Gene Silencing of Phogrin Unveils Its Essential Role in Glucose-Responsive Pancreatic β-Cell Growth

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OBJECTIVE—Phogrin and IA-2, autoantigens in insulin-dependent diabetes, have been shown to be involved in insulin secretion in pancreatic β-cells; however, implications at a molecular level are confusing from experiment to experiment. We analyzed biological functions of phogrin in β-cells by an RNA interference technique.

RESEARCH DESIGN AND METHODS—Adenovirus-mediated expression of short hairpin RNA specific for phogrin (shPhogrin) was conducted using cultured β-cell lines and mouse islets. Both glucose-stimulated insulin secretion and cell proliferation rate were determined in the phogrin-knockdown cells. Furthermore, protein expression was profiled in these cells. To see the binding partner of phogrin in β-cells, coimmunoprecipitation analysis was carried out.

RESULTS—Adenoviral expression of shPhogrin efficiently decreased its endogenous expression in pancreatic β-cells. Silencing of phogrin in β-cells abrogated the glucose-mediated mitogenic effect, which was accompanied by a reduction in the level of insulin receptor substrate 2 (IRS2) protein, without any changes in insulin secretion. Phogrin formed a complex with insulin receptor at the plasma membrane, and their interaction was promoted by high-glucose stimulation that in turn led to stabilization of IRS2 protein. Corroboratively, phogrin knockdown had no additional effect on the proliferation of β-cell line derived from the insulin receptor–knockout mouse.

CONCLUSIONS—Phogrin is involved in β-cell growth via regulating stability of IRS2 protein by the molecular interaction with insulin receptor. We propose that phogrin and IA-2 function as an essential regulator of autocrine insulin action in pancreatic β-cells. ***Diabetes*** **58:682–692, 2009**

Glucose is a principle regulator of pancreatic β-cell survival and growth as well as insulin secretion (1). It is a potent mitogen on pancreatic β-cells and regulates islet β-cell mass through their replication (2). Recent studies have suggested that insulin secreted in response to elevated glucose exerts autocrine/paracrine effects, including promotion of insulin biosynthesis and proliferation of β-cells (3,4). The importance of insulin signaling in maintaining β-cell mass was demonstrated by targeted knockouts of the insulin receptor and insulin receptor substrate 2 (IRS2) (5–8). Although insulin receptor knockout had a restricted effect on β-cell mass (7), its mitogenic function on β-cells was clearly shown by short interfering RNA (siRNA)–based silencing of insulin receptor in β-cell–derived MIN6 cells (9,10). More recently, another pathway was demonstrated showing that glucose metabolism leads to increased β-cell mass through the transcriptional activation of IRS2 (11). Calcium/calmodulin-dependent protein kinases and increased cAMP levels were suggested to contribute to IRS2 expression, and this pathway has been shown to be modulated by the incretin hormone glucagon-like peptide 1 (GLP-1) (12,13). In both cases, IRS2 must be a key mediator for glucose-responsive β-cell growth (14).

Phogrin (IA-2β) and IA-2 (ICA512) are integral glycoproteins localized to dense-core secretory granules in various neuroendocrine cell types and have one inactive protein-tyrosine phosphatase (PTP) domain in the cytoplasmic region (15–18). The targeted deletion of IA-2 or phogrin or both in mice has resulted in mild impairment of glucose-stimulated insulin secretion (GSIS) (19–21). However, it is uncertain whether the alteration is direct or indirect and whether phogrin and IA-2 function at the exocytotic machinery. To address these questions, cultured β-cell lines were used in further studies. Although MIN6 stably overexpressing IA-2 showed a significant increment in both secretory granule number and insulin secretion (22), transient overexpression of phogrin failed to affect GSIS (23) or reduced it (24). Besides gene transduction experiments, interaction of the IA-2 cytoplasmic tail with spectrin and/or syntrophin was found in two-hybrid assay (25). Another function of IA-2 was also proposed, involving the regulation of gene expression in concert with signal transducer and activator of transcription (STAT)5b (26,27). Furthermore, phogrin and IA-2 are able to heterodimerize with other receptor-type PTPs, such as RPTPα, and prevent its activity in a transient fashion (28). Unfortunately, it is still unknown whether all of their interactions physiologically associate with a secretion defect in knockout mice.

IA-2 family members are evolutionally conserved, and the cytoplasmic region, including the PTP core domain, is highly homologous, whereas the luminal region shows lower homology between each of them (29). Although phogrin and IA-2 have similar structures and functions, their expression is regulated distinctly. IA-2 expression increases in accordance with development in rodent tissues (30–32). IA-2 expression in β-cells is influenced by glucose, insulin, cAMP-generating agents, and proinflammatory cytokines (32–34). In contrast, phogrin expression is constant in the developmental stage of islets and is not significantly affected by glucose levels (32).

Because IA-2 expression is changeable and phogrin expression is rather constitutive, we sought to define the

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role of phogrin using pancreatic β-cells. Establishment of stable cell lines expressing short hairpin RNA (shRNA) to reduce phogrin levels prompted us to explore its novel role in β-cell growth. We found that phogrin knockdown led to reduction of the IRS2 protein level and associated growth retardation. Furthermore, we found that phogrin binds to insulin receptor to modify IRS2 stability in β-cells.

RESEARCH DESIGN AND METHODS

Anti-phogrin and anti–IA-2 antibodies were raised in rabbits against the luminal region of phogrin and the luminal region of IA-2, respectively, and were affinity-purified. The guinea pig anti-insulin antibody and the anti–α-tubulin and anti–β-actin mouse monoclonal antibodies were purchased from Sigma. Anti-adaptin, anti–carboxypeptidase E (anti-CPE), anti–calcium-dependent activator protein (anti-CAPS), anti-munc18, anti-dynamin, anti–clathrin heavy chain, anti–phosphatidylinositol 3-kinase (anti-PI3K), anti-Grb2, and anti–extracellular signal–related kinase (anti-ERK) mouse monoclonal antibodies and anti–insulin receptor β-subunit (anti-IRβ) rabbit polyclonal antibodies were from BD Biosciences. Anti-IRS1 and anti-STAT5 rabbit polyclonal antibodies were from Cell Signaling. Anti-IRS2 rabbit polyclonal and anti-epidermal growth factor receptor sheep polyclonal antibodies were from Upstate Biotech. Anti–IGF-IR, anti-IRβ, anti-syntrophin, and anti–5-bromo-2′-deoxyuridine (anti-BrdU) mouse monoclonal antibodies were from Lab Vision, Chemicon, Affinity Bioreagents, and Roche Diagnostics, respectively. Anti–vesicle-associated membrane protein (anti-VAMP2) and anti–green fluorescent protein (anti-GFP) rabbit polyclonal antibodies were from WAKO chemicals and MBL, respectively.

DNA construction. The RNA polymerase III H1 gene promoter was cloned to construct small interfering RNA expressing plasmid vector (pSUPER) according to the reference (35). Oligonucleotides (64-base) corresponding to sense target sequence, hairpin loop, and antisense target sequence were synthesized, annealed together, and then ligated into pSUPER vector. The target sense sequences were as follows: GGTCACCTTACAGAAGCTC (shPhogrin1) (mouse Phogrin mRNA, 244–264), GCCACAACTCACACTACAA (shPhogrin2) (mouse, 1,877–1,895; rat, 1,886–1,904), and GGATACATCCTCACAGGAA (shPhogrin3) (mouse, 1,492–1,510; rat, 1,501–1,519) for phogrin, and GTCTGTATTCAGGATGGCT (shIA2) (mouse, 163–181; rat, 172–190) for IA-2.

Cell culture and transfection. MIN6 cells before passage 25 were cultured in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS) and 50 μmol/l 2-mercaptoethanol. INS-1E cells were cultured in RPMI 1640 with 10% FBS, 10 mmol/l HEPES (pH 7.4), and 50 μmol/l 2-mercaptoethanol. Transfections were performed with Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA). MIN6 cells were transfected with pcDNA3 vector plus pSUPER plasmid, and stable clones were selected in the presence of G418 (36). Isolated colonies of the shPhogrin1-transfected cells were transferred to new culture dishes for propagation (a total of 192 clones: 72 clones in the 1st round, 120 clones in the 2nd round), but only two lines grew up to sufficient scales. Stable cell mixture transfected with empty pSUPER vector was designated as MshV, and individual clones with reduced phogrin expression were designated as MshP (MshP#33 and MshP#44). Insulin-producing cell lines from β-cell–specific insulin receptor–knockout (βIRKO) mice were established as described previously (37). Control cell lines (wild type) were from littermates of βIRKO mice. Mouse pancreatic islets were isolated and cultured as described previously (38).

