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EphA-Ephrin-A-Mediated β Cell Communication Regulates Insulin Secretion from Pancreatic Islets

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SUMMARY

In vertebrates, β cells are aggregated in the form of pancreatic islets. Within these islets, communication between β cells inhibits basal insulin secretion and enhances glucose-stimulated insulin secretion, thus contributing to glucose homeostasis during fasting and feeding. In the search for the underlying molecular mechanism, we have discovered that β cells communicate via ephrin-As and EphAs. We provide evidence that ephrin-A5 is required for glucose-stimulated insulin secretion. We further show that EphA-ephrin-A-mediated β cell communication is bidirectional: EphA forward signaling inhibits insulin secretion, whereas ephrin-A reverse signaling stimulates insulin secretion. EphA forward signaling is downregulated in response to glucose, which indicates that, under basal conditions, β cells use EphA forward signaling to suppress insulin secretion and that, under stimulatory conditions, they shift to ephrin-A reverse signaling to enhance insulin secretion. Thus, we explain how β cell communication in pancreatic islets conversely affects basal and glucose-stimulated insulin secretion to improve glucose homeostasis.

INTRODUCTION

Pancreatic islets are essential for maintaining glucose homeostasis, and defects in their ability to adequately secrete insulin in response to glucose result in diabetes mellitus (Bell and Polonsky, 2001). During embryonic development, β cells initially develop as scattered cells (Grapin-Botton and Melton, 2000). However, as development progresses, they aggregate with other β cells and endocrine cells to form pancreatic islets (Halban, 2004). While glucose metabolism cell-autonomously triggers insulin secretion (Maechler and Wollheim, 2001), communication between β cells suppresses basal insulin secretion but enhances glucose-stimulated insulin secretion (Bosco et al., 1989; Dahl et al., 1996; Halban et al., 1982; Hauge-Evans et al., 1999; Hopcroft et al., 1985; Luther et al., 2006; Maes and Pipeleers, 1984; Meda et al., 1990; Ravier et al., 2005; Yamagata et al., 2002). Cell-cell communication therefore ensures that β cells secrete low amounts of insulin during times of starvation but sufficient amounts of insulin after food uptake. However, the molecular mechanism that allows β cell communication to suppress basal insulin secretion and enhance glucose-stimulated insulin secretion remains elusive.

Here we investigated EphA receptor tyrosine kinases (RTK) and their ephrin-A ligands in pancreatic β cells since EphAs and ephrin-As are involved in various aspects of cell-cell communication (Himanen and Nikolov, 2003; Pasquale, 2005). Ephs and ephrins are classified in A and B subclasses, and most EphAs bind to ephrin-As, while most EphBs bind to ephrin-Bs (Gale et al., 1996). Both EphAs and ephrin-As are localized on the plasma membrane and, therefore, require direct cell-cell contact to bind and activate each other (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; Pasquale, 2005). The signaling pathway downstream of the transmembrane EphA RTKs is referred to as EphA forward signaling and can be exogenously activated by ephrin-A-Fc-fusion proteins (Figure 1A). The signaling pathway downstream of the GPI-anchored ephrin-As is referred to as ephrin-A reverse signaling and can be exogenously activated by EphA-Fc-fusion proteins (Figure 1A). In general, when both signaling pathways are active, EphA-ephrin-A bidirectional signaling takes place (Figure 1A). However, if EphA forward signaling is inhibited by dephosphorylation

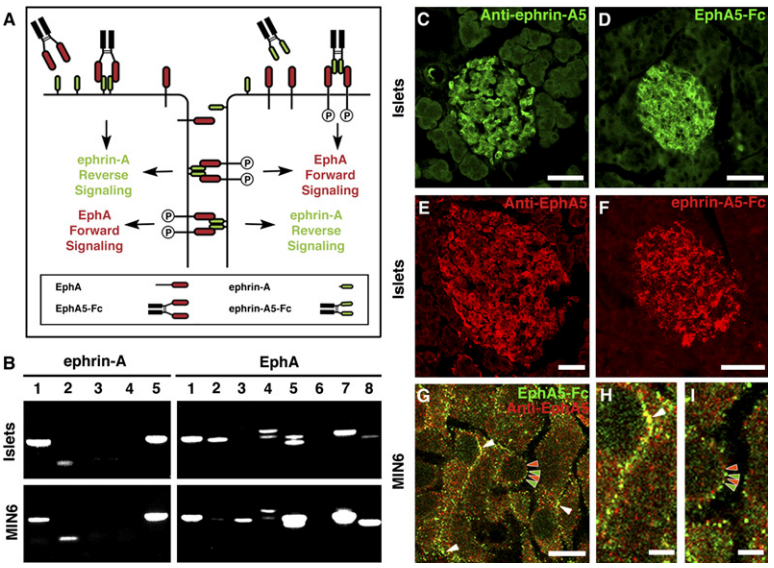

Figure 1. Expression and Localization of Ephrin-As and EphAs in Pancreatic Islets and MIN6 Cells

(A) shows a model of EphA-ephrin-A bidirectional signaling between two adjacent β cells and exogenous activation of ephrin-A reverse signaling (green) and EphA forward signaling (red) by EphA5-Fc- and ephrin-A5-Fc-fusion proteins, respectively. Circled P represents tyrosine phosphorylation.

(B) RT-PCR products for *ephrin-A1* to *-A5* and *EphA1* to *-A8* are shown, performed with mRNA isolated from mouse islets and mouse insulinoma cells (MIN6). Only the upper *EphA4* band represents amplified *EphA4* sequence, whereas both *EphA5* bands represent identical *EphA5* sequences.

(C–F) Confocal images of mouse pancreas sections show an islet surrounded by exocrine pancreatic tissue. Sections were stained with (C) anti-ephrin-A5 antibody, (D) EphA5-Fc that binds to ephrin-As, (E) anti-EphA5 antibody, and (F) ephrin-A5-Fc that binds to EphAs. Scale bars are 50 μm.

