Gene silencing of phogrin unveils its essential role in glucose-responsive pancreatic β-cell growth

Seiji Torii¹⁴, Naoya Saito¹⁴, Ayumi Kawano¹, Ni Hou¹, Kohjiro Ueki², Rohit N. Kulkarni³, and Toshiyuki Takeuchi¹*

¹Secretion Biology Lab, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma 371-8512, ²Department of Metabolic Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, ³Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston MA 02215, USA.

⁴These authors contributed equally to this work.

*Corresponding author:
Toshiyuki Takeuchi MD, Institute for Molecular and Cellular Regulation, Gunma University, Showa 3-39-15, Maebashi, Gunma 371-8512, Japan.
E-mail: tstake@showa.gunma-u.ac.jp

Submitted 1 May 2008 and accepted 5 December 2008.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org
Function of phogrin in β-cell growth

ABSTRACT

Objective - Phogrin and IA-2, autoantigens in insulin-dependent diabetes mellitus, have been shown to be involved in insulin secretion in pancreatic β-cell, 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 shRNA 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. Further, 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 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 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.
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 (IR) and insulin receptor substrate 2 (IRS2) (5-8). Although IR knockout had a restricted effect upon β-cell mass (7), its mitogenic function on β-cells was clearly shown by siRNA-based silencing of IR in β-cell-derived MIN6 cells (9;10). More recently, another pathway was demonstrated 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 GLP1 (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 (SGs) 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 have 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 SG 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 on the regulation of gene expression in concert with STAT5b (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 their interactions physiologically associate with 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 to 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 role of phogrin using pancreatic β-cells. Establishment of stable cell lines expressing 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. Further, we found that phogrin
Function of phogrin in β-cell growth

binds to IR 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 anti-α-tubulin and anti-β-actin mouse monoclonal antibodies were purchased from Sigma. Anti-adaptin, anti-carboxypeptidase E (CPE), anti-CAPS, anti-munc18, anti-dynamin, anti-clathrin heavy chain, anti-P13K, anti-Grb2, and anti-ERK mouse monoclonal antibodies and 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-EGFR sheep polyclonal antibodies were from Upstate Biotech. Anti-IGF1R, anti-IRβ, anti-syntrophin, and anti-BrdU mouse monoclonal antibodies were from Lab Vision, Chemicon, Affinity Bioreagents, and Roche Diagnostics, respectively. Anti-VAMP2 and 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), GCCACAACTCACACTCAA (shPhogrin2) (mouse: 1877-1895, rat: 1886-1904), and GGATACATCCTCACAGGAA (shPhogrin3) (mouse: 1492-1510, rat: 1501-1519) for phogrin, and GTCTGTATTACAGGATGGCT (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 μM of 2-mercaptoethanol. INS-1E cells were cultured in RPMI1640 with 10% FBS, 10 mM HEPES (pH 7.4), and 50 μM 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 βIRKO mice were established as described previously (37). Control cell lines (WT) 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 SmaI and HincII, and then ligated into the promoterless cosmids pAdex vector (pAxcw, TaKaRa Biomedicals, Japan). A full-length mouse phogrin cDNA was transferred to the pAdex vector (pAxCAw). 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 × 10⁶ MIN6
cells/well, ~2 × 10^6 INS-1 cells/well) were seeded into a 6-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, Japan). The infected cells were preincubated in modified Krebs-Ringer buffer (KRB) (39) containing 2 mM glucose for 2 h and then incubated with KRB supplemented with glucose at 25 mM (for MIN6) or 16.8 mM (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).

Assessment of cell growth. After 6 h from adenoviral infection, MIN6 and INS-1E cells were seeded into 6-well plates at 0.5 × 10^6 cells and 1.5 × 10^6 cells per 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 5-bromo-2'-deoxyuridine (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 [3H]thymidine incorporation into islet cells. After 16 h from adenoviral infection, mouse islets were cultured for 48 h. [3H]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 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EGTA, 0.5 mM PMSF, 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 mM sucrose, 20 mM HEPES (pH 7.4), 2 mM MgCl2, 2 mM 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 × g for 15 min to pellet the nuclear and intact plasma membrane fraction. The resultant supernatant was then centrifuged at 12,000 × g for 20 min to separate the heavy 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.