Adenovirus production. H1-RNA promoter and the inserted DNA were cut by *Sma*I and *Hinc*II and then ligated into the promoterless cosmid pAdex vector (pAxcw; TaKaRa Biomedicals, Otsv, Japan). A full-length mouse phogrin cDNA was transferred to the pAdex vector (pAxCAwt). Viral production and propagation was generated using HEK 293 cells. Positive clones were selected by immunofluorescence analysis of MIN6 cells with anti-phogrin antibody. Purified adenoviruses were prepared by CsCl density gradient centrifugation.

Insulin content and secretion. After 6 h from adenoviral infection, cells (~1.5×10^6 MIN6 cells/well, ~2×10^6 INS-1 cells/well) were seeded into a six-well plate for culture. The cells were extracted with acid ethanol for 15 h at 4°C. After clarification of the extracts by centrifugation, the insulin concentration was measured by radioimmunoassay (RIA) (Eiken Chemical, Tokyo, Japan). The infected cells were preincubated in modified Krebs-Ringer buffer (KRB) (39) containing 2 mmol/l glucose for 2 h and then incubated with KRB supplemented with glucose at 25 mmol/l (for MIN6) or 16.8 mmol/l (for INS-1) for 30 min. The cell number and the cellular protein content were measured in all secretion experiments. Similar cell number was maintained among all the samples. Thereby, normalized secreted insulin to either cell number, total cellular proteins, or total insulin content did not show any difference (data not shown).

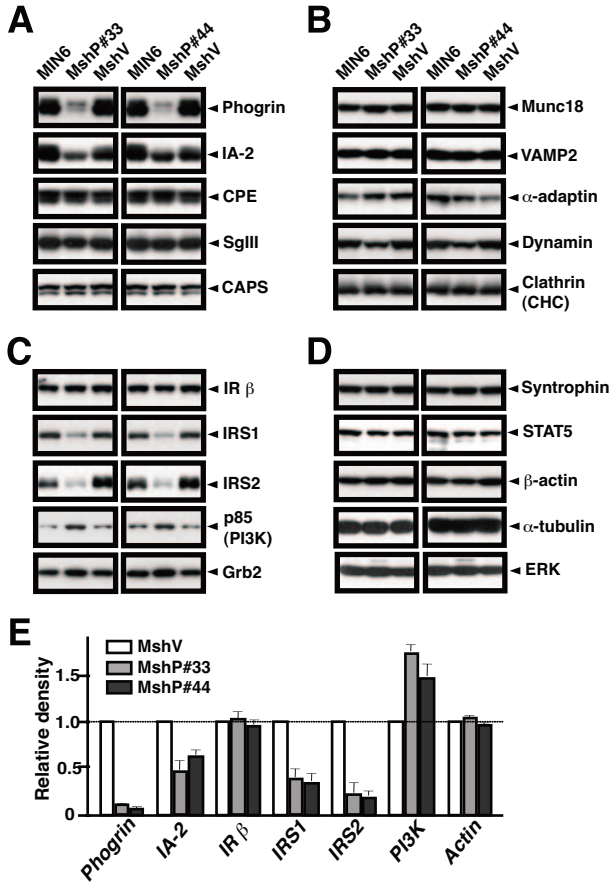

FIG. 1. Expression levels of insulin signaling proteins are changed in phogrin-silencing stable cells. *A–D*: Cell extracts were prepared from MIN6 control cells, shVector-expressing stable cells (MshV), and two distinct shPh1-expressing stable cells (clones 33 and 44). Each extract normalized for total protein content was subjected to SDS-PAGE/immunoblot analysis. Blots were probed with specific antibodies of four categories: secretory granules-associated proteins (*A*), proteins related to vesicle trafficking (*B*), insulin signaling pathway proteins (*C*), and others (*D*). *E*: Intensity of each band was quantified with the densitometric imager, and the results from three independent experiments are presented as fold increases ± SE compared with MshV.

Assessment of cell growth. After 6 h from adenoviral infection, MIN6 and INS-1E cells were seeded into six-well plates at 0.5×10^6 and 1.5×10^6 cells/well, respectively. At an indicated time, cells were collected, and the cell number was measured by a CyQUANT cell proliferation assay kit (Invitrogen). The proliferative activity of infected cells was detected by BrdU incorporation. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to calculate the rate of BrdU-positive cells. DNA synthesis rates were measured by [^{3}H]thymidine incorporation into islet cells. After 16 h from adenoviral infection, mouse islets were cultured for 48 h. [^{3}H]thymidine was added at a final concentration of 1 mCi/ml to pools of 50 islets for an additional 24 h. The proteins and DNA were precipitated with ice-cold 10% trichloroacetic acid and solubilized in 0.3 N NaOH. Aliquots were counted in scintillation fluid and assayed for protein using the Bradford method.

Immunoprecipitation and immunoblotting analyses. Immunoprecipitation and immunoblot analyses were performed as described previously (36). For immunoprecipitation, MIN6 cells were extracted with lysis buffer (20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.5% Nonidet P-40, 1 mmol/l EGTA, 0.5 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Band density was measured by densitometry, quantified using Gel plotting macros of NIH image 1.62 program, and normalized to an indicated sample in the identical membrane.

Subcellular fractionation. Subcellular fractionation was performed as described previously (39). Cells were suspended in buffer containing 250 mmol/l sucrose, 20 mmol/l HEPES (pH 7.4), 2 mmol/l $MgCl_2$, 2 mmol/l EGTA, and the protease inhibitors. The cells were homogenized for 40 strokes by the tight-fitting dounce homogenizer. The total homogenate was centrifuged at $700 \times g$ for 15 min to pellet the nuclear and intact plasma membrane fraction. The resultant supernatant was then centrifuged at $12{,}000 \times g$ for 20 min to separate the heavy