(G–I) Confocal images of a group of MIN6 cells stained for ephrin-As and EphA5 cytoplasmic domain are shown. (G) shows low magnification and (H) and (I) show high magnification of regions in (G). (H) shows contact region between two MIN6 cells with EphA-ephrin-A colocalization (white arrowhead) and (I) shows free surfaces of MIN6 cells with no colocalization between ephrin-As (green arrowheads) and EphA5 (red arrowheads). Scale bars in (G) are 10 μm and in (H) and (I) are 2 μm.

of EphA receptors or, alternatively, truncation of their cytoplasmic parts, ephrin-A reverse signaling occurs without kinase-dependent EphA forward signaling (Holmberg et al., 2000; Parri et al., 2005; Shintani et al., 2006).

In this study, we demonstrate that β cells communicate with each other via EphAs and ephrin-As. We show that EphA forward signaling inhibits basal insulin secretion. In contrast, glucose stimulation attenuates EphA forward signaling by dephosphorylation of EphAs. This allows predominant ephrin-A reverse signaling, which enhances insulin secretion. Our study on pancreatic islets therefore shows that interacting cells coexpressing EphAs and ephrin-As can switch between two opposite states in response to a stimulus. This mechanism enables pancreatic islets to switch from low levels of insulin secretion to high levels of insulin secretion upon glucose stimulation.

RESULTS

Ephrin-A and EphA Expression in Mouse and Human Pancreatic Islets

We investigated the presence of Ephs and ephrins in pancreatic islets as possible candidate molecules involved in cell-cell communication (Figure 1A). First, we detected similar transcription of *ephrin-A*s and *EphA*s in islets and mouse insulinoma cells, MIN6 (Figure 1B). We also detected expression of ephrin-Bs and EphBs (data not shown) but focused on ephrin-As and EphAs due to their strong abundance and similar expression in both islets and MIN6 cells.

Immunostaining mouse pancreas sections showed coexpression of ephrin-A5 and EphA5 proteins in islets, and this expression was stronger in the islets than in the surrounding exocrine tissue (Figures 1C and 1E). A similar expression was observed in the human pancreas (Figures S1A–S1F). Other ephrin-As and EphAs, such as ephrin-A1 and EphA7, were also expressed in mouse pancreatic islets (Figures S2A and S2B).

Next we stained mouse pancreas sections with EphA5-Fc- and ephrin-A5-Fc-fusion proteins, which bind with different affinity to virtually all ephrin-As and EphAs, respectively (Figure 1A; Flanagan and Vanderhaeghen, 1998). The staining showed that islet ephrin-As bind to EphA5 (Figure 1D) and that islet EphAs bind to ephrin-A5 (Figure 1F).

EphA5 and ephrin-As colocalized in regions where MIN6 cells were in contact with each other (Figures 1G and 1H). In line with the findings that EphAs and ephrin-As segregate in motor neurons (Marquardt et al., 2005) and that several transmembrane proteins also segregate in insulinoma cells (Uhles et al., 2003), we observed little colocalization of EphA5 and ephrin-As on the free surfaces of MIN6 cells (Figure 1I). Moreover, based on the EphA5-Fc and ephrin-A5-Fc staining of nonpermeabilized MIN6 cells, we found that ephrin-As were more strongly localized to the plasma membrane compared to the EphAs (Figures S3A and S3B). Conversely, EphAs strongly localized to insulin secretory granules (Figure S3D), whereas ephrin-As did not (Figure S3C).

These results show that EphAs and ephrin-As are coexpressed in β cells and suggest that EphA-ephrin-A

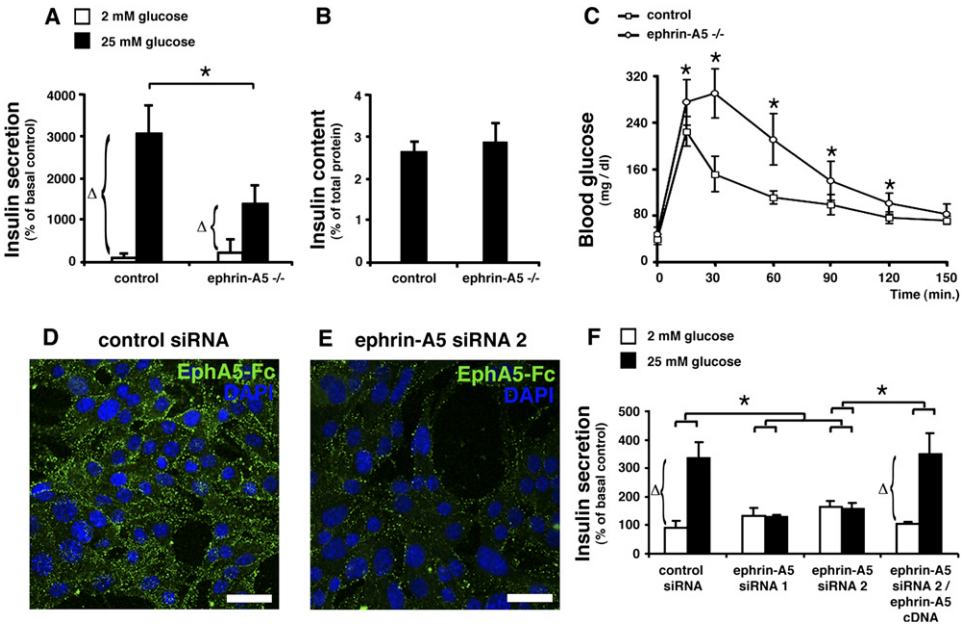

Figure 2. Ephrin-A5 Is Required for Glucose-Stimulated Insulin Secretion

(A) Insulin secretion from male control and ephrin-A5$^{-/-}$ islets at 2 mM glucose (white columns) and 25 mM glucose (black columns) is shown. Secreted insulin is normalized to insulin content and total protein content. Δ indicates the difference between basal and glucose-stimulated insulin secretion. n = 3 experiments.

(B) Insulin content of islets isolated from male control mice and ephrin-A5$^{-/-}$ mice is presented as percent of total protein content. n = 3 experiments.

(C) Glucose-tolerance tests of control and ephrin-A5$^{-/-}$ mice are shown. n = 7 male mice each.

(D) and (E) show EphA5-Fc immunostaining (green) of MIN6 cells transfected with (D) control siRNA and (E) ephrin-A5 siRNA 2. Cell nuclei are stained with DAPI (blue). Scale bars are 20 μm.