Semi-quantitative RT-PCR. Quantitative RT-PCR analysis was performed as described previously (40). PCRs were performed using the following specific primers: phogrin, 5'-AGCCACGGTGACTTGTACAT-3' and 5'-TTGTATGGCCTCCAGCAACTG-3' (237 bp); IRS2, 5'-TATCGCCATCGATGTAGGAG-3' and 5'-GCAGCACTTTACTCTTACCC-3' or 5'-GCAGACTTTACTCTTACC-3' (322 bp). Amplified signals stained with ethidium bromide were quantified by ATTO cool saver system.

Statistical analysis. Results are given as the mean ± standard errors of the means (SEMs), except where indicated otherwise. Differences between groups were analyzed using
Student’s t test. P values less than 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 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 (Online Appendix Fig. S1A). We further produced an antibody specific to IA-2 and obtained a similar result (Fig. S1B). 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, shPh3). Transient expression of each shRNA-plasmid selectively reduced the production of phogrin-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 Methods). Immunoblot analysis with MatN2 confirmed silencing of the endogenous phogrin (Fig. 1A 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 SG-resident proteins and proteins involved in the exo/endocytosis were not altered (Fig. 1A and B). Unexpectedly, we noted that insulin signaling pathway-composing proteins were significantly affected: IRS1 and IRS2 nearly undetectable, PI3K p85 and Grb2 high (Fig. 1C and E). Other proteins were unaffected in expression (Fig. 1D).

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. 2A). 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 a long-term cell cloning. Similarly, specific knockdown of IA-2 was accomplished by adenovirus shIA2 (Fig. 2A). Furthermore, the silencing effects of adenoviral shPhogrin were verified by immunostaining (Fig. 2B). Phogrin expression was reduced at 24 h post-infection and most signals were disappeared at 72 h in the shPh3-expressing MIN6 cells (Fig. 2C). 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. 2D). 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. 3A-C). Conversely, adenovirus-mediated phogrin overexpression did not affect GSIS and insulin content in INS-1E cells (Fig. 3E-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. 2E). No proapoptotic effects were observed at 72 h [5.8 (shVec), 6.7 (shPh1), 6.2 (shPh2), 5.7 (shPh3), 6.8 (shIA2) % cell death rates]. The proliferation rates of shPh1, shPh2, shPh3, and shIA2-infected MIN6 cells, assessed at 72 h post-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. 2F). We further confirmed the similar inhibitory effect of phogrin knockdown on the proliferation of INS-1E cells (Fig. 3D). Consistently, adenoviral phogrin overexpression slightly enhanced the proliferation of INS-1E cells (Fig. 3H). MIN6 cells were not affected by phogrin overexpression (data not shown) perhaps due to a high expression of endogenous protein. To test the effects of Ad-shPhogrin on islet cell replication, mouse islets were incubated with [methyl-3H]thymidine. Phogrin knockdown caused a 36% decrease in [3H]thymidine incorporation into islet cell DNA (Fig. 4A).

Phogrin regulates stability of IRS2 protein.

Recent studies have shown that glucose in the physiological range (5-15 mM) promotes IRS2 expression at the transcriptional level (42), however, chronic exposure to high glucose (HG: >15 mM) 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 response to increasing glucose concentrations from 11 to 25 mM, although the growth rate under the low glucose (LG) culture (< 5 mM) was not influenced (Fig. 4B).
Function of phogrin in \( \beta \)-cell growth

Phogrin forms a complex with insulin receptor. To elucidate the phogrin function, an in vitro binding assay 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 around 95, 100, and 130 kDa were detected in the membrane fraction to bind to GST-phogrin, but not to GST alone (Online Appendix Fig. S2). 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 IR \( \beta \)-subunit (IR\( \beta \)) without its precursor (Fig. 7A and S3A). IR\( \beta \) was not detected by the control antibodies.