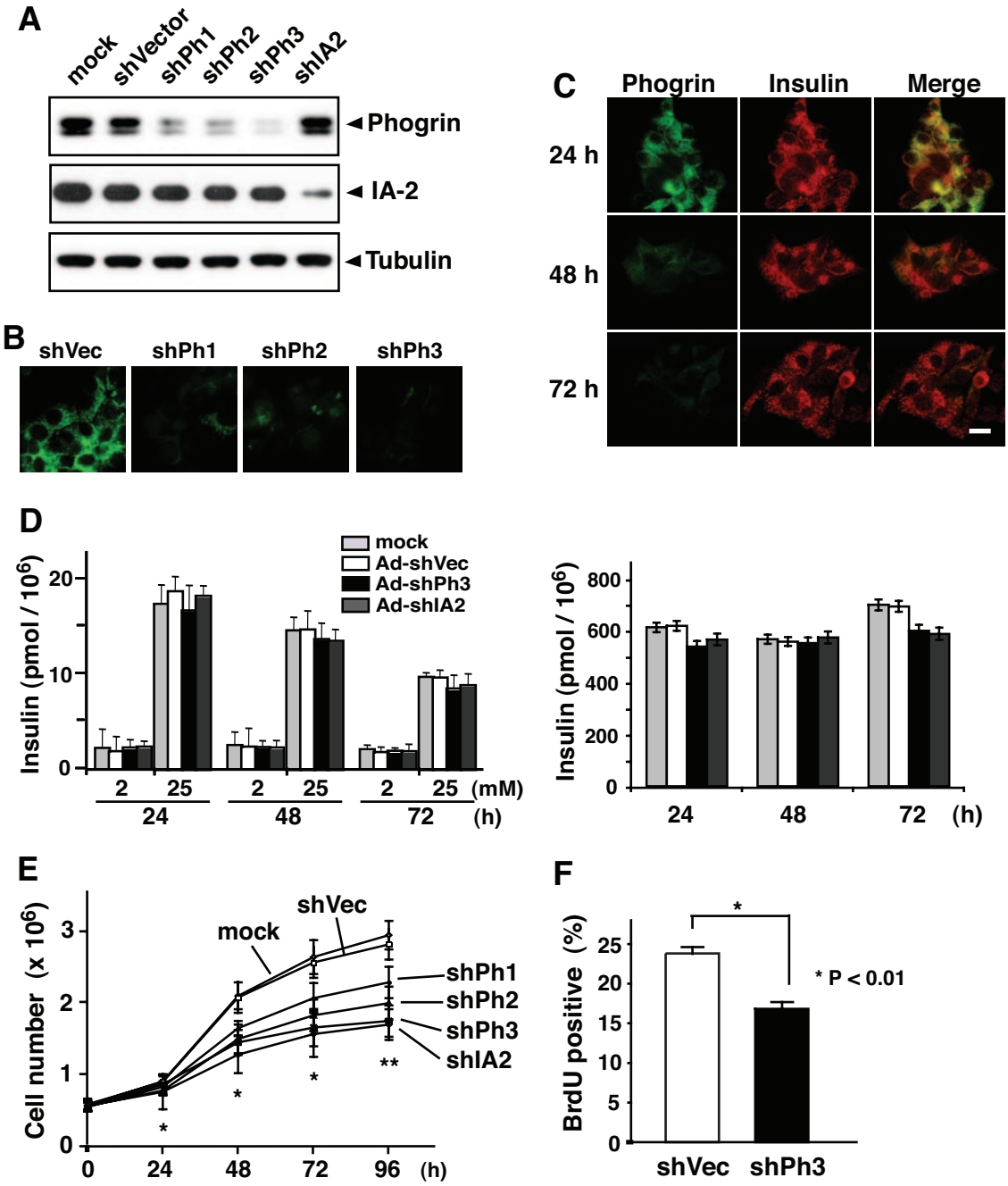

FIG. 2. Effects of shRNA-expressing adenoviruses on GSIS and the proliferation of MIN6 cells. *A*: MIN6 cells were infected with adenoviruses integrating shVector, shPh1, shPh2, shPh3, or shIA2 for 72 h. The expression levels of endogenous phogrin and IA-2 proteins were determined by immunoblotting with specific antibodies for phogrin and IA-2. α-Tubulin is a nontargeting control. *B*: MIN6 cells infected with Ad-shVec, shPh1, shPh2, or shPh3 were immunostained with anti-phogrin antibody. *C*: MIN6 cells expressing shPh3 were fixed at time points 24, 48, and 72 h. Cells were then immunostained with anti-phogrin and anti-insulin antibodies. *D*: The infected cells were incubated for 2 h in modified KRB (2 mmol/l glucose) before high-glucose stimulation. After stimulation for 30 min with 2 or 25 mmol/l glucose, culture media (*left*; secretion) and cells (*right*; content) were collected and subjected to RIA for insulin. Data are given as means ± SE of five independent experiments. *E*: Time course of MIN6 cell proliferation 24–96 h after infection with Ad-shVec, shPh1, shPh2, shPh3, or shIA2. Cell number was measured as indicated in RESEARCH DESIGN AND METHODS. Phogrin-KD or IA2-KD cells grew more slowly than the shVec-infected cells (*$P < 0.001$, **$P < 0.005$). *F*: Cells infected by Ad-shVec or shPh3 for 72 h were assayed for BrdU incorporation by immunofluorescence analysis. Experiments were performed four times ($n = 12$). (Please see http://dx.doi.org/10.2337/db08-0970 for a high-quality digital representation of this figure.)

organelle fraction, including the secretory granules, from the cytoplasmic materials. Fractionates were lysed, and equal proportions of each lysate were subjected to immunoprecipitation and immunoblotting analysis.

Semiquantitative RT-PCR. Quantitative RT-PCR analysis was performed as described previously (40). PCRs were performed using the following specific primers: phogrin, 5′-AGCCACGGTACCTTGTACAT-3′ and 5′-TTGTATGGCTCCAGCAACTG-3′ (237 bp); IRS2, 5′-TATCGCCATCGATGTGAGAGG-3′ and 5′-GTAGCGCTTCACTCTTTCACG-3′ or 5′-GCAGCACTTTACTCTTTCACC-3′ (322 bp). Amplified signals stained with ethidium bromide were quantified by ATTO cool saver system.

Statistical analysis. Results are given as the mean ± SE, except where indicated otherwise. Differences between groups were analyzed using Student's *t* test. *P* values <0.05 were considered statistically significant.

RESULTS

Specific knockdown of Phogrin in β-cell lines using adenovirus-delivered shRNA. We developed a new antibody (MatN2) with a high specificity to phogrin by immunoblotting and immunostaining. To compare the