(F) Insulin secretion is shown from MIN6 cells transfected with control siRNA, ephrin-A5 siRNA 1, or ephrin-A5 siRNA 2 or cotransfected with ephrin-A5 siRNA 2 and *ephrin-A5* cDNA without the targeted *3′-UTR* (= rescue experiment). White columns indicate 2 mM glucose, and black columns indicate 25 mM glucose. Secreted insulin is normalized to insulin content and total protein content. n = 6 experiments.

*$p < 0.05$. All values are means ± SD.

bidirectional signaling may take place between adjacent β cells (Figure 1A).

Ephrin-A5 Is Required for Glucose-Stimulated Insulin Secretion

To investigate whether ephrin-A5, a possible participant in bidirectional signaling between β cells, was required for insulin secretion, we compared control islets with ephrin-A5-deficient islets (ephrin-A5$^{-/-}$) (Figures 2A and 2B). In comparison with control islets, ephrin-A5$^{-/-}$ islets had a significantly reduced glucose-stimulated insulin secretion (compare black columns in Figure 2A), showing that ephrin-A5 is required for normal β cell insulin secretory response to glucose (compare Δs in Figure 2A). In contrast, we did not detect any differences in the insulin content between control and ephrin-A5$^{-/-}$ islets (Figure 2B), thus excluding an effect of ephrin-A5 on insulin production. In line with the insulin-secretion defects (Figure 2A), we also detected impaired glucose tolerance in ephrin-A5-deficient mice (Figure 2C).

To rule out the possibility that the observed defects were due to changes in non-β cells, which are also present in islets, we knocked down ephrin-A5 in MIN6 cells (Figures 2D and 2E), a frequently used model for β cells (Miyazaki et al., 1990). We noticed that ephrin-A5 knockdown led to significantly increased basal insulin secretion (white columns in Figure 2F) as well as significantly reduced glucose-stimulated insulin secretion (black columns in Figure 2F). These results show that ephrin-A5 is involved in suppression of basal insulin secretion and is required for glucose-stimulated insulin secretion. The data further show that ephrin-A5 plays a role in glucose-stimulated insulin secretion, which is not substituted by any of the other ephrin-As expressed in pancreatic β cells.

Opposite Effects of EphA5-Fc and Ephrin-A5-Fc on Insulin Secretion

To uncover the underlying molecular mechanism, we first investigated the effects of ephrin-A reverse signaling and EphA forward signaling on insulin secretion (Figure 3). We used EphA5-Fc to stimulate ephrin-A reverse signaling and ephrin-A5-Fc to stimulate EphA forward signaling (Figures 3A and 3B), and we used Fc fragment as control (Figures S4A and S4B). We showed that EphA5-Fc significantly increased basal and glucose-stimulated insulin secretion (compare EphA5-Fc with Fc in Figure 3A), whereas

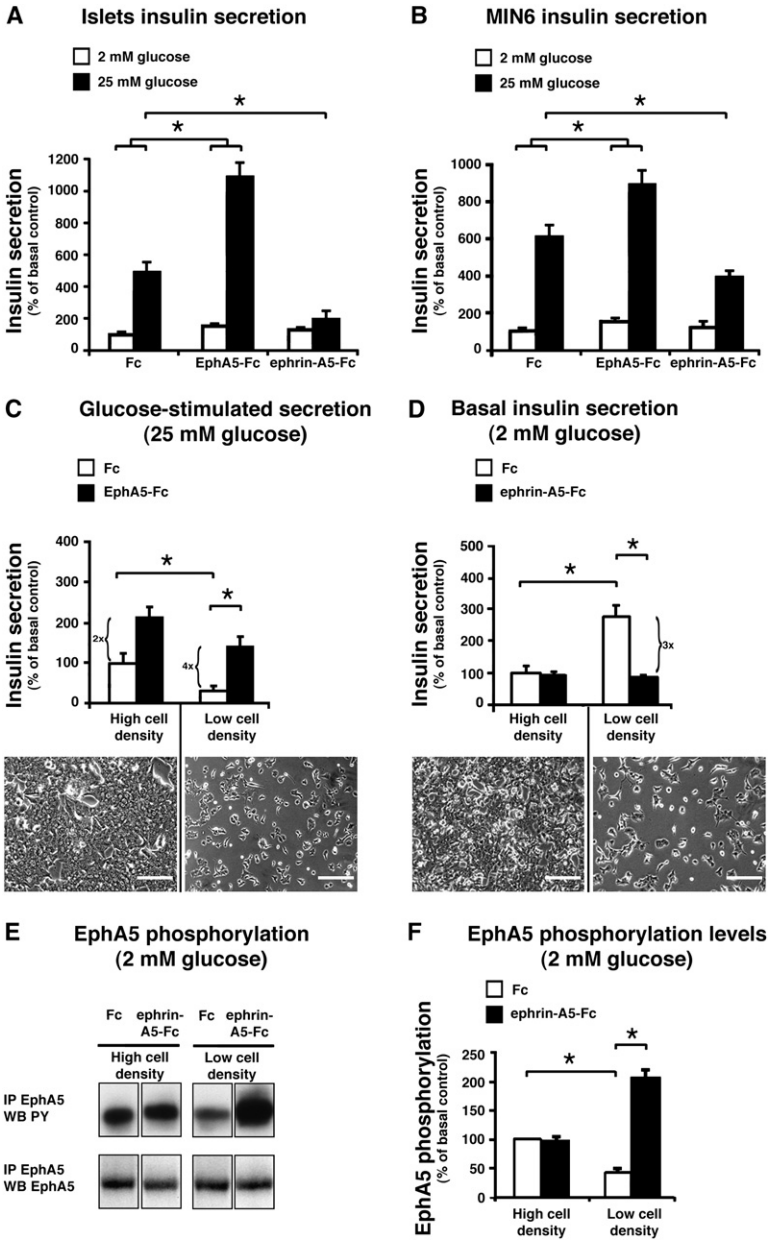

Figure 3. Opposite Effects of EphA5-Fc and Ephrin-A5-Fc on Insulin Secretion

(A) and (B) show insulin secretion from (A) mouse pancreatic islets and (B) MIN6 cells treated with Fc (as control), EphA5-Fc (to activate ephrin-A reverse signaling), and ephrin-A5-Fc (to activate EphA forward signaling) at 2 mM glucose (white columns) and 25 mM glucose (black columns). Insulin secretion is normalized to insulin content and total protein content. n = 3 experiments.