Further, IR\( \beta \) was barely found in the phogrin-immunoprecipitates from the phogrin-knockdown cells (Fig. S3B). These results suggest that phogrin specifically interacts with the mature form of IR. Unexpectedly, IRS2 and IGF-1R were not coimmunoprecipitated with phogrin and IA-2 (Fig. 7A). When MIN6 cells were cultured in LG condition, their interaction was detected at a low level by coimmunoprecipitation (Fig. 7B, 0-h point). Importantly, when cells were placed into the HG culture, IR\( \beta \) content increased dramatically in the phogrin immunoprecipitates, although there was no change in the phogrin and IR expression levels (Fig. 7B). Notably, IRS2 apparently increased during the HG stimulation in MIN6 cells (Fig. 7B, right panel), as previously shown for primary rat \( \beta \)-cells (42). Blockade of SG exocytosis by diazoxide, a potassium channel opener, completely prevented the coimmunoprecipitation of IR (Fig. 7C). Thus, the HG culture promotes molecular interaction of phogrin and IR presumably via the translocation of phogrin into the plasma membrane (PM). To confirm this idea, a conventional fractionation procedure was employed. Under the LG culture, phogrin and peripherally granule-associated Rab27a (39) were mainly distributed in the SG fraction (Fig. 7D). The HG stimulation induced significant redistributions of phogrin and Rab27a from SG to the PM fraction, whereas the plasma membrane-associated IR and syntaxin 1 remained in the PM. Coimmunoprecipitation analyses using each fraction revealed that molecular interaction between phogrin and IR occurred at the PM under the HG condition (Fig. 7D, right lower panel). But, our results do not exclude a possibility that these proteins form a complex on SG that attached to the PM (44).

Finally, we used two \( \beta \)-cell lines established from control (WT) and IR knockout (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 WT β-cells (Fig. 8A). Further, IRS2 protein degradation caused by phogrin knockdown was not observed in IRKO β-cells (Fig. 8B and C). This suggests that regulation of IRS2 stability and β-cell growth by phogrin depends on its interaction with IR.

**DISCUSSION**

Proliferation rates of phogrin-downregulated MIN6 stable cells (Fig. 1) and phogrin-overexpressed stable cells (unpublished data) were reduced and elevated, respectively. Thus, phogrin appeared to be a positive regulator of β-cell growth. Since 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 (Fig. 2-4). However, GSIS stayed unaffected by phogrin or IA-2 knockdown (Fig. 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 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 HG culture (Fig. 4B). Since phogrin was found to form a complex with IR (Fig. 7), and cell growth retardation by phogrin knockdown was not observed in IR-deficient cells (Fig. 8), IR is evidently a functional target of phogrin. Phogrin localizes to insulin-containing SGs, and translocates to the plasma membrane whenever insulin exocytosis is induced (Fig. 7D) (36). Thus, interaction of phogrin with IR is coordinately coupled with the autocrine action of insulin. Indeed, their interaction was strikingly promoted by the HG-stimulated insulin secretion (Fig. 7B-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 (Fig. 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 IR on the plasma membrane (Fig. 7D). 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 SGs 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 PM fraction in our assay may contain the attached granules that had been connected to the PM by lipid bilayers merger. Since IR is reportedly distributed to the
methyl-beta-cyclodextrin-sensitive microdomains of the plasma membrane in HIT-T15 cells (50), it is possible that phogrin and IR colocalize at uncharacterized specific domains equivalent to the SG targeting/fusion sites.

Our data indicate that phogrin interacts with IR, and stabilizes IRS2 protein in β-cells. To this hypothesis, the question “What is the molecular mechanism of this interaction?” arises. As phogrin and IA-2 have an inactive PTP domain in their cytoplasmic tail, it is possible that they bind to IR directly. In fact, the phogrin cytoplasmic fragment could bind to tyrosine-phosphorylated 95-100 kDa proteins in vitro (Fig. S2). Phogrin may regulate the insulin signaling through the tyrosine phosphorylation/dephosphorylation cycle. Insulin or IGF-1 promotes degradation of IRS2 through PI3K, Akt, and mTOR signaling in adipocytes, hepatoma, and embryonic fibroblast cells (51). Also, chronic exposure (> 8 h) to HG and/or IGF-1 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 IR 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. 5E). 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-cell via IR and IRS2. Interestingly, IR and IRS2 mRNA levels reportedly decreased in islets isolated from human type 2 diabetes (53). To prevent the decrease in IR and IRS2 functions, we suggest that phogrin and IA-2 in the β-cells are potential therapeutic targets for treating diabetes.

ACKNOWLEDGMENTS
We would like to thank Dr. P. Maechler (Centre Médical Universitaire) for providing INS-1E cells, and Drs R. Agami (The Netherlands Cancer Institute), M Nishita (Kobe Univ.), S. Mizutani (Gunma Univ.) for technical support. We also thank Dr. M. Hosaka, M. Kosaki, and M. Hosoi for secretarial and technical support. This work was supported by a grant in aid for Young Scientists (to S.T.) and a grant of the Global Center of Excellence program (to T.T.), from the Ministry of Education, Culture, Sports, Science and Technology of Japan. It was also supported in part from the Uehara Memorial Foundation (to S.T.) and U.S. NIH Grant RO1 DK 67536 (to R.N.K.).