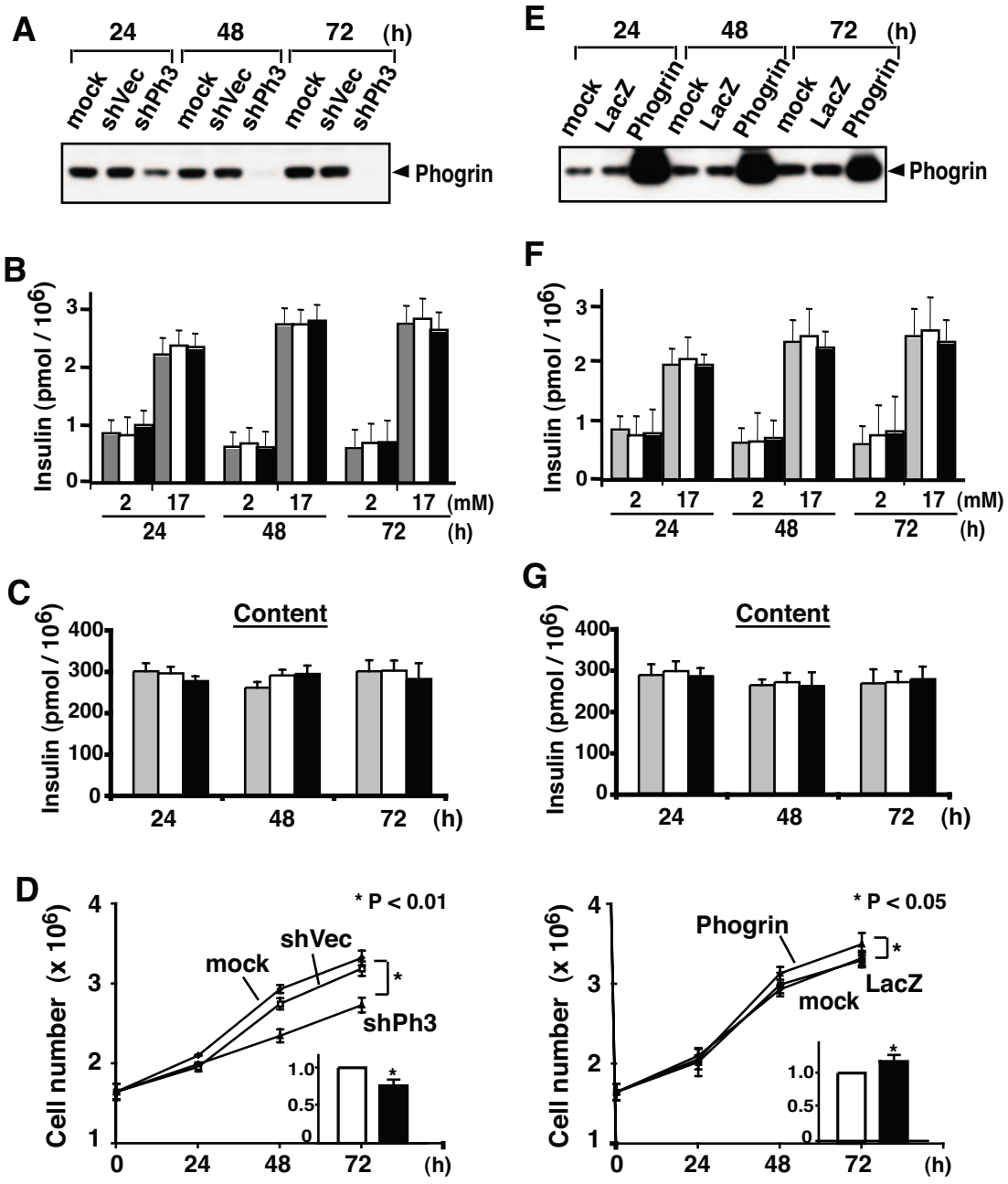

FIG. 3. Effects of phogrin repression and overexpression on INS-1E cells. INS-1E cells were infected with adenoviruses integrating shVector or shPh3 (*A–D*) or infected with adenoviruses expressing β-galactosidase (LacZ) or phogrin (*E–H*). *A* and *E*: Cell extracts were prepared, and each extract was subjected to immunoblotting with anti-phogrin antibodies. The infected cells were incubated in KRB with 2 or 16.8 mmol/l glucose for 30 min. Secreted insulin (*B* and *F*) and intracellular total insulin (*C* and *G*) were measured by RIA and normalized to the total cell number. Data are given as means ± SE of at least four independent experiments. *D* and *H*: Cell growth was assessed as shown in Fig. 2*E* and *F*. *Insets* show the BrdU incorporation rate at 72 h that is presented as the fold increases ± SE compared with control ($n = 3$, $^*P < 0.01$). *B* and *C*: ▨, mock; □, shVec; ■, shPh3. *F* and *G*: ▨, mock; □, LacZ; ■, Phogrin.

phogrin expression in various endocrine cell lines, whole-cell extracts were immunoblotted with MatN2. Phogrin was highly expressed in two pancreatic β-cell lines, MIN6 and βHC9, whereas other cell lines, including rat INS-1, expressed a lower level of phogrin (supplementary Fig. S1*A*, available in an online appendix at http://dx.doi.org/10.2337/db08-0970). We further produced an antibody specific to IA-2 and obtained a similar result (supplementary Fig. S1*B*). However, we noted that IA-2 expression was inconstant and changeable by cell culture conditions (data not shown), which was consistent with the previous observations (32–34). From these results, we chose MIN6 (high expression type) and INS-1 (moderate expression type) for the knockdown study.

We produced three kinds of plasmids that direct the synthesis of shRNAs targeted against the phogrin sequence (shPh1, shPh2, and shPh3). Transient expression of each shRNA plasmid selectively reduced the production of phogrin–enhanced GFP (EGFP) but not the control EGFP (data not shown); however, endogeneous phogrin was not sufficiently decreased in MIN6 cells because DNA transfection is not efficient by conventional transfection methods. Thus, we initially established shRNA-expressing stable cell lines, termed MshP#33 and MshP#44 (see

RESEARCH DESIGN AND METHODS). Immunoblot analysis with MatN2 confirmed silencing of the endogenous phogrin (Fig. 1*A* and *E*). Simultaneously, expression levels of various proteins were also examined, including IA-2 (28), α-adaptin (36), syntrophin (25), STAT5 (27), and CAPS (41), all of which have been reported to interact with IA-2 family proteins directly or indirectly. IA-2 protein levels in MshP#33 and MshP#44 were less than those in control cells; however, other secretory granule–resident proteins and proteins involved in the exo/endocytosis were not altered (Fig. 1*A* and *B*). Unexpectedly, we noted that insulin signaling pathway-composing proteins were significantly affected: IRS1 and IRS2 were nearly undetectable, and PI 3-kinase p85 and Grb2 were high (Fig. 1*C* and *E*). Other proteins were unaffected in expression (Fig. 1*D*).

Because proliferation of MshP#33 and MshP#44 was extremely slow, we found it difficult to keep these cells invariant throughout a number of experiments. Therefore, we generated an adenovirus expression system to achieve high efficiency shRNA expression. MIN6 cell lysates were prepared at 72 h after the infection with adenoviruses bearing shPh1, shPh2, shPh3, shIA2, or control vector. By immunoblotting, endogenous phogrin was reduced by 60∼90% in shPhogrin-expressing cells, and Ad-shPh3 was most effective (Fig. 2*A*). The knockdown effect was specific, because expression levels of IA-2 and α-tubulin were unaffected. Thus, reduction of IA-2 protein in MshP#33 and MshP#44 cells may have resulted from long-term cell cloning. Similarly, specific knockdown of IA-2 was accomplished by adenovirus shIA2 (Fig. 2*A*). Furthermore, the silencing effects of adenoviral shPhogrin were verified by immunostaining (Fig. 2*B*). Phogrin expression was reduced at 24 h after infection and most signals were disappeared at 72 h in the shPh3-expressing MIN6 cells (Fig. 2*C*). Insulin staining patterns were unchanged in these phogrin-knockdown cells.

Phogrin regulates β-cell growth. To assess the involvement of phogrin in the β-cell secretory function, GSIS and total insulin content were determined in MIN6 cells. Knockdown of phogrin or IA-2 did not result in any notable effects on GSIS and insulin content (Fig. 2*D*). Similar results were shown for INS-1E cells, which displayed no significant difference in GSIS and insulin content between shPh3-expressing cells and control cells (Fig. 3*A–C*). Conversely, adenovirus-mediated phogrin overexpression did not affect GSIS and insulin content in INS-1E cells (Fig. 3*E–G*). These observations indicate that insulin secretion in the β-cell lines remained unaltered when phogrin or IA-2 was attenuated or increased.

Next, we determined the cell proliferation rate by measuring cell number. Silencing of phogrin or IA-2 reduced MIN6 cell growth in a time-dependent manner (Fig. 2*E*). No proapoptotic effects were observed at 72 h [5.8 (shVec), 6.7 (shPh1), 6.2 (shPh2), 5.7 (shPh3), and 6.8 (shIA2) % cell death rates]. The proliferation rates of shPh1, shPh2, shPh3, and shIA2-infected MIN6 cells, assessed at 72 h after infection, were 84.0, 77.3, 72.5, and 69.9%, respectively, compared with shVector-infected cells ($P < 0.001$). Moreover, the proliferation rate by BrdU incorporation per DAPI-positive cells revealed a 30% decrease of BrdU incorporation in shPh3-expressing cells (Fig. 2*F*). We further confirmed the similar inhibitory effect of phogrin knockdown on the proliferation of INS-1E cells (Fig. 3*D*). Consistently, adenoviral phogrin overexpression slightly enhanced the proliferation of INS-1E cells (Fig. 3*H*). MIN6 cells were not affected by phogrin overexpression (data not shown) perhaps because of a high expression of endogenous protein. To test the effects of Ad-shPhogrin on islet cell replication, mouse islets were incubated with [methyl-^{3}H]thymidine. Phogrin knockdown caused a 36% decrease in [^{3}H]thymidine incorporation into islet cell DNA (Fig. 4*A*).

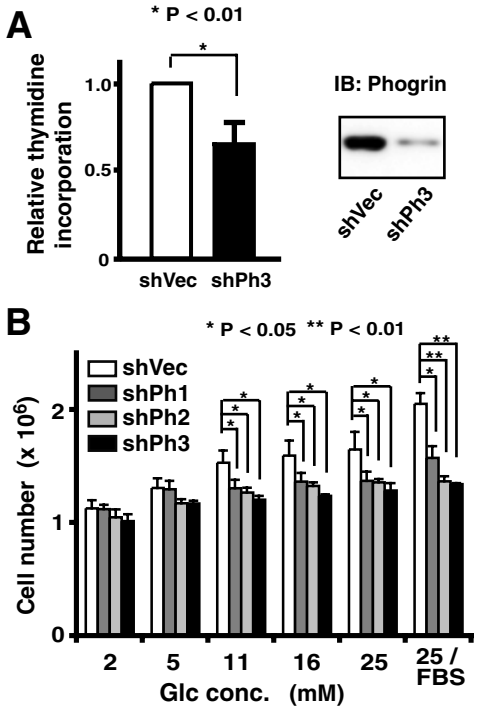

FIG. 4. Phogrin regulates β-cell growth. *A*: Mouse islets were infected with adenoviruses integrating shVector or shPh3. Uptake of [methyl-^{3}H]thymidine into islet cell DNA and total protein concentration were measured. Data are presented as the fold increases ± SE compared with shVector ($n = 5$, *$P < 0.01$). Cell lysates at 80 h were collected and subjected to immunoblot (IB) analysis with phogrin antibody (*right*). *B*: MIN6 cells were infected with adenoviruses integrating shVector, shPh1, shPh2, or shPh3. After 24 h, cells were placed in DMEM containing different glucose concentrations and cultured for another 24 h. Cell growth was assessed as shown in Fig. 2*E*. Data are given as means ± SE of four independent experiments.