(C) Glucose-stimulated insulin secretion from MIN6 cells at high cell density (lower left image) and low cell density (lower right image) is shown after treatment with Fc (white columns) or EphA5-Fc (black columns). n = 4 experiments. Scale bars are 100 μm.

(D) Basal insulin secretion from MIN6 cells at high cell density and low cell density after treatment with Fc (white columns) or ephrin-A5-Fc (black columns) is shown. n = 3 experiments. Scale bars are 100 μm.

(E) Detection of tyrosine-phosphorylated EphA5 (PY) and total EphA5 in western blots after EphA5 immunoprecipitation from lysates of MIN6 cells that were grown at high and low cell density and kept under basal conditions for 1 hr with Fc or with ephrin-A5-Fc is shown.

(F) EphA5 tyrosine phosphorylation, relative to total EphA5 protein, is shown in a histogram for the experiment shown in (E). n = 2 experiments.

*$p < 0.05$. All values are means ± SD.

ephrin-A5-Fc significantly decreased glucose-stimulated insulin secretion from mouse pancreatic islets (compare ephrin-A5-Fc with Fc in Figure 3A).

Islets consist mainly of β cells but also harbor other cell types that might have been affected in these experiments. Therefore, we tested the role of ephrin-A reverse signaling and EphA forward signaling in MIN6 cells and obtained similar results (Figure 3B). Moreover, EphA7-Fc and ephrin-A1-Fc also conversely affected glucose-stimulated insulin secretion (Figure S2C). Finally, we showed that EphA5-Fc increased, and that ephrin-A5-Fc suppressed, glucose-stimulated insulin secretion from human pancreatic islets (Figure S1G).

EphA5-Fc Partially Rescues Glucose-Stimulated Insulin Secretion in Cells with Reduced Cell-Cell Contact

Glucose-stimulated insulin secretion is decreased in situations of reduced β cell communication (Luther et al., 2006; Maes and Pipeleers, 1984; Meda et al., 1990). Therefore we asked whether EphA5-Fc restored glucose-stimulated insulin secretion in MIN6 cells with reduced cell-cell contact (Figure 3C). We first showed that nonconfluent cells ("low cell density") had less glucose-stimulated insulin secretion compared to confluent cells ("high cell density"; compare white columns in Figure 3C). We then showed that EphA5-Fc partially restored

glucose-stimulated insulin secretion in these cells (compare last two columns in Figure 3C), suggesting that endogenous ephrin-A reverse signaling is required for enhancing glucose-stimulated insulin secretion.

Ephrin-A5-Fc Fully Rescues Suppression of Basal Insulin Secretion in Cells with Reduced Cell-Cell Contact

We noticed that ephrin-A5-Fc decreased glucose-stimulated insulin secretion but did not decrease basal insulin secretion from islets and MIN6 cells (Figures 3A and 3B). We hypothesized that basal insulin secretion could not be decreased because it was maximally suppressed by endogenous EphA-ephrin-A interactions.

As shown in Figure 3D, nonconfluent MIN6 cells had significantly enhanced basal insulin secretion (compare white columns) as well as reduced EphA5 phosphorylation levels compared to confluent cells (Figure 3E; compare white columns in Figure 3F). Importantly, ephrin-A5-Fc decreased basal insulin secretion in these cells to a level characteristic of confluent MIN6 cells (compare last two columns in Figure 3D). Consistent with the idea that this inhibition was due to exogenous activation of EphA forward signaling, ephrin-A5-Fc significantly increased EphA5 phosphorylation levels in the nonconfluent cells (Figure 3E; compare last two columns in Figure 3F). These results therefore suggest that endogenous EphA forward signaling is required for inhibiting basal insulin secretion.

Ephrin-A Reverse Signaling Stimulates Insulin Secretion

The previous experiments strongly suggested that ephrin-A reverse signaling enhanced insulin secretion (Figure 3). Here we used single primary β cells to stimulate ephrin-A reverse signaling with EphA5-Fc, without simultaneously affecting endogenous EphA forward signaling (Figure 4A).

We expressed GFP-tagged insulin in primary mouse β cells and stimulated these cells with glucose in the presence or absence of EphA5-Fc (Figure 4A). We then monitored the secretory events in single β cells by using total internal reflection-fluorescence microscopy (TIR-FM; Movies S1 and S2). As shown in Figure 4B, EphA5-Fc treatment significantly increased fusion events of newly recruited insulin secretory granules (white columns).

Glucose-stimulated insulin secretion, in response to a sudden increase in glucose concentration, follows a biphasic time course consisting of a rapid first phase followed by a sustained second phase (Rorsman and Renstrom, 2003). We found that EphA5-Fc increased the number of secretory events during both phases of insulin secretion with a stronger effect on the second phase (compare Figures 4C and 4D). We conclude that ephrin-A reverse signaling stimulates insulin secretion.

EphA Forward Signaling Suppresses Insulin Secretion

The experiments, shown in Figure 3, strongly suggested that EphA forward signaling suppressed insulin secretion. Here we used TIR-FM of single ephrin-A5-Fc-treated primary β cells to provide evidence of a suppressive effect of EphA forward signaling (Figure 4E; Movies S3 and S4). As shown in Figure 4F, ephrin-A5-Fc significantly reduced the number of fusion events of newly recruited insulin secretory granules (white columns). In addition, ephrin-A5-Fc reduced the number of secretory events during both phases of insulin secretion with a stronger effect on the second phase (compare Figures 4G and 4H).

As additional evidence of an inhibitory effect of EphA forward signaling on insulin secretion, a dominant-negative EphA5 protein (DN-EphA5) lacking its cytoplasmic domain was expressed in MIN6 cells (Figure S5A). This DN-EphA5 binds to ephrin-As and is expected to induce ephrin-A reverse signaling and inhibit EphA forward signaling (Gao et al., 1998). In contrast, overexpression of a full-length EphA5 protein (WT EphA5) is expected to induce both ephrin-A reverse signaling and EphA forward signaling (Figure S5B). In support of an inhibitory effect of EphA forward signaling, we found that DN-EphA5 increased basal and glucose-stimulated insulin secretion, whereas WT EphA5 overexpression decreased glucose-stimulated insulin secretion in confluent MIN6 cells (Figure S5C). We conclude that EphA forward signaling suppresses insulin secretion.