Abbreviations:
SG, secretory granule; shRNA, short hairpin RNA; siRNA, short interfering RNA; cAMP, cyclic adenosine monophosphate; IR, insulin receptor; IRS2, insulin receptor substrate 2; PTP, protein tyrosine phosphatase; GSIS, glucose-stimulated insulin secretion; βIRKO, β-cell specific IR-knockout; BrdU, 5-bromo-2′-deoxyuridine; EGFP, enhanced green fluorescent protein; LG, low glucose; HG, high glucose; MOI, multiplicity of
Function of phogrin in β-cell growth

infection; RIA, radioimmuno assay; PM, plasma membrane

REFERENCES

1. Heit JJ, Karnik SK, Kim SK: Intrinsic regulators of pancreatic beta-cell proliferation. *Annu Rev Cell Dev Biol* 22:311-338, 2006
2. Alonso LC, Yokoe T, Zhang P, Scott DK, Kim SK, O'Donnell CP, Garcia-Ocana A: Glucose infusion in mice: a new model to induce beta-cell replication. *Diabetes* 56:1792-1801, 2007
3. Leibiger IB, Leibiger B, Berggren PO: Insulin feedback action on pancreatic beta-cell function. *FEBS Lett* 532:1-6, 2002
4. Muller D, Huang GC, Amiel S, Jones PM, Persaud SJ: Identification of insulin signaling elements in human beta-cells: autocrine regulation of insulin gene expression. *Diabetes* 55:2835-2842, 2006
5. Burks DJ, White MF: IRS proteins and beta-cell function. *Diabetes* 50 Suppl 1:S140-145, 2001
6. Kulkarni RN: New insights into the roles of insulin/IGF-I in the development and maintenance of beta-cell mass. *Rev Endocr Metab Disord* 6:199-210, 2005
7. Otani K, Kulkarni RN, Baldwin AC, Krutzfeldt J, Ueki K, Stoffel M, Kahn CR, Polonsky KS: Reduced beta-cell mass and altered glucose sensing impair insulin-secretory function in betaIRKO mice. *Am J Physiol Endocrinol Metab* 286:E41-49, 2004
8. Ueki K, Okada T, Hu J, Liew CW, Assmann A, Dahlgren GM, Peters JL, Shackman JG, Zhang M, Artner I, Satin LS, Stein R, Holzenberger M, Kennedy RT, Kahn CR, Kulkarni RN: Total insulin and IGF-I resistance in pancreatic beta cells causes overt diabetes. *Nat Genet* 38:583-588, 2006
9. Ohsugi M, Cras-Meneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA: Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. *J Biol Chem* 280:4992-5003, 2005
10. Diao J, Asghar Z, Chan CB, Wheeler MB: Glucose-regulated glucagon secretion requires insulin receptor expression in pancreatic alpha-cells. *J Biol Chem* 280:33487-33496, 2005
11. Terauchi Y, Takamoto I, Kubota N, Matsui J, Suzuki R, Komeda K, Hara A, Toyoda Y, Miwa I, Aizawa S, Tsutsumi S, Tsubamoto Y, Hashimoto S, Eto K, Nakamura A, Noda M, Tobe K, Aburatani H, Nagai R, Kadowaki T: Glucokinase and IRS-2 are required for compensatory beta cell hyperplasia in response to high-fat diet-induced insulin resistance. *J Clin Invest* 117:246-257, 2007
12. Jhala US, Canettieri G, Srecaot RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M: cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev* 17:1575-1580, 2003
13. Park S, Dong X, Fisher TL, Dunn S, Omer AK, Weir G, White MF: Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. *J Biol Chem* 281:1159-1168, 2006
14. Rhodes CJ: Type 2 diabetes—a matter of beta-cell life and death? *Science* 307:380-384, 2005
15. Wasmeier C, Hutton JC: Molecular cloning of phogrin, a protein-tyrosine phosphatase homologue localized to insulin secretory granule membranes. *J Biol Chem* 271:18161-18170, 1996
16. Lu J, Li Q, Xie H, Chen ZJ, Borovitskaya AE, Maclaren NK, Notkins AL, Lan MS: Identification of a second transmembrane protein tyrosine phosphatase, IA-2beta, as an autoantigen in insulin-dependent diabetes mellitus: precursor of the 37-kDa tryptic fragment. Proc Natl Acad Sci USA 93:2307-2311, 1996

