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Par14 Protein Associates with Insulin Receptor Substrate 1 (IRS-1), Thereby Enhancing Insulin-induced IRS-1 Phosphorylation and Metabolic Actions

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Background: Par14 is a parvulin-type peptidyl-prolyl *cis/trans* isomerase homologous with Pin1, but its functions remain largely unknown.
Results: Par14 markedly enhanced insulin signaling by associating with IRS-1, and hepatic overexpression of Par14 normalized hyperglycemia in diabetic mice.
Conclusion: Par14 exhibits an insulin-sensitizing effect.
Significance: This is the first report showing the roles of Par14 in metabolism and signal transduction.

Pin1 and Par14 are parvulin-type peptidyl-prolyl *cis/trans* isomerases. Although numerous proteins have been identified as Pin1 substrates, the target proteins of Par14 remain largely unknown. Par14 expression levels are increased in the livers and embryonic fibroblasts of Pin1 KO mice, suggesting a compensatory relationship between the functions of Pin1 and Par14. In this study, the association of Par14 with insulin receptor substrate 1 (IRS-1) was demonstrated in HepG2 cells overexpressing both as well as endogenously in the mouse liver. The analysis using deletion-mutated Par14 and IRS-1 constructs revealed the N-terminal portion containing the basic domain of Par14 and the two relatively C-terminal portions of IRS-1 to be involved in these associations, in contrast to the WW domain of Pin1 and the SAIN domain of IRS-1. Par14 overexpression in HepG2 markedly enhanced insulin-induced IRS-1 phosphorylation and its downstream events, PI3K binding with IRS-1 and Akt phosphorylation. In contrast, treating HepG2 cells with Par14 siRNA suppressed these events. In addition, overexpression of Par14 in the insulin-resistant ob/ob mouse liver by adenoviral transfer significantly improved hyperglycemia with normalization of hepatic *PEPCK* and *G6Pase* mRNA levels, and gene suppression of Par14 using shRNA adenovirus significantly exacerbated the glucose intolerance in Pin1 KO mice. Therefore, although Pin1 and Par14 associate with different portions of IRS-1, the prolyl *cis/trans* isomerization in multiple sites of IRS-1 by these isomerases appears to be critical for efficient insulin receptor-induced IRS-1 phosphorylation. This process is likely to be one of the major mechanisms regulating insulin sensitivity and also constitutes a potential therapeutic target for novel insulin-sensitizing agents.

Peptidyl-prolyl *cis/trans* isomerases play a role in protein folding by catalyzing the *cis/trans* rotation around the Xaa-Pro peptide bonds in target proteins (1–4). The peptidylprolyl isomerase family consists of at least three subfamilies as follows: cyclophilins, FK506-binding proteins, and parvulins. Parvulin, a small prolyl isomerase, the activity of which is not inhibited by either cyclosporin A or FK506 (5), consists of two isoforms in mammals, termed Pin1 and Par14 (Fig. 1*A*) (4, 6, 7). Pin1 and Par14 share a highly conserved isomerase domain in their C termini. The N-terminal WW domain of Pin1 is involved in the recognition of specific motifs containing phosphorylated Pro-directed Ser/Thr (Ser(P)/Thr-Pro), and numerous target proteins have been reported (8). In contrast, the N-terminal domain of Par14 is a basic domain having no homology with the WW domain of Pin1, which indicates the target proteins of Par14 differ from those of Pin1. In turn, Par14 reportedly binds to the AT-rich segments of DNA via its basic N terminus (9), which suggests a role in the process of transcription initiation. Besides DNA binding, Par14 reportedly binds to preribosomal

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ribonucleoprotein particles (10), and it is contained in the preribosomal ribonucleoprotein complexes with fibronectin, p160 (Myb binding), p58 cyclin-dependent kinase, and α- and β-tubulin (11). Not only the target proteins but also the physiological roles of Par14 have been poorly elucidated, compared with those of Pin1. However, because the expression levels of Par14 were reportedly up-regulated in mouse embryonic fibroblasts (MEFs)[3] from Pin1 KO mice, it appears that Par14 compensates for some functions of Pin1. Indeed, depletion of Par14 with siRNA inhibited growth of $Pin1^{-/-}$ MEFs, far more strongly than in control MEFs (12). Thus, Par14 has been proposed to compensate, at least partially, for loss of Pin1-mediating roles in cell cycle regulation and chromatin remodeling. However, it is puzzling that no study has been conducted to date using Par14 KO mice, even though precise evaluation of the tissue-specific roles of Pin1 or Par14, individually, would require conditional KO mice of either or both of these isomerases.

However, we reported that Pin1 expression is higher in the fed than in the fasted state, or it is elevated by high fat diet feeding. In addition, it was revealed that Pin1 associates with IRS-1, a major substrate of the insulin receptor. IRS-1 is phosphorylated via activation of the insulin receptor and recruits several signaling proteins, including PI 3-kinase into a multicomponent complex via interaction between their phosphotyrosine-containing motifs and the Src homology 2 domains of downstream signaling molecules (13). PI 3-kinase activation in response to the association with IRS-1 or IRS-2 is considered to play a critical role in insulin-induced glucose metabolism in muscle, fat, and liver tissues (14–17). Our previous study revealed Pin1 to increase the phosphorylation level of IRS-1 and thereby enhance insulin sensitivity and adipogenesis (18).

Here, we present evidence that Par14 also plays a critical role as a positive regulator of insulin action, despite having a binding domain different from that of IRS-1. This is the first report, to our knowledge, showing the roles of Par14 in metabolism and signal transduction, and the fact that hepatic overexpression of Par14 exerted a marked normalizing effect on the hyperglycemia in diabetic mice raises the possibility that these two isoforms of parvulin might be promising target molecules for treating diabetes mellitus. We speculate that the functions of Par14 are not limited to insulin signaling, and they may well extend to numerous signal transduction molecules. We thus anticipate that this will be a promising research field.

EXPERIMENTAL PROCEDURES

Materials—Anti-Par14 antibody was generated by immunizing rabbits with the peptide GDLGWMTRGSMVGPFQEAAFALPVSGMDKPVFTDPPVKTKFGYHIIMVEGRK (amino acids 105–156 of human Par14). Anti-FLAG tag, anti-hemagglutinin (HA) tag, and anti-actin antibodies were purchased from Sigma. The antibodies against IRS-1, IRS-2, Akt, phospho-Akt (Thr-308, Ser-473), actin, and phosphorylated tyrosine (4G10) were purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies were obtained from GE Healthcare. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui (Tokyo, Japan). Fetal bovine serum was purchased from Biowest. All other reagents were of analytical grade.

Cell Culture and Expression Vectors—HepG2 hepatoma cells and human embryonic kidney (HEK293) cells were grown in DMEM containing 10% (v/v) fetal calf serum at 37 °C in 5% CO_2 in air. The myc-tobacco etch virus-FLAG (MEF) tag cassette was generated by DNA synthesis and inserted into cloning sites in the mammalian expression vector pcDNA3 (Invitrogen) yielding pcDNA3-MEF, as reported previously (19). To create the N-terminal MEF-tagged IRS-1 construct, human IRS-1 cDNA was inserted into pcDNA3-MEF. Transfection of these expression vectors was performed by the lipofection method using FuGENE X-tremeGENE HP DNA transfection reagent (Roche Applied Science).