Downstream Targets of EphA-Ephrin-A Signaling and Their Impact on Insulin Secretion

It has been shown that destabilization of F-actin enhances insulin secretion, whereas its stabilization inhibits insulin secretion (Orci et al., 1972). This led to the hypothesis that dense cortical F-actin limits access of insulin secretory granules to the plasma membrane. However, since complete F-actin depolymerization inhibits glucose-stimulated insulin secretion (Li et al., 1994), certain actin filaments appear to be required for the secretory process.

Because ephrin-A reverse signaling and EphA forward signaling, were shown to rearrange F-actin in several cell types (Marquardt et al., 2005; Parri et al., 2005), we tested whether EphA5-Fc and ephrin-A5-Fc also changed the F-actin state of mouse insulinoma cells (Figures 5A–5E). We found that EphA5-Fc resulted in F-actin rearrangements with a subtle decrease in F-actin intensity under basal conditions (compare Figures 5A and 5B), consistent with its stimulatory effect on insulin secretion. In contrast, ephrin-A5-Fc increased F-actin polymerization in nonconfluent clusters of MIN6 cells under basal and stimulatory conditions (compare Figures 5C and 5D), consistent with its inhibitory effect on insulin secretion.

EphAs modulate activity of Rac1, a Rho-GTPase involved in F-actin remodeling and endocytosis (Marston et al., 2003; Shamah et al., 2001; Zhuang et al., 2007; Zimmer et al., 2003). Since Rac1 is also involved in glucose-stimulated insulin secretion (Kowluru and Veluthakal, 2005; Li et al., 2004), we investigated whether EphA5-Fc and ephrin-A5-Fc modulated Rac1 activity in confluent MIN6 cells. In line with previous reports (Kowluru and Veluthakal, 2005; Li et al., 2004), glucose stimulation

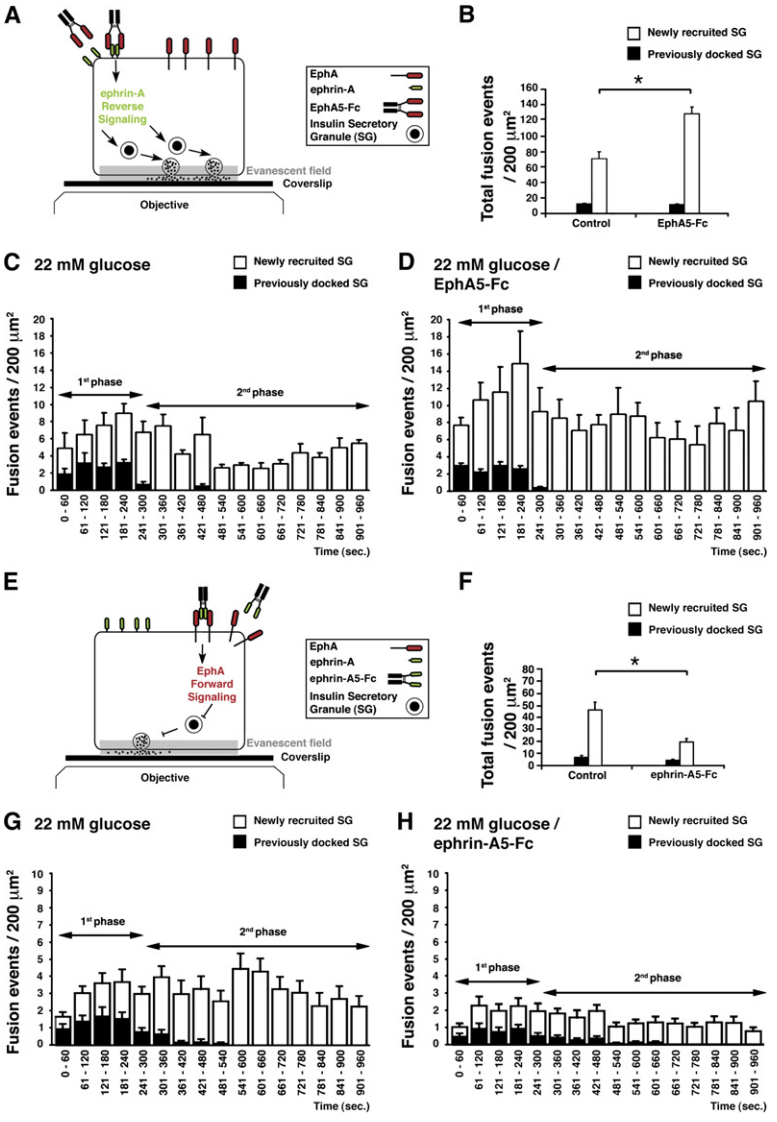

Figure 4. Opposite Effects of Ephrin-A Reverse Signaling and EphA Forward Signaling on Insulin Secretory Granule Fusion

(A) Model shows activation of ephrin-A reverse signaling by EphA5-Fc in a single insulin-GFP-expressing mouse pancreatic β cell. Total internal reflection-fluorescence microscopy (TIR-FM) was used to detect fusion of insulin secretory granules (SG) with the plasma membrane.

(B) Histogram shows all secretory fusion events in single β cells detected during 16 min treatment with 22 mM glucose in the absence (control) or presence of EphA5-Fc. Fusion events of previously docked SG (black columns) and newly recruited SG (white columns) were normalized to the cell area. n = 5–7 cells each.

(C) and (D) show time course of the fusion events shown in (B). Cells were treated with 22 mM glucose in the absence (C) or presence (D) of EphA5-Fc.

(E) Model shows activation of EphA forward signaling by ephrin-A5-Fc in a single insulin-GFP-expressing mouse pancreatic β cell. TIR-FM was used to detect fusion of SG with the plasma membrane.

(F) Histogram showing all secretory fusion events in single β cells detected during 16 min treatment with 22 mM glucose in the absence (control) or presence of ephrin-A5-Fc. n = 20–21 cells each.