17. Solimena M, Dirkx R, Jr., Hermel JM, Pleasic-Williams S, Shapiro JA, Caron L, Rabin DU: ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules. Embo J 15:2102-2114, 1996

18. Drake PG, Peters GH, Andersen HS, Hendriks W, Moller NP: A novel strategy for the development of selective active-site inhibitors of the protein tyrosine phosphatase-like proteins islet-cell antigen 512 (IA-2) and phogrin (IA-2beta). Biochem J 373:393-401, 2003

19. Kubosaki A, Gross S, Miura J, Saeki K, Zhu M, Nakamura S, Hendriks W, Notkins AL: Targeted disruption of the IA-2beta gene causes glucose intolerance and impairs insulin secretion but does not prevent the development of diabetes in NOD mice. Diabetes 53:1684-1691, 2004

20. Saeki K, Zhu M, Kubosaki A, Xie J, Lan MS, Notkins AL: Targeted disruption of the protein tyrosine phosphatase-like molecule IA-2 results in alterations in glucose tolerance tests and insulin secretion. Diabetes 51:1842-1850, 2002

21. Kubosaki A, Nakamura S, Notkins AL: Dense core vesicle proteins IA-2 and IA-2beta: metabolic alterations in double knockout mice. Diabetes 54 Suppl 2:S46-51, 2005

22. Harashima S, Clark A, Christie MR, Notkins AL: The dense core transmembrane vesicle protein IA-2 is a regulator of vesicle number and insulin secretion. Proc Natl Acad Sci USA 102:8704-8709, 2005

23. Emmanouilidou E, Teschemacher AG, Pouli AE, Nicholls LI, Seward EP, Rutter GA: Imaging Ca2+ concentration changes at the secretory vesicle surface with a recombinant targeted cameleon. Curr Biol 9:915-918, 1999

24. Doi A, Shono T, Nishi M, Furuta H, Sasaki H, Nanjo K: IA-2beta, but not IA-2, is induced by ghrelin and inhibits glucose-stimulated insulin secretion. Proc Natl Acad Sci USA 103:885-890, 2006

25. Ort T, Maksimova E, Dirkx R, Kachinsky AM, Berghs S, Froehner SC, Solimena M: The receptor tyrosine phosphatase-like protein ICA512 binds the PDZ domains of beta2-syntrophin and nNOS in pancreatic beta-cells. Eur J Cell Biol 79:621-630, 2000

26. Trajkovski M, Mziaut H, Altkruger A, Ouwendijk J, Knoch KP, Muller S, Solimena M: Nuclear translocation of an ICA512 cytosolic fragment couples granule exocytosis and insulin expression in {beta}-cells. J Cell Biol 167:1063-1074, 2004

27. Mziaut H, Trajkovski M, Kersting S, Ehninger A, Altkruger A, Lemaitre RP, Schmidt D, Saeger HD, Lee MS, Drechsel DN, Muller S, Solimena M: Synergy of glucose and growth hormone signalling in islet cells through ICA512 and STAT5. Nat Cell Biol 8:435-445, 2006

28. Gross S, Blanchetot C, Schepens J, Albet S, Lammers R, den Hertog J, Hendriks W: Multimerization of the protein-tyrosine phosphatase (PTP)-like insulin-dependent diabetes mellitus autoantigens IA-2 and IA-2beta with receptor PTPs (RPTPs). Inhibition of RPTPalpha enzymatic activity. J Biol Chem 277:48139-48145, 2002

29. Cai T, Krause MW, Odenwald WF, Toyama R, Notkins AL: The IA-2 gene family: homologs in Caenorhabditis elegans, Drosophila and zebrafish. Diabetologia 44:81-88, 2001

30. Roberts C, Roberts GA, Lobner K, Bearzatto M, Clark A, Bonifacio E, Christie MR: Expression of the protein tyrosine phosphatase-like protein IA-2 during pancreatic islet development. J Histochem Cytochem 49:767-776, 2001

31. Shimizu S, Saito N, Kubosaki A, SungWook S, Takeyama N, Sakamoto T, Matsumoto Y,
Function of phogrin in β-cell growth

Saeki K, Matsumoto Y, Onodera T: Developmental expression and localization of IA-2 mRNA in mouse neuroendocrine tissues. *Biochem Biophys Res Commun* 288:165-171, 2001