Animals—C57BL/6J ob/ob mice (obtained from The Oriental Yeast Co., Ltd.) or Pin1 KO mice (20) were housed under climate-controlled conditions with a 12:12-h light/dark cycle and were provided standard food and water *ad libitum*. All protocols were approved by the Institutional Review Board of Hiroshima University.

Preparation of Adenoviruses Expressing HA-tagged Par14 Protein, Par14, IRS-1, IRS-2, and LacZ—To create the C-terminal HA-tagged Par14 construct, human Par14 cDNA-HA was inserted into pcDNA3 (Invitrogen). Then the coding portion of HA-tagged Par14 was isolated from pcDNA3-Par14-HA. The recombinant adenoviruses containing the cDNA coding for HA-tagged Par14 were then constructed. Similarly, recombinant adenoviruses expressing Par14, IRS-1 and IRS-2, and LacZ were generated, and the adenovirus encoding LacZ served as a control. Recombinant adenoviruses were used for adenoviral gene transfer into HepG2 cells and the livers of ob/ob mice as described previously (21). The HepG2 cells and mice were used for the experiments 48 and 96 h, respectively, after adenoviral transfection.

Immunoprecipitation and Immunoblotting—For the immunoprecipitation experiments, HepG2 cells or mouse liver tissues were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 500 μM Na_3VO_4, 100 KIU/ml aprotinin, 20 μg/ml PMSF, 10 μg/ml leupeptin and 5 μg/ml pepstatin. The lysates were centrifuged at 15,000 $\times$ *g* for 30 min at 4 °C, and the supernatants were incubated for 4 h at 4 °C with the antibody and then for 1 h with 30 μl of protein A-and G-Sepharose beads. The anti-FLAG M2 affinity gel from Sigma (catalogue number A2220) was used for the immunoprecipitation of cellular extracts from cells transfected with a FLAG-tagged protein. The pellets were washed five times with 1 ml of lysis buffer, resuspended in Laemmli sample buffer, and subjected to SDS-PAGE. Western blot analysis was carried out. In brief, 10 μg of protein lysates were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes in a transfer buffer consisting of 20 mM Tris-HCl, 150 mM glycine, and 20% methanol. The membranes were blocked with 3% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and

[3] The abbreviations used are: MEF, mouse embryonic fibroblast; Pin1, peptidyl-prolyl *cis/trans* isomerase 1; IRS, insulin receptor substrate; SREBP, sterol regulatory element-binding protein; FAS, fatty-acid synthase; ACC, acetyl-CoA carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; Glc-6-Pase, glucose-6-phosphatase; PI, phosphatidylinositol; CRE, cAMP-response element.

incubated with specific antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies, and subjected to immunoblotting using the SuperSignal West Pico Chemiluminescence System (Thermo Fisher Scientific, Rockford, IL). The results of several immunoblots were quantitatively analyzed using LAS-3000 mini (FujiFilm).

Preparation of Glutathione S-Transferase (GST)-Par14 Fusion Protein—The cDNAs encoding full-length human Par14 and five deletion mutants of Par14 (amino acids 1–29 (basic domain), 30–103 (peptidylprolyl isomerase domain), and 1–30, 1–50, and 1–75) were subcloned into a pGEX-4T-1 vector (Amersham Biosciences), which was used to transform *Escherichia coli* BL21 (Promega). Transformed cells were grown to an A_{600} of 0.6 in LB medium supplemented with 0.1 mg/ml ampicillin and stimulated for 3 h with 1.0 mM isopropyl β-D-thiogalactopyranoside. GST fusion proteins were conjugated to glutathione-Sepharose 4B (Amersham Biosciences) and used for GST pulldown experiments.

GST Pulldown—HepG2 cells overexpressing IRS-1 by adenoviral transfer were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 500 μM Na_3VO_4, 100 KIU/ml aprotinin, 20 μg/ml PMSF, 10 μg/ml leupeptin, and 5 μg/ml pepstatin. The lysates were centrifuged at 15,000 × *g* for 30 min at 4 °C, and the supernatants (2 mg/ml protein concentration) were incubated with 1 ml of glutathione-Sepharose 4B for 1 h at 4 °C to remove nonspecifically bound proteins, then incubated with purified GST alone, GST-Par14, and GST-Par14 deletion mutant proteins for 1 h, and finally washed six times with lysis buffer. Glutathione-Sepharose 4B beads were boiled in Laemmli sample buffer, which was used for the SDS-PAGE and immunoblotting.

RNA Interference—HepG2 cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen). siRNAs were synthesized (Invitrogen) with the following sequences (sense strands): Par14 siRNA-1 (5′-GCCUUGCCUGUAAGUGGGAUGGAUA-3′) and Par14 siRNA-2 (5′-AAAGUCUGGGAUGAGAUUCAAUGAA-3′). Briefly, siRNA was mixed with RNAiMAX in Opti-MEM. Then the siRNA mixture was added to the medium of HepG2 cells in 24- or 6-well plates. After incubation for 48 h, the cells were stimulated with 100 nM insulin for the indicated times.

Preparation of RNAi Adenovirus Vectors for Mouse Par14 and Control LacZ—RNAi adenovirus vectors for mouse Par14, 5′-ACAGTGCTGACAAGAAGTCT-3′, and for control LacZ were constructed with a BLOCK-iT™ U6 RNAi entry vector kit and a BLOCK-iT™ adenoviral RNAi expression system (Invitrogen) according to the manufacturer's protocol. Each adenovirus was amplified in HEK293 cells and concentrated using an Adenopure™ adenovirus purification kit (Puresyn). Recombinant RNAi adenoviruses were injected into the Pin1 KO mice, and 96 h later, these mice were used for the experiments.

Intraperitoneal Glucose and Pyruvate Tolerance Tests—Following an overnight fast, mice received an intraperitoneal injection of glucose or pyruvate (2 mg/g body weight). Blood glucose levels were determined at base line and over time as shown in the figures.

Quantitative Real Time Reverse Transcription-PCR—Total RNAs from tissues were isolated using Sepasol reagent, according to the manufacturer's instructions (Nakalai Tesque, Kyoto, Japan). Template cDNA was synthesized from total RNA using Verso cDNA synthesis kits (Thermo Fisher Scientific K.K.). Quantitative real time-PCR was performed using a SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara, Japan) on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primers were designed as follows: mouse *SREBP-1*, tctgctttggaacctcatcc and gccaggttagaagcagcaag (209 bp); mouse *ACC*, gagaggggtcaagtccttcc and ctgctgccgtcataagacaa (199 bp); mouse *FAS*, ttgctggcactacagaatgc and aacagcctcagagcgacaat (199 bp); mouse *PEPCK*, agcctttggtcaacaactgg and tgccttcggggttagttatg (215 bp); mouse *G6Pase*, tcgtggctggagtcttgtca and ggctggcaaagggtgtagtg (170 bp).

Statistical Analysis—Results are expressed as means ± S.E., and significance was assessed using one-way analysis of variance, unless otherwise indicated.

RESULTS

Par14 Associates with IRS-1 via Its Basic Domain and N-terminal Half of the Isomerase Domain—The structures of Par14 and Pin1 are shown in Fig. 1*A*. Par14 with the C-terminal HA tag, IRS-1, and LacZ were overexpressed by the indicated combinations in HepG2 cells, using adenoviral gene transfer, and the expressed IRS-1 and HA-tagged Par14 in the cell lysates were detected by immunoblotting (Fig. 1, *B* and *C*). HA-tagged Par14 was detected in the anti-IRS-1 immunoprecipitates from the cell lysates expressing HA-tagged Par14 and IRS-1 but not from those expressing HA-tagged Par14 and LacZ (Fig. 1*B*). Similarly, IRS-1 was detected in the anti-HA immunoprecipitates from the cell lysates expressing HA-tagged Par14 and IRS-1 but not from those expressing IRS-1 and LacZ (Fig. 1*C*). In contrast, there was no detectable association between IRS-2 and Par14 (Fig. 1*D*).