(G) and (H) show time course of fusion events shown in (F). Cells were treated with 22 mM glucose in the absence (G) or presence (H) of ephrin-A5-Fc. *p < 0.05. All values are means ± SEM.

significantly enhanced Rac1 activity (Fc; Figures 5F and 5G). Consistent with its stimulatory effects on insulin secretion, we found that EphA5-Fc stimulated Rac1 activity (compare Fc with EphA5-Fc in Figure 5G), whereas, consistent with its inhibitory effect on insulin secretion, ephrin-A5-Fc inhibited Rac1 activity (compare Fc with ephrin-A5-Fc in Figure 5G). In addition, both EphA5-Fc and ephrin-A5-Fc required Rac1 activity for their effects on glucose-stimulated insulin secretion (compare DN-Rac1 with empty vector in Figure 5H).

Since Eph-ephrin signaling was shown to affect connexin localization and gap junction communication (Davy et al., 2006; Mellitzer et al., 1999), we also investigated whether connexin-36, the gap junction protein of β cells (Ravier et al., 2005), was required for the effects of Eph-ephrin signaling on insulin secretion (Figure S6). We showed that the stimulatory effect of EphA5-Fc required connexin-36 (compare Fc with EphA5-Fc in Figure S6C), whereas an inhibitory effect of ephrin-A5-Fc was still detected in the absence of this gap junction protein (compare Fc with ephrin-A5-Fc in Figure S6C).

Taken together, these results show that EphA-ephrin-A-signaling events affect several β cell components required for regulating insulin secretion: F-actin, Rac1, and connexin-36.

Glucose-Induced Dephosphorylation of EphA5

We then investigated the hypotheses that, under basal conditions, EphA forward signaling was active in order to suppress insulin secretion (Figure 6A) and that glucose stimulation attenuated EphA forward signaling by EphA dephosphorylation, thus allowing predominance of ephrin-A reverse signaling with a stimulatory outcome for insulin secretion (Figure 6B).

Supporting this hypothesis, EphA5 phosphorylation levels were higher at a low glucose concentration (2 mM)

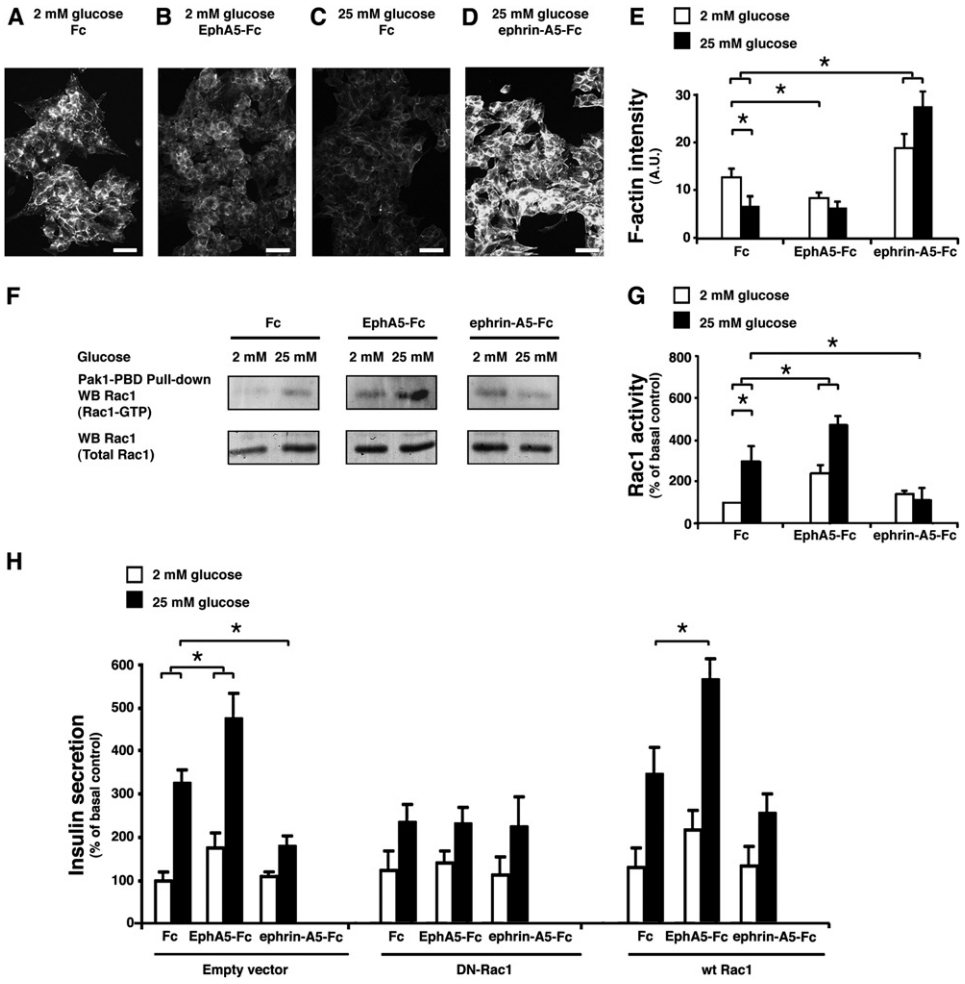

Figure 5. Opposite Effects of EphA5-Fc and Ephrin-A5-Fc on F-Actin and Rac1 Activity

(A–D) F-actin staining of MIN6 cells treated for 10 min with (A) 2 mM glucose and Fc, (B) 2 mM glucose and EphA5-Fc, (C) 25 mM glucose and Fc, (D) 25 mM glucose and ephrin-A5-Fc is shown. Scale bars are 20 μm.

(E) Quantification of F-actin fluorescence intensity in MIN6 cells treated for 10 min with Fc, EphA5-Fc, and ephrin-A5-Fc at 2 mM glucose (white columns) and 25 mM glucose (black columns) is shown. n = 10 images of each coverslip (n = 3) were quantified.

(F) Detection of active Rac1-GTP (upper bands) and total Rac1 (lower bands) in western blots is shown. Cells were treated for 10 min with Fc, EphA5-Fc, and ephrin-A5-Fc at 2 mM glucose and 25 mM glucose.

(G) Corresponding histograms of Rac1 activity, relative to total Rac1 protein, are shown. n = 3 experiments.

(H) Insulin secretion from MIN6 cells transfected with empty vector, *DN-Rac1*, and WT *Rac1* after treatment with Fc, EphA5-Fc, and ephrin-A5-Fc at 2 mM glucose (white columns) and 25 mM glucose (black columns) is shown. n = 3 experiments.