32. Lobner K, Steinbrenner H, Roberts GA, Ling Z, Huang GC, Piquer S, Pipeleers DG, Seissler J, Christie MR: Different regulated expression of the tyrosine phosphatase-like proteins IA-2 and phogrin by glucose and insulin in pancreatic islets: relationship to development of insulin secretory responses in early life. *Diabetes* 51:2982-2988, 2002

33. Seissler J, Nguyen TB, Aust G, Steinbrenner H, Scherbaum WA: Regulation of the diabetes-associated autoantigen IA-2 in INS-1 pancreatic beta-cells. *Diabetes* 49:1137-1141, 2000

34. Steinbrenner H, Nguyen TB, Wohlrab U, Scherbaum WA, Seissler J: Effect of proinflammatory cytokines on gene expression of the diabetes-associated autoantigen IA-2 in INS-1 cells. *Endocrinology* 143:3839-3845, 2002

35. Brummelkamp TR, Bernards R, Agami R: A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296:550-553, 2002

36. Torii S, Saito N, Kawano A, Zhao S, Izumi T, Takeuchi T: Cytoplasmic transport signal is involved in phogrin targeting and localization to secretory granules. *Traffic* 6:1213-1224, 2005

37. Kulkarni RN, Winnay JN, Daniels M, Bruning JC, Flier SN, Hanahan D, Kahn CR: Altered function of insulin receptor substrate-1-deficient mouse islets and cultured beta-cell lines. *J Clin Invest* 104:R69-75, 1999

38. Hou N, Torii S, Saito N, Hosaka M, Takeuchi T: Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases. *Endocrinology* 149:1654-1665, 2008

39. Torii S, Takeuchi T, Nagamatsu S, Izumi T: Rab27 effector granuphilin promotes the plasma membrane targeting of insulin granules via interaction with syntaxin 1a. *J Biol Chem* 279:22532-22538, 2004

40. Kinoshita T, Imamura J, Nagai H, Shimotohno K: Quantification of gene expression over a wide range by the polymerase chain reaction. *Anal Biochem* 206:231-235, 1992

41. Cai T, Fukushige T, Notkins AL, Krause M: Insulinoma-Associated Protein IA-2, a Vesicle Transmembrane Protein, Genetically Interacts with UNC-31/CAPS and Affects Neurosecretion in Caenorhabditis elegans. *J Neurosci* 24:3115-3124, 2004

42. Lingohr MK, Briaud I, Dickson LM, McCuaig JF, Alarcon C, Wicksteed BL, Rhodes CJ: Specific regulation of IRS-2 expression by glucose in rat primary pancreatic islet beta-cells. *J Biol Chem* 281:15884-15892, 2006

43. Briaud I, Dickson LM, Lingohr MK, McCuaig JF, Lawrence JC, Rhodes CJ: Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in beta-cells. *J Biol Chem* 280:2282-2293, 2005

44. Tsuboi T, Zhao C, Terakawa S, Rutter GA: Simultaneous evanescent wave imaging of insulin vesicle membrane and cargo during a single exocytotic event. *Curr Biol* 10:1307-1310, 2000

45. Henquin JC, Nenquin M, Szollosi A, Kubosaki A, Louis Notkins A: Insulin secretion in islets from mice with a double knockout for the dense core vesicle proteins islet antigen-2 (IA-2) and IA-2beta. *J Endocrinol* 196:573-581, 2008