Then the cell lysates prepared from the mouse liver were subjected to immunoprecipitation with anti-IRS-1 antibody or control IgG. As shown in Fig. 1*E*, Par14 was detected in the anti-IRS-1 but not in the control IgG or the anti-IRS-2 immunoprecipitate (Fig. 1, *E* and *F*), indicating an endogenous association between IRS-1 and PAR14. To examine the involvement of Ser/Thr phosphorylation in IRS-1 in the association with Par14, HepG2 cells were treated with 1 μg/ml okadaic acid for 1 h (Fig. 1*G*) or 100 nM insulin for 5 min (Fig. 1*H*), and the association between endogenous IRS-1 and Par14 was then examined. Treatment with okadaic acid reduced the mobility shift of IRS-1 on SDS-PAGE indicating increased phosphorylations of IRS-1, although the association between IRS-1 and Par14 was not significantly altered (Fig. 1*G*). Similarly, insulin treatment for 5 min had no significant effect on the amount of IRS-1 associated with PAR14 (Fig. 1*H*). These results raise the possibility that Par14 associates with IRS-1, although independently of the Ser/Thr phosphorylation in IRS-1.

To identify the domain in Par14 involved in the association with IRS-1, a full-length or various deletion mutants of Par14 were bacterially produced as GST fusion proteins. These GST fusion proteins or GST alone conjugated with glutathione beads were incubated with the cell lysates of HepG2 cells over-

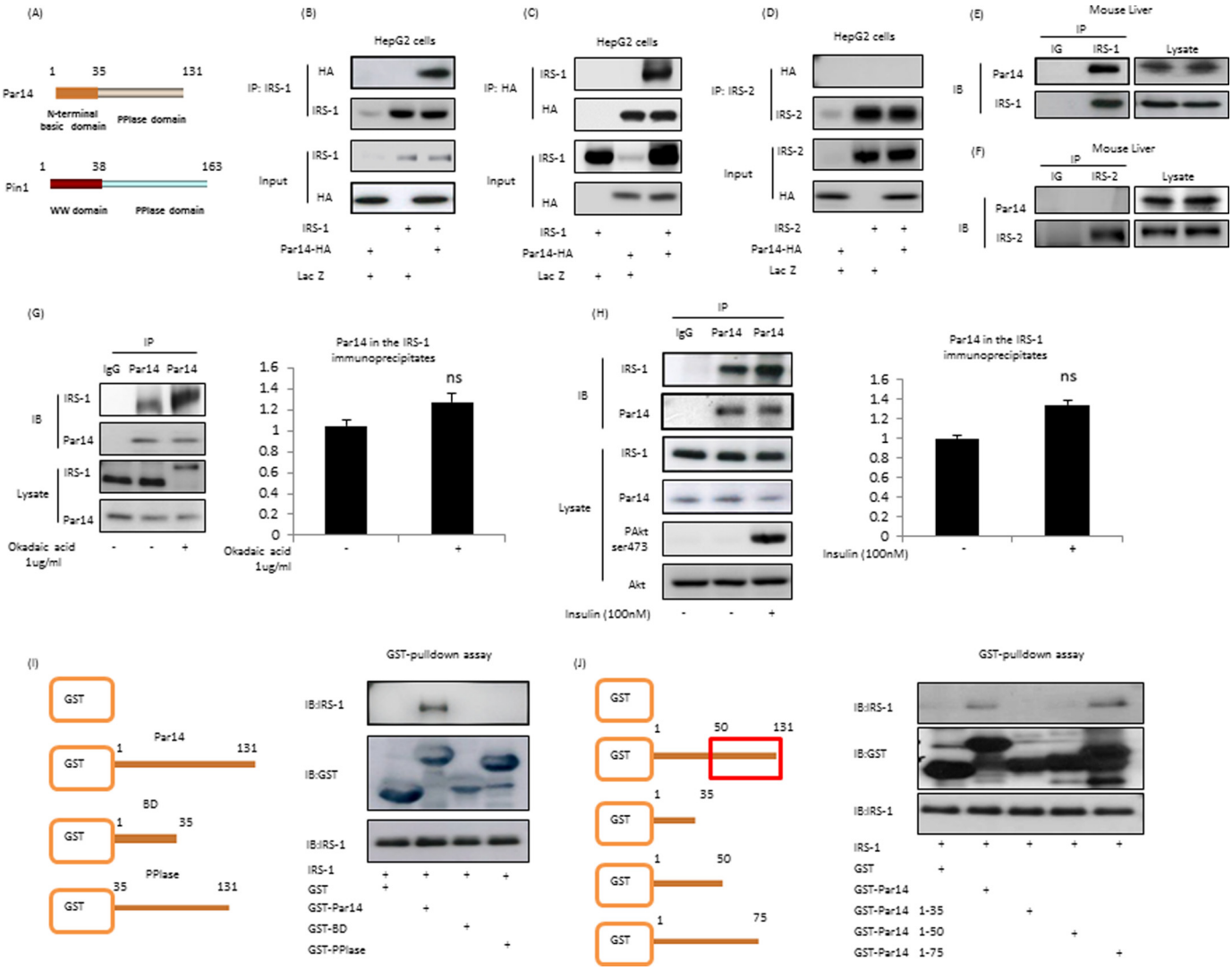

FIGURE 1. **Par14 associates with IRS-1.** *A,* structures of Par14 and Pin1 both possess the peptidylprolyl isomerase domains in their C termini. *B,* IRS-1, Par14 with the C-terminal HA tag, and LacZ were overexpressed in HepG2 cells using adenoviral gene transfer. The cell lysates were immunoprecipitated (*IP*) with anti-HA or anti-IRS1 antibody, and immunoblotting (*IB*) with anti-HA antibody was subsequently performed. *C,* IRS-1, Par14 with the C-terminal HA tag, and LacZ were overexpressed in HepG2 cells using adenoviral gene transfer. The cell lysates were immunoprecipitated with anti-HA antibody, and immunoblotting with anti-IRS-1 or anti-HA antibody was subsequently performed. *D,* IRS-2, Par14 with the C-terminal HA tag, and LacZ were overexpressed in HepG2 cells using adenoviral gene transfer. The cell lysates were immunoprecipitated with anti-IRS-2 antibody, and immunoblotting with anti-HA or anti-IRS-2 antibody was subsequently performed. *E,* cell lysates from mouse livers were immunoprecipitated with anti-IRS-1 or control antibody and immunoblotted with anti-Par14 or anti-IRS-1 antibody. *F,* cell lysates from mouse livers were immunoprecipitated with anti-IRS-2 or control antibody and immunoblotted with anti-Par14 or anti-IRS-2 antibody. *G* and *H,* HepG2 cells were treated with 1 μg/ml okadaic acid for 1 h or 100 nM insulin for 5 min, and the association between endogenous IRS-1 and Par14 was then examined. Representative immunoblotting data are shown. The results of immunoblots from six independent experiments were quantitatively analyzed and are presented as means (±S.E.). *ns,* not significant. *I* and *J,* cell lysates from HepG2 cells overexpressing IRS-1 were prepared and incubated with glutathione beads conjugated with GST alone or GST containing either the full-length or the illustrated portion of Par14. The proteins associated with GST proteins were immunoblotted with anti-IRS-1 antibody. Representative immunoblotting data from three independent experiments are shown.

expressing IRS-1 by adenoviral gene transfer. As shown in Fig. 1*I*, although GST full-length Par14 bound to IRS-1, the domain of GST-peptidylprolyl isomerases or GST-basic domain failed to bind. The experiments using further GST fusion proteins revealed amino acids 1–75, but not 1–50, of Par14 to be capable of binding to IRS-1 (Fig. 1*J*). Thus, the basic domain (amino acids 1–29) is not sufficient, although the basic domain plus the N-terminal half of the isomerase domain of Par14 is necessary for the association with IRS-1.