*$p < 0.05$. All values are means ± SD.

compared to all depicted time points after glucose stimulation (25 mM; Figures 6C and 6D). In islets, the presence of phosphorylated EphA5 decreased shortly after glucose stimulation (Figure 6C). However, when the islets were brought back to low glucose concentration, EphA5 phosphorylation reached basal levels again (Figure 6C).

As with islets, the amount of phosphorylated EphA5 in MIN6 cells decreased shortly after glucose stimulation (Figure 6D), and bringing back glucose-stimulated MIN6 cells to a low glucose concentration fully restored basal EphA5 phosphorylation (Figure 6D). We also noticed that ephrin-A5-Fc significantly increased EphA5 phosphorylation in nonconfluent MIN6 cells (Figures 3E, 3F, 6E, and 6F). Thus, EphA5 phosphorylation correlates with inhibition of insulin secretion.

To demonstrate that EphA5 was not the only EphA to be dephosphorylated in β cells, we performed experiments with other members of the EphA-receptor family and observed a similar dephosphorylation upon glucose stimulation (data not shown). We also showed that 10 μM peroxovanadate (PV), a PTP inhibitor, effectively prevented glucose-induced EphA5 dephosphorylation (Figure 6G). Therefore, these results suggest that, under basal conditions, EphA forward signaling is active in order to inhibit

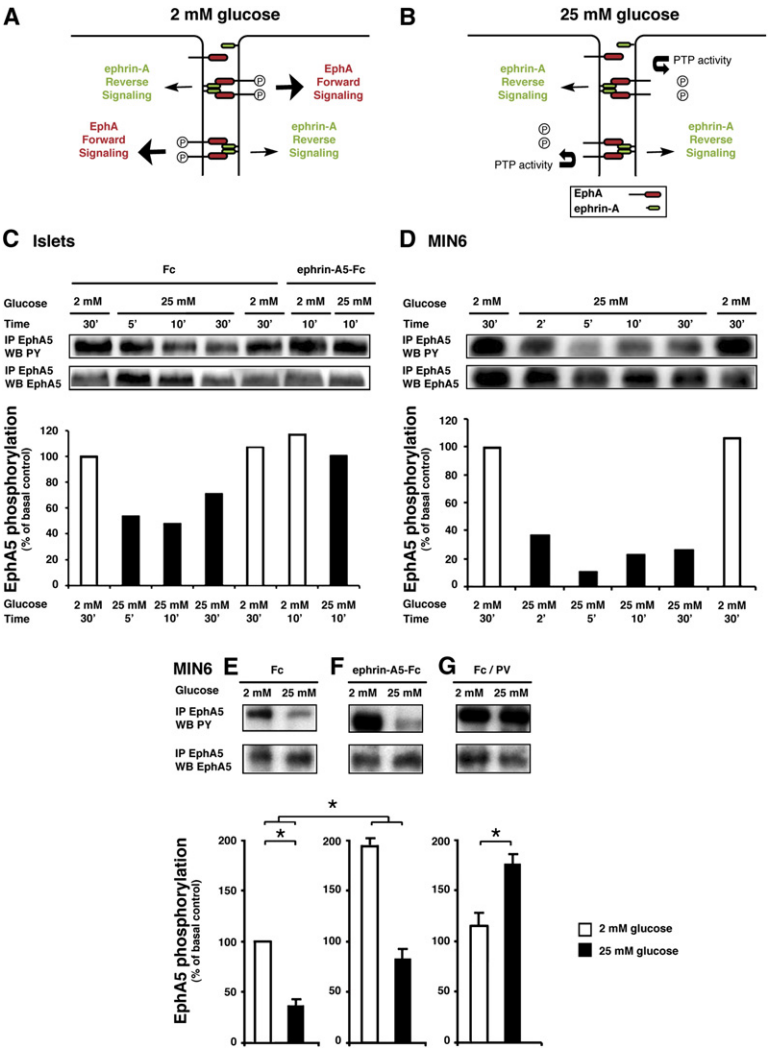

Figure 6. Glucose-Induced Dephosphorylation of EphA5

(A and B) Model showing how glucose changes the outcome of EphA-ephrin-A bidirectional signaling. (A) At low glucose concentration, EphA forward signaling is active. (B) Upon glucose stimulation, EphA forward signaling is attenuated by EphA dephosphorylation involving a protein tyrosine phosphatase (PTP) activity.

(C–G) Detection of tyrosine-phosphorylated EphA5 (PY) and total EphA5 in western blots after immunoprecipitation of EphA5 from cell lysates is shown. The EphA5 phosphorylation level relative to total EphA5 protein is shown in histograms. 2 mM glucose (white columns) and 25 mM glucose (black columns) are shown.

(C) Pancreatic islets were treated with 2 mM glucose for 30 min, then with 25 mM glucose for 5, 10, and 30 min; this was followed by a switch from 25 mM to 2 mM glucose for 30 min. Islets treated with ephrin-A5-Fc for 10 min at 2 mM and 25 mM glucose are also shown.

(D) MIN6 cells were treated with 2 mM glucose for 30 min and then with 25 mM glucose for 2, 5, 10, and 30 min, followed by a switch from 25 mM to 2 mM glucose for 30 min.

(E–G) MIN6 cells were treated for 5 min with (E) Fc, (F) ephrin-A5-Fc, and (G) Fc + 10 μM PV (to inhibit the glucose-induced EphA5 dephosphorylation) at 2 mM and 25 mM glucose. n = 2 experiments. *p < 0.05. All values are means ± SD.

insulin secretion (Figure 6A) and that, upon glucose stimulation, EphA forward signaling is downregulated by dephosphorylation (Figure 6B).

EphA5 Dephosphorylation Is Required for Glucose-Stimulated Insulin Secretion

It was previously shown that treatment of islets with 10 μM PV strongly reduced glucose-stimulated insulin secretion (Gogg et al., 2001). Based on our experiments, we hypothesized that PV was inhibitory, in part, by preventing glucose-induced EphA dephosphorylation (Figure 7A). After confirming in islets and MIN6 cells that 10 μM PV suppressed glucose-stimulated insulin secretion (Figures 7D and 7E), we showed that insulin secretion could be partially rescued in PV-treated MIN6 cells by overexpression of DN-EphA5 (Figure 7B; compare Empty vector/PV with DN-EphA5/PV in Figure 7E). In contrast, WT EphA5 reduced glucose-stimulated insulin secretion in PV-treated MIN6 cells even further (Figure 7C; compare empty vector/PV with WT EphA5/PV in Figure 7E). We conclude that downregulation of EphA forward signaling is essential for glucose-stimulated insulin secretion.