46. Mziaut H, Kersting S, Knoch KP, Fan WH, Trajkovski M, Erdmann K, Bergert H, Ehehalt F, Saeger HD, Solimena M: ICA512 signaling enhances pancreatic beta-cell proliferation by
regulating cyclins D through STATs. Proc Natl Acad Sci U S A 105:674-679, 2008
47. Pouli AE, Emmanouilidou E, Zhao C, Wasmeier C, Hutton JC, Rutter GA: Secretory-granule
dynamics visualized in vivo with a phogrin-green fluorescent protein chimaera. Biochem J
333:193-199, 1998
48. Taraska JW, Perrais D, Ohawa-Imaizumi M, Nagamatsu S, Almers W: Secretory granules
are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. Proc Natl
Acad Sci U S A 100:2070-2075, 2003
49. Vo YP, Hutton JC, Angleson JK: Recycling of the dense-core vesicle membrane protein
phogrin in MIN6 beta-cells. Biochem Biophys Res Commun 324:1004-1010, 2004
50. Uhles S, Moede T, Leibiger B, Berggren PO, Leibiger IB: Isoform-specific insulin receptor
signaling involves different plasma membrane domains. J Cell Biol 163:1327-1337, 2003
51. Rui L, Fisher TL, Thomas J, White MF: Regulation of insulin/insulin-like growth factor-1
signaling by proteasome-mediated degradation of insulin receptor substrate-2. J Biol Chem
276:40362-40367, 2001
52. Hennige AM, Burks DJ, Ozcan U, Kulkarni RN, Ye J, Park S, Schubert M, Fisher TL, Dow
MA, Leshan R, Zakaria M, Mossa-Basha M, White MF: Upregulation of insulin receptor
substrate-2 in pancreatic beta cells prevents diabetes. J Clin Invest 112:1521-1532, 2003
53. Gunton JE, Kulkarni RN, Yim S, Okada T, Hawthorne WJ, Tseng YH, Roberson RS, Ricordi
C, O'Connell PJ, Gonzalez FJ, Kahn CR: Loss of ARNT/HIF1beta mediates altered gene
expression and pancreatic-islet dysfunction in human type 2 diabetes. Cell 122:337-349, 2005
Function of phogrin in β-cell growth

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 (Clone #33 and #44). Each extract normalized for total protein content was subjected to SDS-PAGE/Immunoblot analysis. Blots were probed with specific antibodies of 4 categories; (A) secretory granules-associated proteins, (B) proteins related to vesicle trafficking, (C) insulin signaling pathway proteins, (D) others. (E) Intensity of each band was quantified with the densitometric imager and the results from three independent experiments are presented as a fold increase ± SEM compared to MshV.
Function of phogrin in β-cell growth

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 non-targeting 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 mM glucose) before HG stimulation. After stimulation for 30 min with 2 mM or 25 mM glucose, culture media (left panel) and cells (right panel) were collected and subjected to RIA for insulin. Data are given as means ± SEMs of five independent experiments. E: Time course of MIN6 cell proliferation 24-96 h post-infection with Ad-shVec, shPh1, shPh2, shPh3, or shIA2. Cell number was measured as indicated in Methods. Phogrin-KD or IA2-KD cells grew 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).
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). Cell extracts were prepared and each extract was subjected to immunoblotting with anti-phogrin antibodies (A and E). The infected cells were incubated in KRB with 2 mM or 16.8 mM 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 ± SEMs of at least four independent experiments. Cell growth was assessed as shown in Fig. 2E and 2F (D and H). Insets show the BrdU incorporation rate at 72 h that is presented as the fold increase ± SEMs compared to control (n = 3, * P < 0.01).
Fig. 4. Phogrin regulates β-cell growth. A: Mouse islets were infected with adenoviruses integrating shVector or shPh3. Uptake of [methyl-3H]thymidine into islet cell DNA and total protein concentration were measured. Data are presented as the fold increase ± SEMs compared to shVector (n = 5, * P < 0.01). Cell lysates at 80 h were collected and subjected to immunoblot analysis with phogrin antibody (right panel). 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. 2E. Data are given as means ± SEMs of four independent experiments.
**Function of phogrin in β-cell growth**

**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. Following 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 a fold increase ± SEM compared to 5 moi of MshV.  
*C:* INS-1 cells were infected with increasing MOI 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-1R.  
*D:* Isolated mouse islets were infected with Ad-shVec or shPh3, and then cultured for up to 5 d. 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.
Function of phogrin in β-cell growth

A

|          | Ad-shVec | Ad-shPh3 |
|----------|----------|----------|
|          | 24 36 48 60 72 | 24 36 48 60 72 |
| 99       | Phogrin   |          |
| 52       |          |          |
| 208      | IRS2     |          |
| 208      | IRS1     |          |
| 208      | pro-IR   |          |
| 119      | IRb      |          |
| 99       | ERK1/2   |          |

B

|          | shPh3 | shVec | shIA2 |
|----------|-------|-------|-------|
|          | 5 10 15 | 5 10 15 | 5 10 15 |
| 10       | Phogrin |          |          |
| 2.5      | IA-2   |          |          |
| 2.5      | IRS2   |          |          |
| 2.5      | IRb    |          |          |
| 2.5      | IRS1   |          |          |
| 2.5      | IGF-1R |          |          |
| 2.5      | p85(PI3K) |      |          |
| 2.5      | Grb2   |          |          |
| 2.5      | Shc    |          |          |
| 2.5      | p70S6K |          |          |
| 2.5      | SHP-2  |          |          |
| 2.5      | EGFR   |          |          |
| 2.5      | Dynamin|          |          |
| 2.5      | VAMP2  |          |          |