Par14 Associates with the Portion of IRS-1 Proximal to the C Terminus—Next, to identify the portion of IRS-1 associating with Par14, the cDNAs coding for seven deletion mutants of IRS-1 containing the C-terminal FLAG tag were generated as the first step and inserted into the pcDNA3 expression vector (Fig. 2*A*). HA-tagged Par14 and one of these seven deletion IRS-1 mutants with the C-terminal FLAG tag were co-overexpressed, and the cell lysates were immunoprecipitated with anti-FLAG antibody and subjected to immunoblotting with anti-HA tag antibody. As shown in Fig. 2*B*, amino acids 553–713 (D-5) and amino acids 711–970 (D-6) of IRS-1 associated with Par14, as detected in the anti-HA immunoprecipitates (Fig. 2*B*). To determine further limited binding sites in IRS-1, smaller deletion mutants, *i.e.* D5-1, D5-2, D6-0, D6-1, D6-2, D6-3, and D6-4, consisting of ~110 amino acids from the 553–

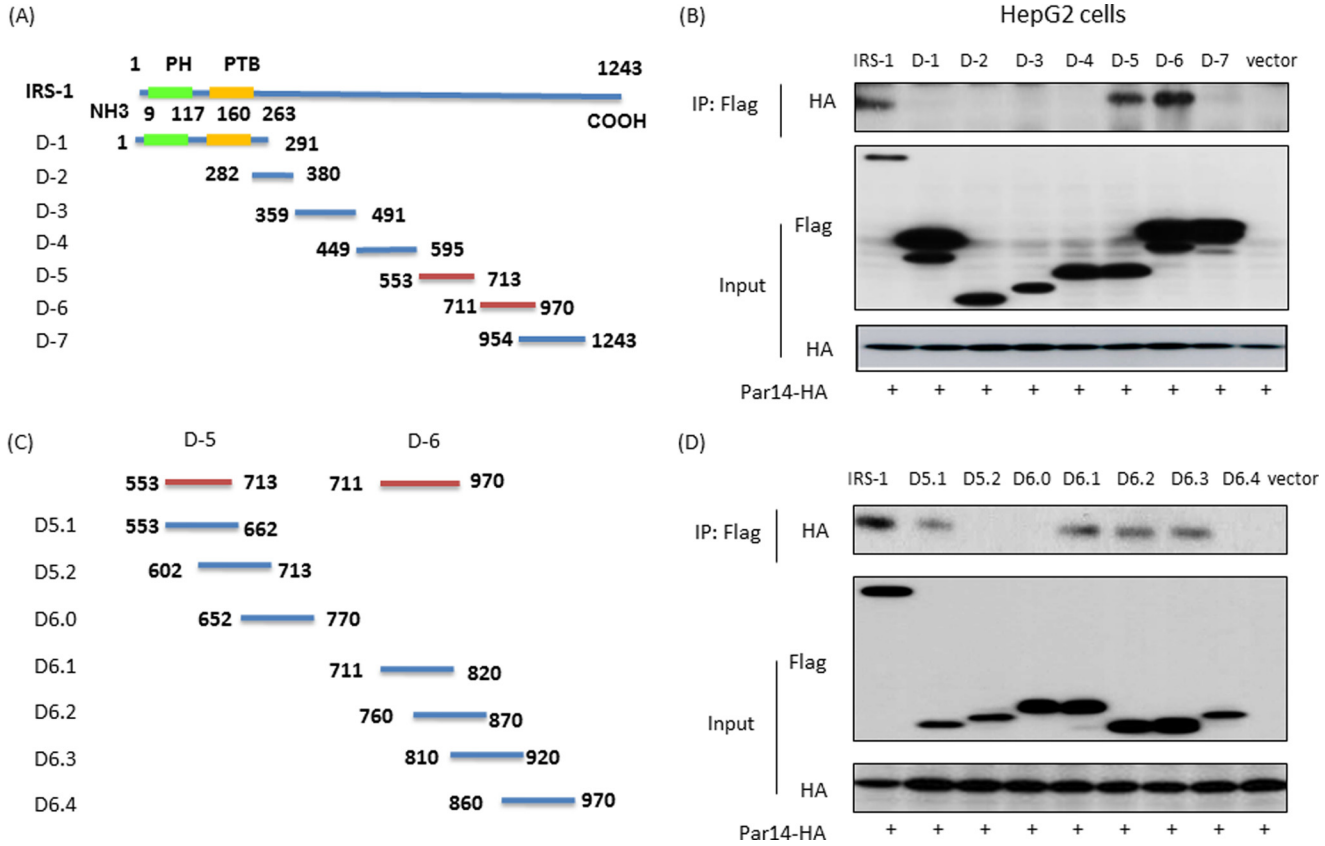

FIGURE 2. **Two portions of IRS-1 are involved in the association with Par14.** *A,* structure of IRS-1 and seven deletion mutants of IRS-1. *B,* HA-tagged Par14 and one of full-length and seven deletion mutants of IRS-1 containing the C-terminal FLAG tag were co-overexpressed, and the cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody and immunoblotted with anti-HA tag antibody. *C* and *D,* smaller deletion mutants containing the ~110 amino acids in the 553–970-amino acid portion of IRS-1 were generated, and their associations with Par14 were examined. Representative immunoblotting data from four independent experiments are shown.

970 segment of the IRS-1 protein, as well as the D-5 and D-6 portions, were generated (Fig. 2*C*), and their abilities to associate with Par14 were examined. As shown in Fig. 2*D*, D5-1, but not D5-2, bound to Par14, suggesting amino acids 553–601 to be critical for the association with IRS-1. As for the other sites examined, D6-1, D6-2, and D6-3 were capable of binding to Par14, raising the possibility that the amino acids spanning ~770–860 of IRS-1 are involved in the association with IRS-1.

Overexpression of Par14 Enhanced Insulin-induced IRS-1 Phosphorylation, p85 Binding with IRS-1, and Akt Phosphorylation—To investigate the effect of Par14 on insulin signaling, Par14 or control LacZ was overexpressed in HepG2 cells, and the cells were stimulated with insulin for the indicated periods (Fig. 3). The expression levels of IRS-1, p85, and Akt were not changed by this PAR14 overexpression. The tyrosine phosphorylation of IRS-1 and the amount of the p85 subunit of PI 3-kinase associated with IRS-1 were shown to be increased by insulin stimulation, and the degrees of these insulin-induced increases were markedly higher in the Par14-overexpressing cells than in those overexpressing LacZ (Fig. 3, *A–C*). The phosphorylations of Ser-473 and Thr-308 in Akt, located downstream from PI 3-kinase activation, were also significantly enhanced by Par14 overexpression (Fig. 3, *D–F*). Then the effects of full-length PAR14 and its deletion mutants were investigated. The deletion mutants consisting of amino acids 1–75 or 35–131 failed to affect the insulin-induced IRS-1 and Akt phosphorylations (Fig. 3, *G–J*).