Because glucose is a potent regulator of β-cell growth and both MIN6 and INS-1 cells are glucose responsive, we examined whether phogrin modulates the glucose-dependent mitogenesis in MIN6 cells. MIN6 growth was abolished by the phogrin knockdown in response to increasing glucose concentrations from 11 to 25 mmol/l, although the growth rate under the low-glucose culture (<5 mmol/l) was not influenced (Fig. 4*B*).

Phogrin regulates stability of IRS2 protein. Previous reports suggested that insulin/IGF-I signaling (IIS) provides β-cell mass (6). To address the function of phogrin, we investigated IIS-associated activity and expression of growth-related molecules. Phogrin knockdown in MIN6 cells resulted in a marked reduction in IRS2 protein level (Fig. 5*A*), as seen in shPhogrin stable cell lines (Fig. 1). Reduction of the IRS2 level was evident in phogrin-knockdown cells >48 h after infection, whereas such reduction was undetected in shVector-infected cells even at 72 h (Fig. 5*A*). IRS2 reduction was partially dependent on the multiplicity of infection (MOI) number of adenoviruses (Fig. 5*B*). In addition, insulin receptor was slightly decreased in the cells having a higher degree of phogrin knockdown (Fig. 5*A* and *B*). On the other hand, the silencing of IA-2 protein caused a modest decrease in IRS2 protein but a marked reduction of an insulin receptor level

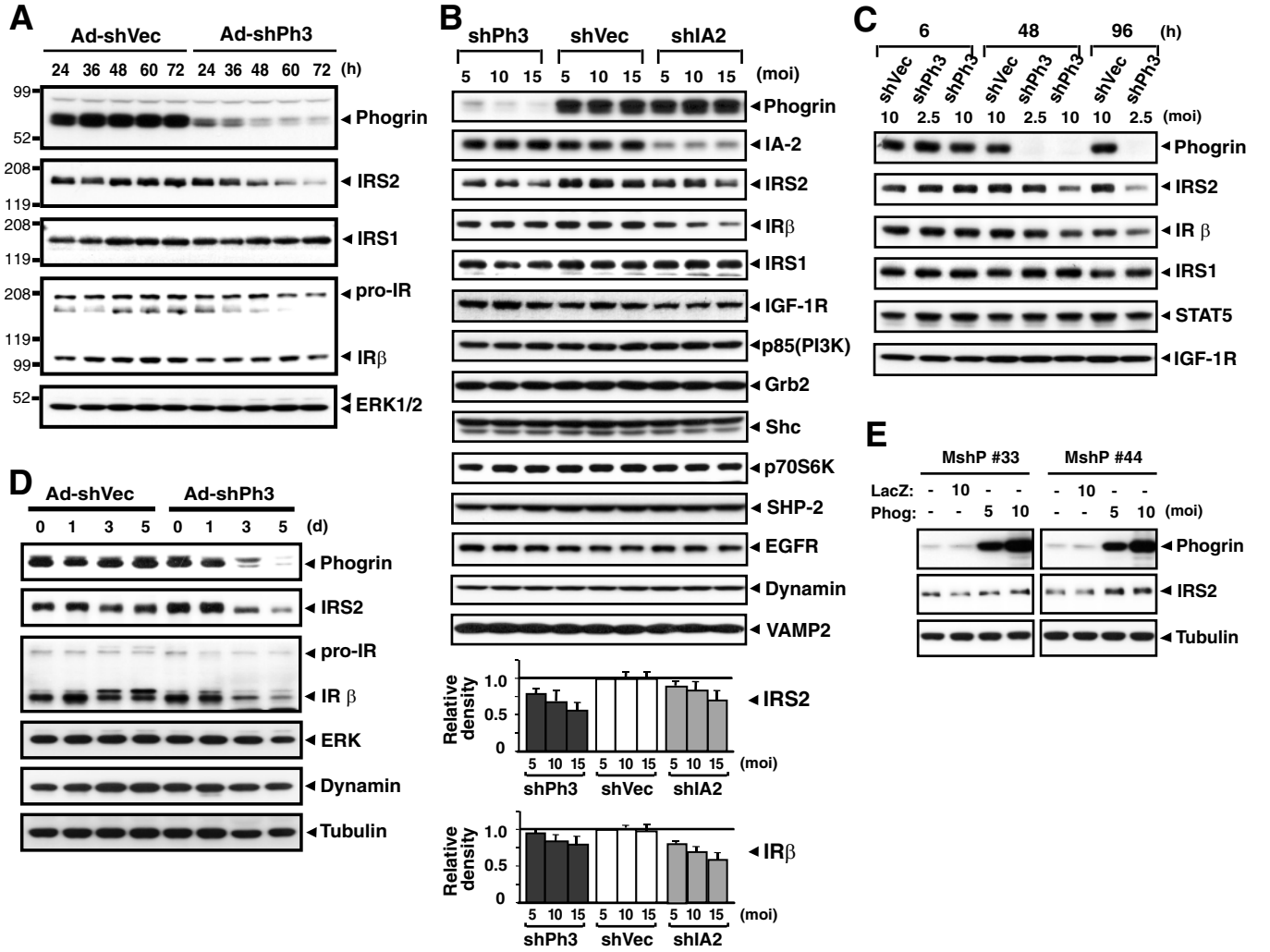

FIG. 5. Effects of phogrin repression on various protein levels. *A*: MIN6 cells were infected with 15 MOI of adenoviruses integrating shVector or shPh3 and then cultured for up to 72 h with medium changes every 24 h. Cell extracts were prepared at every 12-h time point, and each extract normalized for total protein content was subjected to SDS-PAGE/immunoblot analysis. Blots were probed with specific antibodies as shown on the *right*. *B*: MIN6 cells were infected with increasing MOI of adenoviruses integrating shVector, shPh3, or shIA2. After culture for 48 h, cell extracts were prepared and analyzed by immunoblotting. Blots were probed with specific antibodies as shown on the *right*. Intensity of each band was quantified with the densitometric imager, and the results from three independent experiments are presented as fold increases ± SE compared with 5 MOI of MshV. *C*: INS-1 cells were infected with increasing MOIs of adenoviruses integrating shVector or shPh3 and then cultured for up to 96 h. Cell extracts were analyzed by immunoblotting with antibodies to phogrin, IRS2, IRβ, IRS1, STAT5, and IGF-IR. *D*: Isolated mouse islets were infected with Ad-shVec or shPh3 and then cultured for up to 5 days. Each extract normalized for total protein content was subjected to SDS-PAGE/immunoblot analysis. *E*: Stables cells (33 and 44) were infected with adenoviruses expressing LacZ or phogrin at MOI of 5 or 10. Cell extracts were analyzed by immunoblotting with anti-phogrin and anti-IRS2 antibodies.

(Fig. 5*B*). No significant changes were noted in other proteins, including IRS1 and other unrelated proteins, suggesting the specific reduction of IRS2 and insulin receptor. Similar reduction was also observed in INS-1E cells (Fig. 5*C*) and mouse islets (Fig. 5*D*) in a time-dependent manner. Furthermore, adenoviral phogrin overexpression caused a partial recovery of IRS2 level in shPhogrin stable cell lines (Fig. 5*E*), suggesting that phogrin specifically regulates IRS2 expression in β-cells.