C

|          | 6 | 48 | 96 |
|----------|---|----|----|
|          | 10 | 2.5 | 10 |
| 10       | Phogrin |      |      |
| 2.5      | IRS2  |      |      |
| 2.5      | IRb   |      |      |
| 2.5      | IRS1  |      |      |
| 2.5      | STAT5 |      |      |
| 2.5      | IGF-1R |     |      |

D

|          | Ad-shVec | Ad-shPh3 |
|----------|----------|----------|
|          | 0 1 3 5 0 1 3 5 | 0 1 3 5 0 1 3 5 |
| 99       | Phogrin   |          |
| 52       |          |          |
| 208      | IRS2     |          |
| 208      | pro-IR   |          |
| 119      | IRb      |          |
| 99       | ERK1/2   |          |
|          |          |          |

E

|          | MshP #33 | MshP #44 |
|----------|----------|----------|
|          | LacZ: 10 5 | LacZ: 10 5 |
|          | Phog: 5 10 | Phog: 5 10 |
| 50       | Phogrin  |          |
| 200      | IRS2    |          |
| 200      | Tubulin |          |

20
Function of phogrin in β-cell growth

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 and IRS2. Data are presented as a fold increase ± SEMs compared to 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 and IRS2. The intensity of each band was quantified with the densitometric imager and the results are presented as a fold increase ± SEMs compared to uninfected control cells (time 0). C: MIN6 cells were infected with adenoviruses integrating shVector, shPh3 or shIA2. After 36 h, cells were cultured for more 20 h in the presence or absence of 10 μM lactacystin, 20 μM MG-132, 10 μg/ml pepstatin A, or 5 mM 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 a fold increase ± SEMs compared to 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, PI3K (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 ± SEM (n=3).
**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, IGF-1R (*left panel*). Right panels show the immunoprecipitated level of phogrin or IA-2. 5% of each immunoprecipitate and the original lysates (10 μg) were analyzed by immunoblotting. Asterisk: Immunogloblin.  
**B**: MIN6 cells were stimulated with serum-free DMEM containing HG for an indicated time, after pre-incubation of LG for 2 h. Cell extracts were prepared and an equal amount of each extract was immunoprecipitated with anti-phogrin antibody, then the amount of IR in each precipitate was determined by immunoblotting (*left panel*). The intensity of each band was quantified with the densitometric imager and the coimmunoprecipitation (co-IP) levels are presented as a fold increase ± SEMs compared to LG incubation (time 0). Cell extracts were analyzed by immunoblotting with antibodies to IRβ, IRS2, and phogrin (*right panels*).  
**C**: MIN6 cells were stimulated for 1.5 h with HG in the presence or absence of 200 μM diazoxide. Interaction of phogrin with IR was assessed by the coimmunoprecipitation analysis as in B (*left panel and right graph*).  
**D**: MIN6 cells were incubated with serum-free DMEM containing LG or HG for 4 h. The cells were extracted and fractioned by the method described in Methods. Equal proportions of the fractions were immunoprecipitated with anti-phogrin antibody, then the amount of IR in each precipitate was determined by immunoblotting (*right lower panels*). Equal proportions of the fractions were analyzed by immunoblotting with antibodies for IRβ, syntaxin 1, phogrin, Rab27a, or IRS2 (*left panels*). The subcellular fractions are designated as follows: T, total homogenate; PM, plasma membrane; SG, secretory granules; Cyt, cytosol. The amount of phogrin in the PM or the SG was quantitated using densitometry and quantification of its translocation from SG to PM are shown in a bar graph (n=3).
Function of phogrin in β-cell growth

A

B

C

D

|                | LG | HG |
|----------------|----|----|
| IRb            | ![Image] | ![Image] |
| Syntaxin1      | ![Image] | ![Image] |
| Phogrin        | ![Image] | ![Image] |
| Rab27a         | ![Image] | ![Image] |
| IRS2           | ![Image] | ![Image] |

Transfer rate (PM / SG %)

LG PHOGRIN HG PHOGRIN

IP: anti-Phogrin
Figure 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 increase ± SEMs compared to 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. 6D. Accumulative data are presented as a percentage of the IRS2 protein levels at time 0 as means ± SEM (n=3).