Subsequently, to examine the effect of PAR14 on IRS-1 dephosphorylation, IRS-1 phosphorylation levels after insulin removal were investigated in HepG2 cells overexpressing LacZ or PAR14 (Fig. 3*K*). Insulin-induced IRS-1 phosphorylations were time-dependently reduced in both cell types, and quantitative analysis revealed no significant difference in the dephosphorylation velocity according to PAR14 overexpression. Thus, it is likely that PAR14 enhances IRS-1 phosphorylation but does not affect dephosphorylation.

Gene Silencing of Par14 Reduced Insulin Signaling—To examine the role of endogenously expressed Par14 in insulin signaling, HepG2 cells were treated with Par14 siRNA-1 or control siRNA. As shown in Fig. 4, *A* and *D*, endogenous Par14 was reduced by this siRNA treatment, although the expression levels of IRS-1, p85, and Akt showed no changes in response to PAR14 siRNA treatment. Insulin-induced tyrosine phosphorylation of IRS-1, association of the p85 subunit of PI 3-kinase with IRS-1, and phosphorylations of Ser-473 and Thr-308 in Akt were all significantly reduced in the cells treated with Par14 siRNA-1, as compared with control siRNA (Fig. 4, *A–F*). Essentially, the same results were obtained with Par14 siRNA-2 (Fig. 4, *I–L*), although the gene silencing effect of siRNA-2 was somewhat weaker than that of siRNA-1. In addition, the reduction in

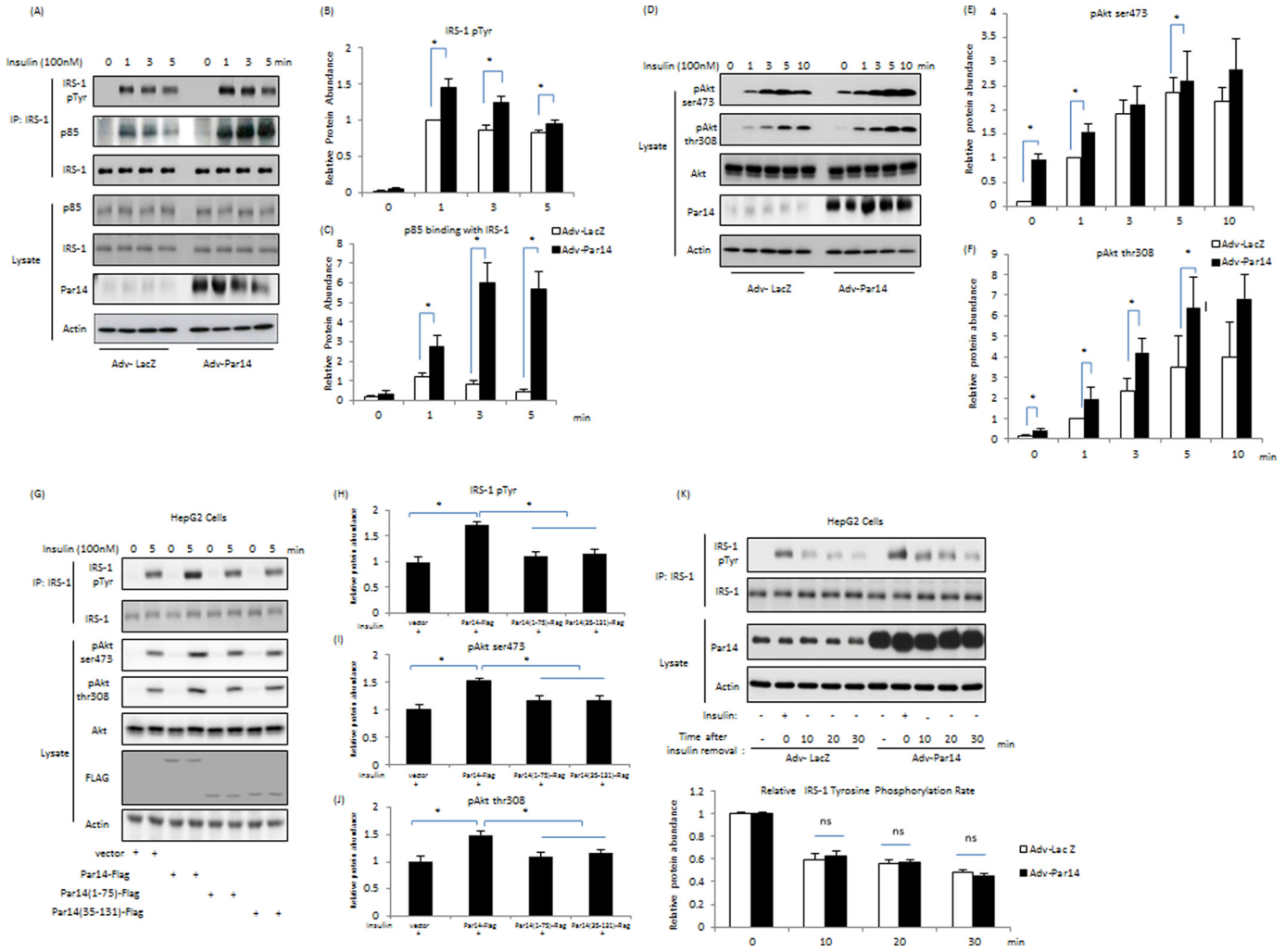

FIGURE 3. **Overexpression of Par14 enhanced insulin signaling.** Par14 or control LacZ was overexpressed in HepG2 cells, and the cells were then stimulated with insulin at the indicated time points. Next, lysates were prepared from HepG2 cells. The tyrosine phosphorylation of IRS-1, association of the p85 subunit of PI 3-kinase with IRS-1 (*A–C*), phosphorylations of Ser-473 and Thr-308 in Akt (*D–F*), as well as p85, IRS-1, Par14, and actin in the cell lysates were investigated and quantitatively analyzed. *G–J,* full-length, deletion mutants consisting of amino acids 1–75 or 29–103 or control LacZ were overexpressed in HepG2 cells and insulin-induced phosphorylations of IRS-1 and Akt were examined. *K,* HepG2 cells overexpressing Par14 or control LacZ were stimulated with insulin for 1 min, and the medium was changed to one free of serum and insulin. *ns*, not significant. Then the phosphorylation levels of IRS-1 were examined at the indicated periods after the switch to the insulin-free medium. Quantitative analysis is shown in the *right panel* with the maximal phosphorylation level before insulin removal represented as 1. The results from four independent experiments are presented as means (±S.E.), from four independent experiments (*, $p < 0.05$). Representative immunoblotting data are shown. *IP*, immunoprecipitation.

insulin-induced IRS-1 phosphorylation was more marked with treatment using both PAR14 and Pin1 siRNAs than with PAR14 siRNA alone (Fig. 4, *K–N*).

Adenoviral Transfer of Par14 Normalized Hyperglycemia of ob/ob Mice—The ob/ob mouse is a well established rodent model of obesity, diabetes mellitus, and hyperlipidemia due to a genetic abnormality of leptin. Par14 or control LacZ was overexpressed in the livers of ob/ob mice by adenoviral gene transfer (Fig. 5*A*). Hyperglycemia of ob/ob mice showed remarkable normalization with hepatic Par14 overexpression with reduced expressions of gluconeogenic genes such as *PEPCK* and *G6Pase* (Fig. 5, *B* and *C*). *SREBP-1* and *FAS* mRNAs were increased by Par14 overexpression (Fig. 5, *D* and *E*), although the mRNA level of *ACC* was unaltered (Fig. 5*F*).