Recent studies have shown that glucose in the physiological range (5–15 mmol/l) promotes IRS2 expression at the transcriptional level (42); however, chronic exposure to high glucose (>15 mmol/l) decreases IRS2 protein levels via proteasomal degradation in β-cells (43). We examined whether IRS2 protein is reduced either by transcriptional regulation or by protein modulation in the phogrin-knockdown cells. Phogrin mRNA was almost silenced at 24 h, and its decline was faster than that of protein, as expected (Fig. 6*A* and *B*). In contrast, IRS2 protein was decreased starting 48 h later, whereas IRS2 mRNA level was barely affected even at 72 h (Fig. 6*A* and *B*). This suggests that phogrin regulates the IRS2 expression at the protein level but not at the mRNA level. Consistently, IRS2 reduction was completely blocked by the proteasomal inhibitors lactacystin and MG-132 (Fig. 6*C*). Thus, IRS2 protein appears unstable and is rapidly degraded by the proteasome in the phogrin-knockdown cells. Inhibitors for autophagic/lysosomal protein degradation had virtually no effect on this event (Fig. 6*C*). Furthermore, IRS2 protein stability was assessed by treatment of the translational inhibitor cycloheximide (42). IRS2 protein levels in the phogrin-knockdown cells decayed more rapidly than those in control cells (Fig. 6*D*). Taken together, phogrin plays an essential role in the stability of IRS2 protein but not in its gene transcription.

Phogrin forms a complex with insulin receptor. To elucidate the phogrin function, an in vitro binding assay

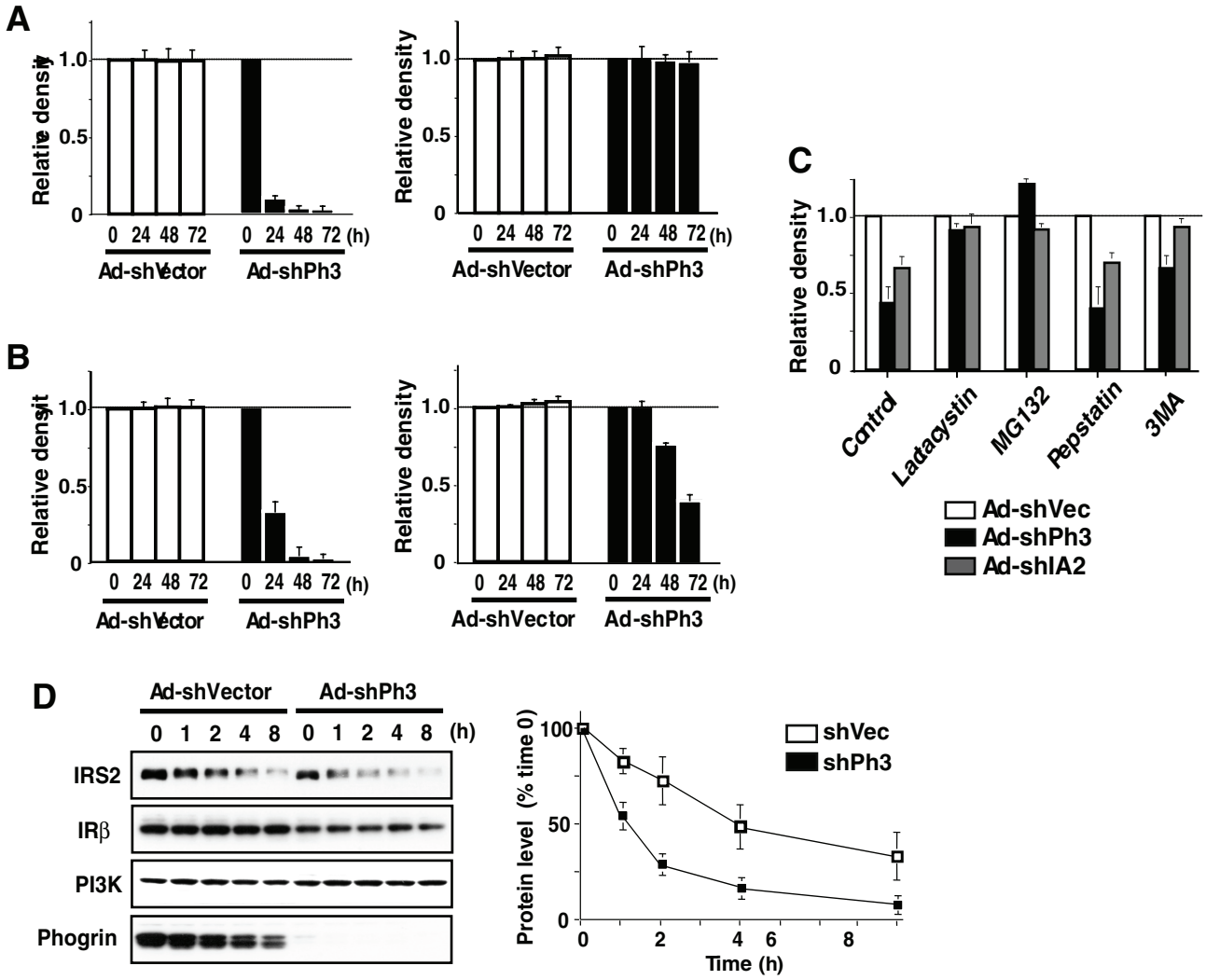

FIG. 6. Phogrin regulates IRS2 protein level. MIN6 cells infected with adenoviruses integrating shVector or shPh3 were divided into two dishes and cultured for up to 72 h with medium changes every 24 h. *A*: Total RNAs were prepared at every 24-h time point, and each RNA was analyzed by a quantitative RT-PCR analysis with probes for phogrin mRNA (*left panel*) and IRS2 mRNA (*right panel*). Data are presented as fold increases ± SE compared with uninfected control cells (time 0). Experiments were performed three times in duplicate. *B*: Cell extracts were prepared at every 24-h time point, and each extract normalized for total protein content was analyzed by immunoblotting with antibodies to phogrin protein (*left panel*) and IRS2 protein (*right panel*). The intensity of each band was quantified with the densitometric imager, and the results are presented as fold increases ± SE compared with uninfected control cells (time 0). *C*: IRS2 protein. MIN6 cells were infected with adenoviruses integrating shVector, shPh3, or shIA2. After 36 h, cells were cultured for 20 h more in the presence or absence of 10 μmol/l lactacystin, 20 μmol/l MG-132, 10 μg/ml pepstatin A, or 5 mmol/l 3-methyladenine (3MA). Cell extracts were analyzed by immunoblotting with anti-IRS2 antibody. The intensity of each band was quantified with the densitometric imager, and the results are presented as fold increases ± SE compared with shVector. *D*: MIN6 cells were infected with adenoviruses integrating shVector or shPh3. After 40 h, cells were incubated with the translational inhibitor cycloheximide (10 μg/ml) for 0–8 h, and then IRS2, IRβ, PI 3-kinase (p85), and phogrin protein levels were measured by immunoblotting. Accumulative data are presented as a percentage of the IRS2 protein levels at time 0 as means ± SE (n = 3).

was first performed. Sodium vanadate-treated MIN6 cell extracts were incubated with bacterially expressed GST-phogrin, and bound proteins were detected by immunoblotting with anti–phospho-tyrosine antibodies. Specific bands ~95, 100, and 130 kDa were detected in the membrane fraction to bind to GST-phogrin but not to GST alone (supplementary Fig. S2, available in the online appendix). The result indicates that tyrosine-phosphorylated transmembrane proteins are candidates as the phogrin-binding molecules. Coimmunoprecipitation experiments using MIN6 cell lysates revealed that both phogrin and IA2 immunoprecipitates contained a significant amount of insulin receptor β-subunit (IRβ) without its precursor (Fig. 7*A*; supplementary Fig. S3*A*, available in the online appendix). IRβ was not detected by the control antibodies. Furthermore, IRβ was barely found in the phogrin immunoprecipitates from the phogrin-knockdown cells (Fig. S3*B*). These results suggest that phogrin specifically interacts with the mature form of insulin receptor. Unexpectedly, IRS2 and IGF-IR were not coimmunoprecipitated with phogrin and IA-2 (Fig. 7*A*). When MIN6 cells were cultured in low-glucose condition, their interaction was detected at a low level by coimmunoprecipitation (Fig. 7*B*, 0-h point). Importantly, when cells were placed into the high-glucose culture, IRβ content increased dramatically in the phogrin immunoprecipitates, although there was no change in the phogrin and insulin receptor expression levels (Fig. 7*B*). Notably, IRS2 apparently increased during the high-glucose stimulation in MIN6 cells (Fig. 7*B*, *right panel*), as previously shown for primary rat β-cells (42). Blockade of secretory granule exocytosis by diazoxide, a potassium channel opener, completely prevented the coimmunoprecipitation of insulin receptor (Fig. 7*C*).

