater in the liver of mice with hepatic GRAIL deficiency than in that of control mice, although this difference did not achieve statistical significance.

In summary, by generating mice with hepatic GRAIL deficiency, we have shown that this E3 ubiquitin ligase in the liver is important for normal glucose and lipid metabolism in living animals. Our study has thus uncovered a physiological role for GRAIL in a cell type other than T lymphocytes. The mechanism by which GRAIL influences the expression of genes in the liver remains to be elucidated. Further investigation of this mechanism may shed light on the pathophysiology of metabolic diseases such as type 2 diabetes or dyslipidemia.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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