Adenoviral Transfer of Par14 shRNA into Pin1 KO Mouse Livers Induced Glucose Intolerance—To investigate the role of Par14 endogenously expressed in the liver, the adenovirus expressing Par14 shRNA was generated and then injected into Pin1 KO mice. As shown in Fig. 6*A*, treatment with this shRNA adenovirus reduced endogenous Par14 by ~70–80%, as compared with the normal level, in Pin1 KO mouse livers (Fig. 6*A*). In the Par14 shRNA-treated mice, fasting glucose levels were not significantly changed (Fig. 6*B*), although glucose and pyruvate tolerance tests revealed significant sustained hyperglycemia after the injection of glucose or pyruvate, as compared with the control shRNA adenovirus treatment (Fig. 6, *D* and *E*). The hepatic mRNA levels of *PEPCK* and *G6Pase* were significantly higher in the livers treated with Par14 shRNA than in those treated with control shRNA (Fig. 6*C*). In addition, as expected, expressions of *SREBP-1* and its target genes were down-regulated by shPar14 treatment (Fig. 6, *F–H*). These results suggest the physiological importance of Par14 in hepatic metabolism.

DISCUSSION

Although many groups, including ours, have reported the functions of Pin1 (4, 18, 22–24), the cellular functions of Par14

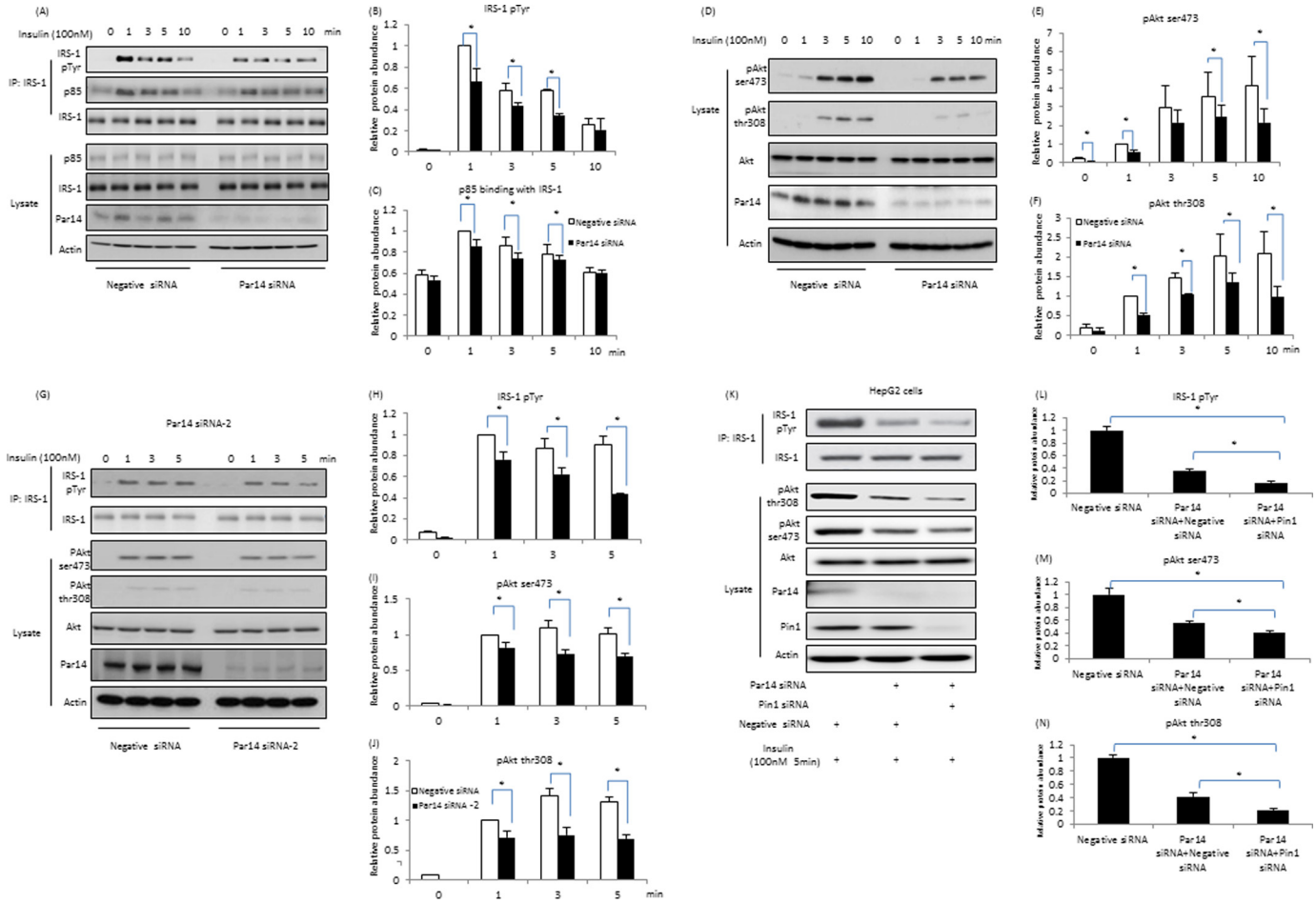

FIGURE 4. **Gene silencing of Par14 using siRNA suppressed insulin signaling.** HepG2 cells were treated with Par14 siRNA-1 or control siRNA and stimulated with insulin for the indicated periods. Insulin-induced tyrosine phosphorylation of IRS-1, association of the p85 subunit of PI 3-kinase with IRS-1 (*A–C*), and phosphorylations of Ser-473 and Thr-308 in Akt (*D–F*) were investigated. HepG2 cells were treated with Par14 siRNA-2 or control siRNA and similar results were obtained (*G–J*). HepG2 cells were treated with control siRNA, Par14 siRNA, or Par14 siRNA plus Pin1 siRNA, and insulin-induced phosphorylation of IRS-1, association of the p85 subunit of PI 3-kinase with IRS-1, and phosphorylations of Ser-473 and Thr-308 in Akt at 3 min after insulin stimulation were investigated (*K–N*). The quantitatively analyzed results of four independent experiments are presented as means (±S.E.) from four independent experiments (*, $p < 0.05$). Representative immunoblotting data are shown.

remain largely unknown. Nevertheless, because the Par14 expression level is significantly elevated in MEFs as well as the livers of Pin1 KO mice (data not shown), it is reasonable to speculate that Par14 plays compensatory roles in some, but very probably not all, Pin1-target protein actions in $Pin1^{-/-}$ mice. In this study, we found that Par14 also associates with IRS-1. This was demonstrated by immunoprecipitation experiments with overexpressed Par14 and IRS-1 as well as endogenous proteins. Interestingly, unlike the case of Pin1, the domains responsible for the association with Par14 were present in two portions of IRS-1 rather close to the C terminus, *i.e.* those encompassing amino acids 553–612 and 770–860, respectively. Considering the basic N-terminal domain of Par14, the acidic portion of these domains of IRS-1 might be the binding site, but further study is required to determine the consensus sequences that are recognized by Par14. In addition, in contrast to the WW domain of Pin1, which binds only to Ser(P)/Thr-Pro motifs, phosphorylation of Ser or Thr does not appear to be necessary for efficient binding with Par14, an observation also supported by the finding that treatment of the cells with okadaic acid did not significantly affect the association between Par14 and IRS-1. Thus, although the binding capacity of Pin1 depends strictly on the phosphorylation states of its substrates, it appears that Par14 constitutively binds to and isomerizes the substrates, irrespective of the phosphorylation states of these substrates. It was also demonstrated that insulin-induced IRS-1 phosphorylation and its downstream signal transductions such as the PI 3-kinase association with IRS-1 and Akt phosphorylation were all enhanced in Par14-overexpressing cells while being reduced in Par14 siRNA-treated cells. The suppressive effects exerted by Par14 were shown to be additive to that associated with Pin1 siRNA, suggesting their functions to be similar but independent, probably due to their different binding sites in IRS-1.