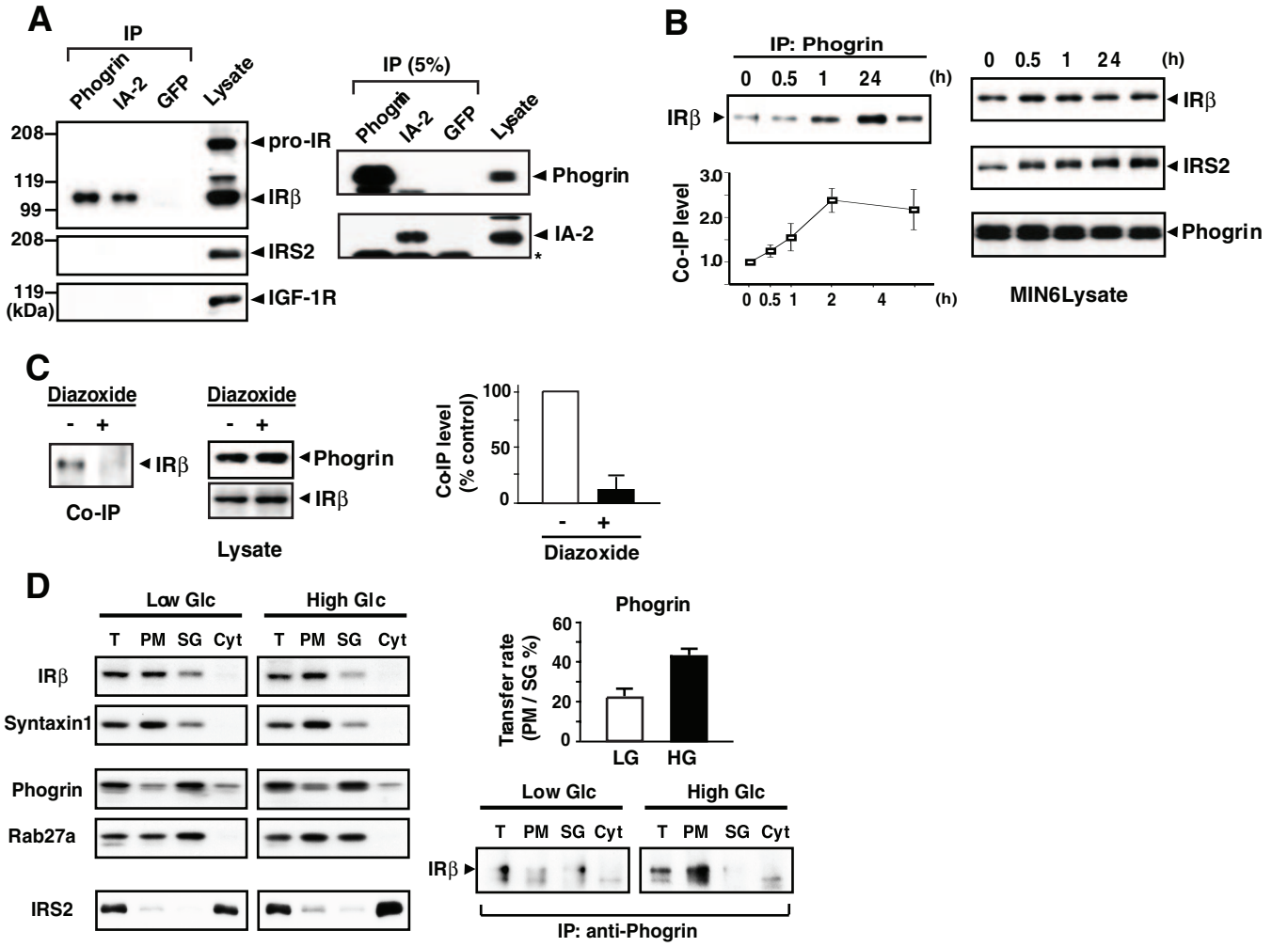

FIG. 7. Phogrin interacts with insulin receptor. *A*: MIN6 cell extracts (4 mg each) were incubated with anti-phogrin, anti–IA-2, or anti-GFP antibodies. Each immunoprecipitate and an aliquot of the original lysates (15 μg) were analyzed by immunoblotting with antibodies against IRβ, IRS2, and IGF-IR (*left*). *Right panels* show the immunoprecipitated level of phogrin or IA-2. Five percent of each immunoprecipitate and the original lysates (10 μg) were analyzed by immunoblotting. *Immunogloblin. *B*: MIN6 cells were stimulated with serum-free DMEM containing high glucose for an indicated time, after preincubation of low glucose for 2 h. Cell extracts were prepared, an equal amount of each extract was immunoprecipitated with anti-phogrin antibody, and then the amount of insulin receptor in each precipitate was determined by immunoblotting (*left*). The intensity of each band was quantified with the densitometric imager, and the coimmunoprecipitation (co-IP) levels are presented as fold increases ± SE compared with low-glucose incubation (time 0). Cell extracts were analyzed by immunoblotting with antibodies to IRβ, IRS2, and phogrin (*right*). *C*: MIN6 cells were stimulated for 1.5 h with high glucose in the presence or absence of 200 μmol/l diazoxide. Interaction of phogrin with insulin receptor was assessed by the coimmunoprecipitation analysis as in *B* (*left panel* and *bottom graph*). *D*: MIN6 cells were incubated with serum-free DMEM containing low glucose or high glucose for 4 h. The cells were extracted and fractioned by the method described in RESEARCH DESIGN AND METHODS. Equal proportions of the fractions were immunoprecipitated with anti-phogrin antibody, and then the amount of insulin receptor in each precipitate was determined by immunoblotting (*right bottom panels*). Equal proportions of the fractions were analyzed by immunoblotting with antibodies for IRβ, syntaxin 1, phogrin, Rab27a, or IRS2 (*left*). The subcellular fractions are designated as follows: T, total homogenate; PM, plasma membrane; SG, secretory granules; Cyt, cytosol. The amount of phogrin in the plasma membrane or the secretory granule was quantitated using densitometry and quantification of its translocation from secretory granule to plasma membrane are shown in a bar graph ($n = 3$).

Thus, the high-glucose culture promotes molecular interaction of phogrin and insulin receptor presumably via the translocation of phogrin into the plasma membrane. To confirm this idea, a conventional fractionation procedure was used. Under the low-glucose culture, phogrin and peripherally granule-associated Rab27a (39) were mainly distributed in the secretory granule fraction (Fig. 7*D*). The high-glucose stimulation induced significant redistributions of phogrin and Rab27a from secretory granule to the plasma membrane fraction, whereas the plasma membrane-associated insulin receptor and syntaxin 1 remained in the plasma membrane. Coimmunoprecipitation analyses using each fraction revealed that molecular interaction between phogrin and insulin receptor occurred at the plasma membrane under the high-glucose condition (Fig. 7*D*, *right bottom panel*). However, our results do not exclude a possibility that these proteins form a complex on secretory granule that attached to the plasma membrane (44).

Finally, we used two β-cell lines established from control (wild-type) and IRKO mice for proliferation assay. BrdU incorporation assay revealed that silencing of phogrin failed to show the growth retardation of IRKO β-cells, whereas a 33% decrease in growth rate was observed for wild-type β-cells (Fig. 8*A*). Furthermore, IRS2 protein degradation caused by phogrin knockdown was not observed in IRKO β-cells (Fig. 8*B* and *C*). This suggests that regulation of IRS2 stability and β-cell growth by phogrin depends on its interaction with insulin receptor.