Regarding the mechanism underlying Par14- or Pin1-induced enhancement of IRS-1 tyrosine phosphorylation, we can suggest two possibilities. A previous study using nuclear magnetic resonance structural analyses revealed that Pin1 accelerates the *cis/trans* conversion rate by more than 1000-fold (25). Very recently, proline isomer-specific antibodies recognizing the *cis*-form and the *trans*-form in Tau were developed, and it was clearly shown that Pin1 shifts the equilibrium between the *cis*- and *trans*-conformations of the proline in Tau toward the

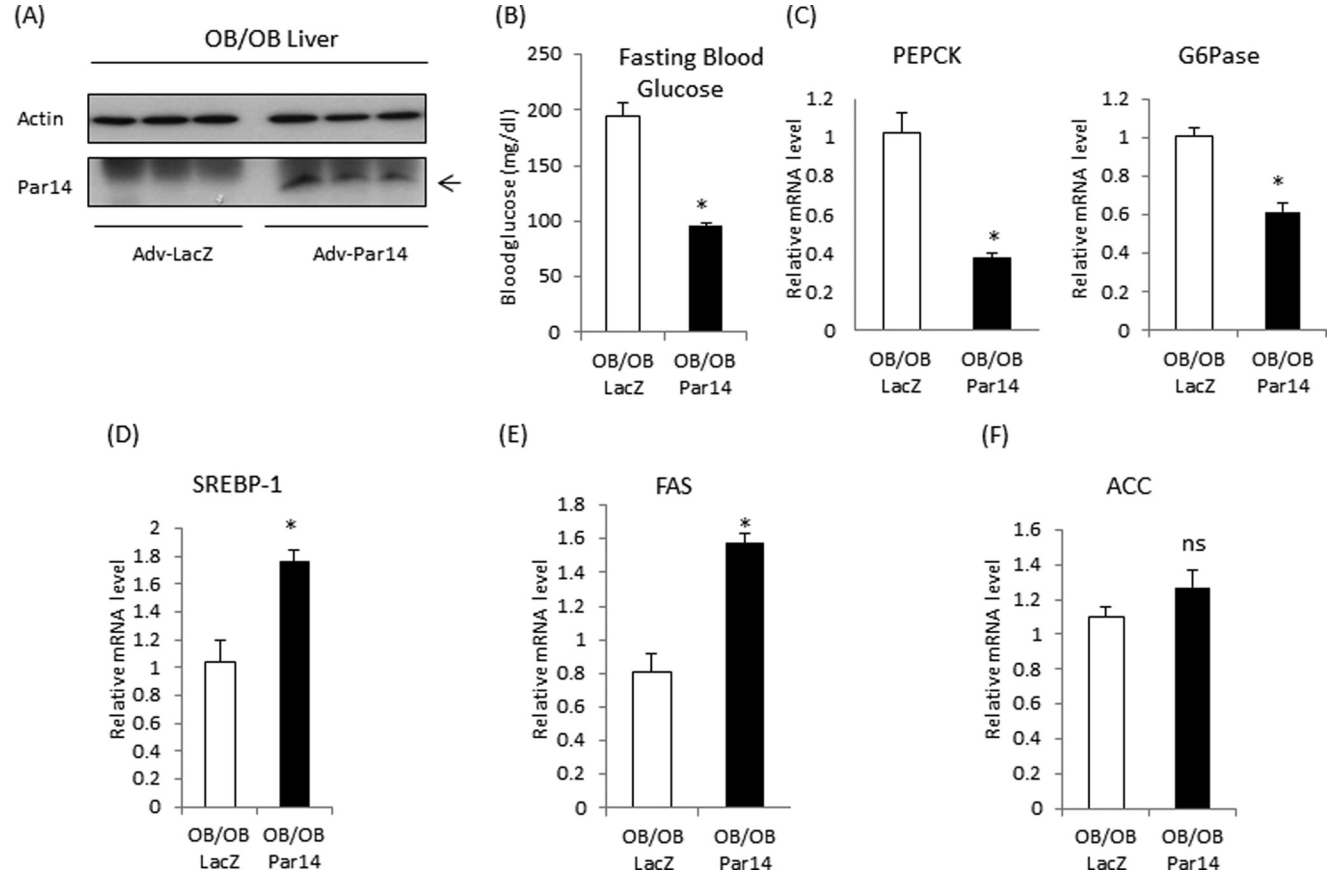

FIGURE 5. **Hepatic overexpression of Par14 normalized hyperglycemia in ob/ob mice.** Par14 or control LacZ was overexpressed in the livers of ob/ob mice using adenoviral transfer (six mice for each Par14 or LacZ). *A,* cell lysates of the livers overexpressing Par14 or control LacZ were subjected to immunoblotting with antibodies against anti-actin or with anti-Par14 antibody. Representative immunoblotting data of three mice from each Par14 or LacZ group are shown. *B–F,* fasting glucose concentrations and mRNA levels of PEPCK, Glc-6-Pase, SREBP-1, FAS, and ACC were measured in ob/ob mouse livers overexpressing Par14 or control LacZ. The results are presented as means (±S.E.), from six independent experiments (*, $p < 0.05$). *ns*, not significant. *IP*, immunoprecipitation.

trans-conformation (26). Taking these previous reports into consideration, one of our speculations is that Pin1 or Par14 increases the ratio of IRS-1 with a particular Xaa-Pro bond in the *trans*-conformation and that such an IRS-1 molecule may have higher affinity for the insulin receptor than IRS-1 with the *cis*-conformation. Alternatively, the process of frequent shifts between the *cis*- and *trans*-conformations in IRS-1 itself might increase the affinity of IRS-1 for the activated insulin receptor. However, the speed of IRS-1 dephosphorylation was not affected by Par14 overexpression. Another possibility is that the association with Par14 or a conformational change of the Xaa-Pro bond may affect the phosphorylation status of some serine residue(s) in IRS-1. To date, a number of specific serine and threonine phosphorylation sites in IRS-1 have been identified and are suggested to be molecular mechanisms linking inflammation, endoplasmic reticulum stress, and obesity with insulin resistance (27–30). However, confusingly, some serine and threonine phosphorylation sites have the ability to both decrease and enhance insulin signaling, depending on the circumstances (31). Some serine residues, specifically 612, 632, 636, 662, 731, and 789, located near or within the portions associated with Par14, reportedly modulate IRS-1 tyrosine phosphorylation (32–35). We found that the phosphorylation of Ser-636 was not altered by Par14 overexpression in HepG2 cells (data not shown), but this possibility cannot be ruled out in terms of the effects on other serine phosphorylations.

In good agreement with these *in vitro* findings, adenoviral gene transfer of Par14 into the liver markedly normalized the hyperglycemia characteristic of insulin-resistant ob/ob mice in which insulin-induced IRS-1 phosphorylation is reportedly impaired (30), although it cannot be ruled out that Par14 has other target molecules in addition to IRS-1 that function to lower blood glucose levels. The gene expressions involved in glucose metabolism also tended to normalize, with lowering of the blood glucose concentration, in the Par14-expressing livers of ob/ob mice. Furthermore, Par14 shRNA adenovirus, prepared to reduce endogenous Par14 expression, reduced Par14 expression in the liver by 70 – 80% and significantly exacerbated the glucose intolerance in Pin1 KO mice. Up-regulation of the mRNAs of *SREBP-1* and its downstream genes, *FAS* and *ACC*, by Par14 overexpression and their suppression by Par14 siRNA treatment are also attributable to the enhancing effects of Par14 on insulin signaling. These metabolic effects were not present in wild-type mice (data not shown), probably due to incomplete elimination of the Par14 and a compensatory increase in the Pin1 expression level. Although it would be necessary to prepare Par14 KO mice to further elucidate the individual effects of Pin1 and Par14, it is reasonable to consider Par14 to have in

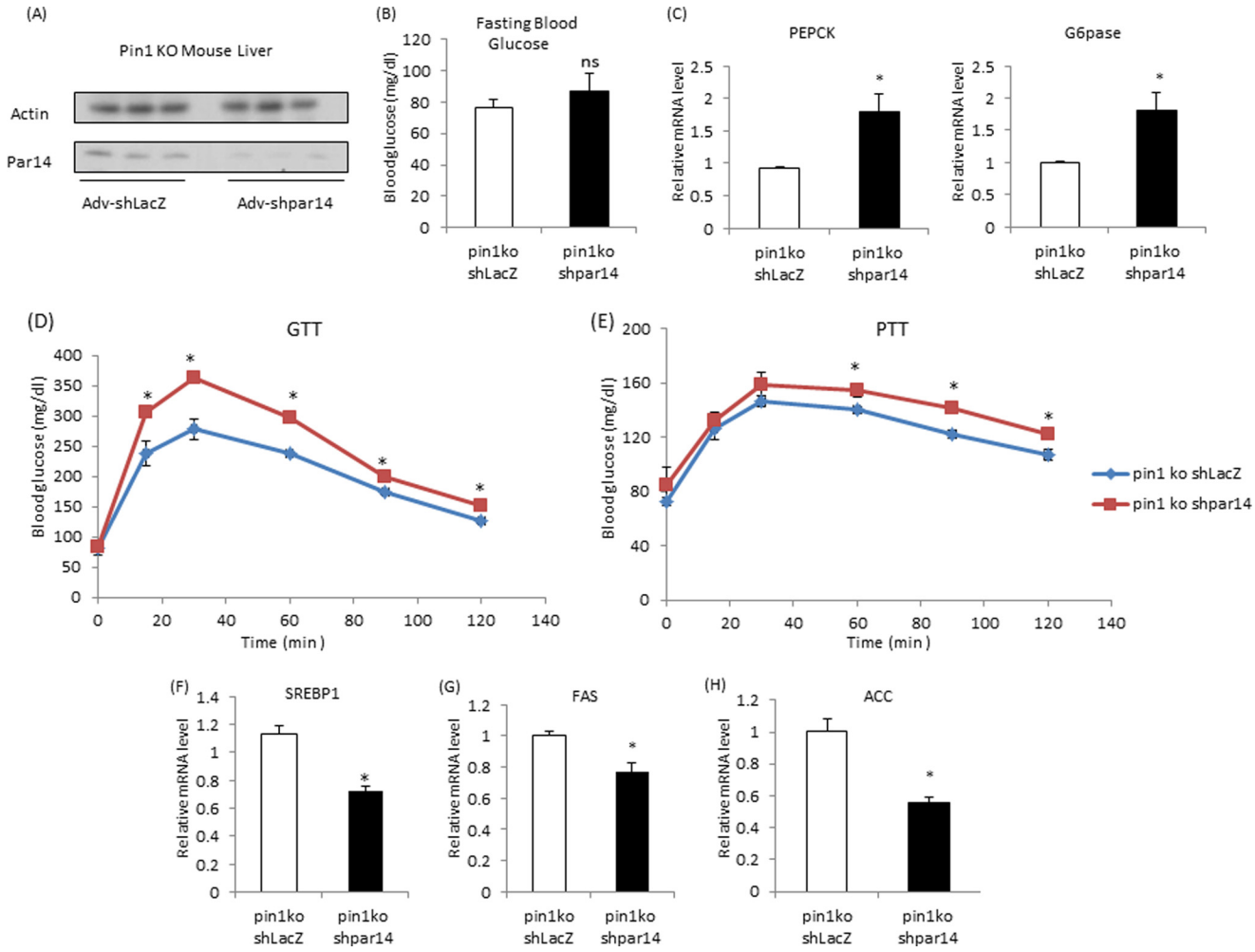

FIGURE 6. **Gene silencing of Par14 in the Pin1 KO mouse livers induced glucose intolerance.** The Par14 and control shRNA adenoviruses were introduced into Pin1 KO mice (six mice for each Par14 and control shRNA) (*A*). Par14 expression was determined by immunoblotting using anti-Par14 antibody. *B,* fasting insulin concentrations in control and Par14 shRNA-treated mice. *ns,* not significant. *C,* hepatic PEPCK and Glc-6-Pase (*G6pase*) mRNA levels were determined by real time PCR. *D,* for the glucose tolerance test, glucose at 2 mg/kg was injected into control and Par14 shRNA-treated Pin1 KO mice, and blood glucose concentrations were measured, as indicated. *E,* for pyruvate tolerance test, pyruvate at 2 mg/kg was injected into control and Pin1 KO mice, and blood glucose concentrations were measured, as indicated. *F–H,* hepatic SREBP-1, ACC, and FAS mRNA levels were determined by real time PCR. Representative immunoblotting data are shown. The results are presented as means (±S.E.), from six independent experiments (*, $p < 0.05$).

common with Pin1 the feature of functioning with IRS-1 as a positive regulator of glucose tolerance and insulin actions.

Pin1 binds to Crtc2 and suppresses CRE transcriptional activity, which leads to the suppression of hepatic gluconeogenesis (36). In the case of Pin1, both mechanisms via IRS-1 and CRTC2 associations apparently contribute to the hypoglycemic effect *in vivo* and eventually lead to the incorporation of nutrients into cells such as hepatocytes or adipocytes. However, no effect of Par14 on the association with Crtc2 or on the suppression of CRE transcriptional activity was detected (data not shown). Although it may be inferred from the effects on CRE transcriptional activity that Par14 has a weaker glucose lowering effect *in vivo* based on the difference in their impacts on CRE transcriptional activity, it is likely that Par14 binds to IRS-1, irrespective of the phosphorylation status of IRS-1, in contrast to the association of Pin1 with only Ser-phosphorylated IRS-1. Thus, it is unclear whether Pin1 or Par14 exhibits the stronger effect in terms of the potential lowering of blood glucose levels.

At present, much remains unclear about the functions as well as the target proteins of Par14. This report is the first to demonstrate the effect of Par14 on a signal transduction molecule in the cytosol. In addition, it was strongly suggested that the prolyl *cis/trans* conformational changes of IRS-1, induced by Pin1 and Par14, are critical for efficient phosphorylation by the insulin receptor. This mechanism may provide insights into developing agents for treating metabolic disorders and other diseases.

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