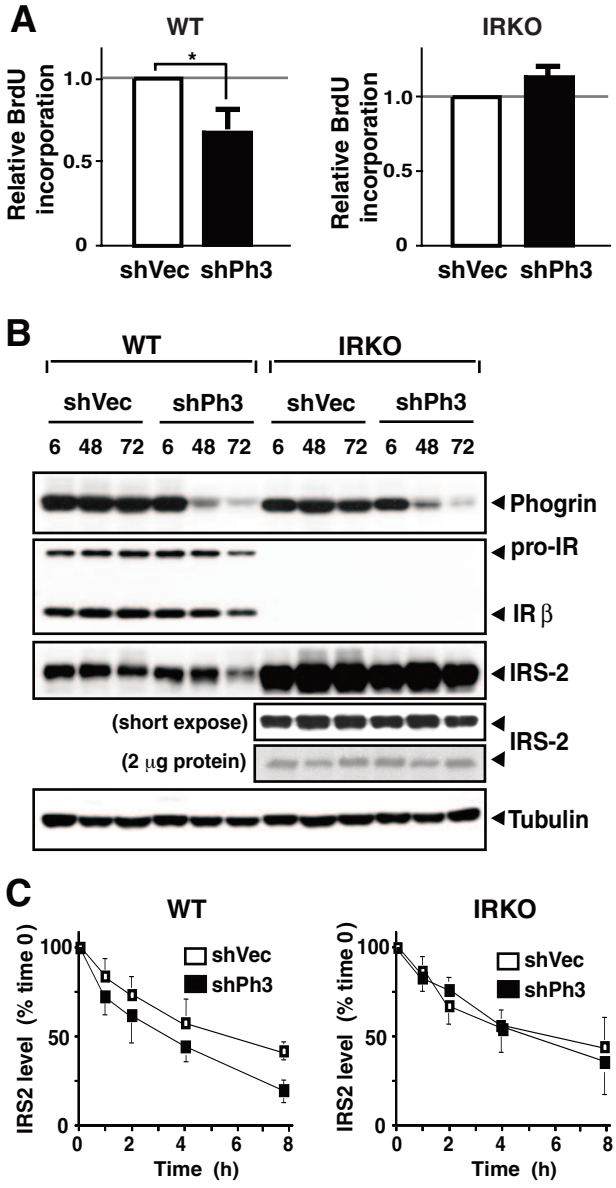

FIG. 8. Phogrin silencing had no effect on the proliferation of IRKO β-cells. *A*: Wild-type (WT) or insulin receptor–knockout (IRKO) β-cells were infected by adenoviruses of shVector or shPh3 for 48 h and were then assayed for BrdU incorporation by immunofluorescence analysis. Data are presented as the fold increases ± SE compared with shVector (n = 9, *P < 0.05). *B*: Cell extracts were prepared at every 24-h time point, and each extract normalized for total protein content was subjected to SDS-PAGE/immunoblot analysis. *C*: IRS2 protein stability in WT or IRKO β-cells was assessed as in Fig. 6*D*. Accumulative data are presented as a percentage of the IRS2 protein levels at time 0 as means ± SE (n = 3).

DISCUSSION

Proliferation rates of phogrin-downregulated MIN6 stable cells (Fig. 1) and phogrin-overexpressed stable cells (S. Torii and T. Takeuchi, unpublished data) were reduced and elevated, respectively. Thus, phogrin appeared to be a positive regulator of β-cell growth. Because studies using stable cells seem inadequate for evaluation of primary functions of phogrin, we generated adenoviruses expressing shRNA. As expected, silencing of phogrin by Ad-shPhogrin caused a marked retardation of cell growth in both cultured cell lines and mouse islets (Figs. 2–4). However, GSIS stayed unaffected by phogrin or IA-2 knockdown (Figs. 2 and 3). Previous studies have shown that the deletion of IA-2 or phogrin gene or in combination in mice resulted in mild impairment of GSIS but did not affect β-cell mass (19–21). However, more recent studies have suggested that islets from double knockout mice failed to show any secretion defect (45), and β-cell regeneration was reduced in partially pancreatectomized ICA512 (IA-2) knockout mice (46). Therefore, it is possible that the alteration in GSIS in knockout mice is indirect and that β-cell mass is recovered by compensatory function of other genes. Our present results support the notion that the primary function of phogrin is a regulation of β-cell growth but not of insulin secretion.

Glucose is a potent mitogen for β-cells and regulates various cellular dynamics, including insulin secretion and nutritional metabolism (1). Although several signaling molecules such as PKC have been thought to be a mediator for glucose-induced β-cell growth (1), recent studies have indicated a novel pathway in which the autocrine/paracrine function of secreted insulin promotes β-cell proliferation (3,4). We demonstrated that β-cell growth retardation induced by phogrin knockdown was observed under the high-glucose culture (Fig. 4*B*). Because phogrin was found to form a complex with insulin receptor (Fig. 7) and because cell growth retardation by phogrin knockdown was not observed in insulin receptor–deficient cells (Fig. 8), insulin receptor is evidently a functional target of phogrin. Phogrin localizes to insulin-containing secretory granules and translocates to the plasma membrane whenever insulin exocytosis is induced (Fig. 7*D*) (36). Thus, interaction of phogrin with insulin receptor is coordinately coupled with the autocrine action of insulin. Their interaction was strikingly promoted by the high glucose-induced insulin secretion (Fig. 7*B–D*). In other words, phogrin regulates the glucose-induced β-cell growth through modulating the autocrine insulin signaling. Insulin secretion was never influenced by overexpression or repression of phogrin (Figs. 2 and 3), thereby, modulation of autocrine effects by phogrin is not intervened by extracellular insulin dosage.

Our subcellular fractionation experiments indicate that phogrin interacts with insulin receptor on the plasma membrane (Fig. 7*D*). However, EGFP-tagged phogrin in MIN6 or PC12 cells did not spread to whole plasma membrane under the evanescent microscopy observation (44,47,48), suggesting that it remains on secretory granules during exocytotic events. On the other hand, the experimental result that phogrin antibody in culture medium accessed to the cell surface phogrin protein in MIN6 cells (49) suggests that phogrin positively reaches the cell surface. Thus, the plasma membrane fraction in our assay may contain the attached granules that had been connected to the plasma membrane by lipid bilayers merger. Because insulin receptor is reportedly distributed to the methyl-β-cyclodextrin–sensitive microdomains of the plasma membrane in HIT-T15 cells (50), it is possible that phogrin and insulin receptor colocalize at uncharacterized specific domains equivalent to the secretory granule targeting/fusion sites.

Our data indicate that phogrin interacts with insulin receptor and stabilizes IRS2 protein in β-cells. This hypothesis raises a question: What is the molecular mechanism of this interaction? Because phogrin and IA-2 have an inactive PTP domain in their cytoplasmic tail, it is possible that they bind to insulin receptor directly. In fact, the phogrin cytoplasmic fragment could bind to tyrosine-phosphorylated 95- to 100-kDa proteins in vitro (supple-

mentary Fig. S2). Phogrin may regulate the insulin signaling through the tyrosine phosphorylation/dephosphorylation cycle. Insulin or IGF-I promotes degradation of IRS2 through PI 3-kinase, Akt, and mTOR signaling in adipocytes, hepatoma, and embryonic fibroblast cells (51). Also, chronic exposure (>8 h) to high glucose and/or IGF-I induces proteasomal degradation of IRS2 in β-cells (43). These proposed signaling pathways may be mediated by phogrin function for stabilization of IRS2.

Ablation of IRS2 in β-cells led to a decrease in β-cell mass and an increase in islet apoptosis (5). In contrast, IRS2 expression in mice prevented diabetes by promoting β-cell growth (52). Furthermore, IRS2 expression induced by glucose, GLP-1, and other signaling proteins has been shown to contribute to their regulatory functions for proliferation, indicating that IRS2 is a master regulator of β-cell growth (14). Our data further indicate that IRS2 protein is stabilized under the control of phogrin and insulin receptor interaction for its cell growth regulation. This is because silencing of phogrin resulted in a marked reduction of IRS2 without change in other protein levels. Furthermore, adenoviral overexpression of phogrin caused a partial recovery of IRS2 level in MshP#33 and MshP#44 cells (Fig. 5*E*). Thus, phogrin functions as a primary regulator of secreted insulin-mediated β-cell growth by stabilizing IRS2 protein.

To respond to hyperglycemia, β-cells proliferate and expand to compensate for increased insulin secretion demand (14). The present observations suggested a novel mechanism in which phogrin contributes to glucose-induced proliferation of β-cells via insulin receptor and IRS2. Interestingly, insulin receptor and IRS2 mRNA levels reportedly decreased in islets isolated from human type 2 diabetes (53). To prevent the decrease in insulin receptor and IRS2 functions, we suggest that phogrin and IA-2 in the β-cells are potential therapeutic targets for treating diabetes.

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