DISCUSSION

The Mystery of β Cell Communication

A few dozen to a few thousand β cells form the core of pancreatic islets (Halban, 2004). The presence of islets and β cell aggregates in all vertebrates underlines the importance of β cell-β cell interactions. Various experiments over the last three decades have shown that β cell communication inhibits basal insulin secretion and enhances glucose-stimulated insulin secretion; therefore, it contributes to glucose homeostasis (Bosco et al., 1989; Dahl et al., 1996; Halban et al., 1982; Hauge-Evans et al., 1999; Hopcroft et al., 1985; Luther et al., 2006; Maes and Pipeleers, 1984; Meda et al., 1990; Ravier et al., 2005; Yamagata et al., 2002). However, it remained an open question how β cell communication exerted opposite effects on

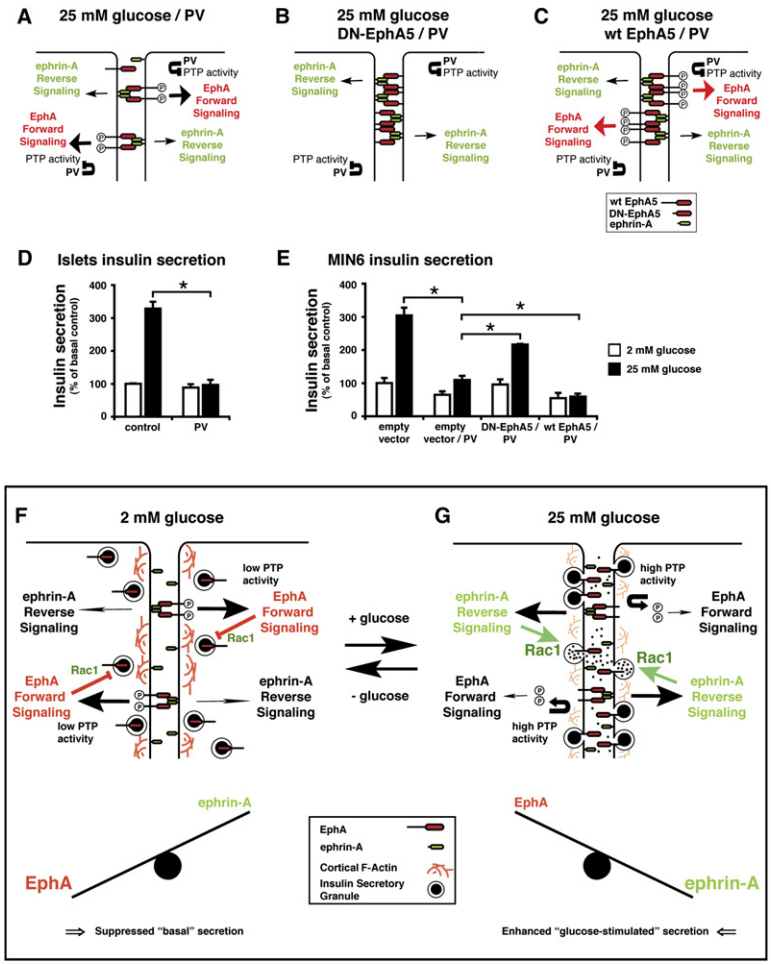

Figure 7. EphA Dephosphorylation Is Required for Insulin Secretion

(A) Model shows that PV blocks a PTP activity, which normally dephosphorylates EphAs at high glucose concentration.

(B) Model shows that overexpression of EphA5 that is lacking its cytoplasmic domain (DN-EphA5) rescues inhibition of EphA forward signaling in PV-treated cells at high glucose concentration.

(C) Model shows that overexpression of WT EphA5 potentiates EphA forward signaling in PV-treated cells.

(D) Insulin secretion from mouse pancreatic islets treated with Fc in the absence or presence of 10 μM PV at 2 mM glucose (white columns) and 25 mM glucose (black columns) is shown. Secreted insulin is normalized to insulin content and total protein content. n = 3 experiments. *$p < 0.05$. All values are means ± SD.

(E) shows insulin secretion from MIN6 cells transfected with an empty vector, *DN-EphA5*, or WT *EphA5* in the absence or presence of 10 μM PV at 2 mM glucose (white columns) and 25 mM glucose (black columns). Secreted insulin is normalized to insulin content and total protein content. n = 2 experiments. *$p < 0.05$. All values are means ± SD.

(F and G) Model. (F) At low glucose concentration, interactions between EphAs (red) and ephrin-As (green) on adjacent β cell plasma membranes suppress insulin secretion. (G) Upon glucose stimulation, a PTP activity dephosphorylates EphAs, and EphA-ephrin-A interactions stimulate insulin secretion.

insulin secretion that were dependent on glucose concentrations.

EphA-Ephrin-A-Mediated Cell-Cell Communication and Its Effects on Insulin Secretion

Here, we discovered that β cells communicate with each other via ephrin-As and EphAs. Based on our results, we propose a model that explains for the first time how β cell communication exerts opposite effects on insulin secretion, dependent on glucose concentrations: at a low glucose concentration (Figure 7F), EphA forward signaling is predominant and suppresses insulin secretion. In contrast, at a high glucose concentration (Figure 7G), EphAs are dephosphorylated via PTP activity. This dephosphorylation shifts the balance from EphA forward signaling toward ephrin-A reverse signaling, which enhances insulin secretion (Figure 7G).

We showed that EphAs and ephrin-As are segregated within mouse insulinoma cells and are able to functionally interact with the ephrin-As and EphAs of adjacent cells. We discovered that a large portion of EphAs was localized on insulin secretory granules, suggesting that EphAs access the β cell plasma membrane when insulin is released with different outcomes that are dependent on glucose concentration. At low glucose concentrations, EphAs are phosphorylated, and they inhibit further insulin secretion (negative feedback; Figure 7F). In contrast, at high glucose concentrations, EphAs are dephosphorylated and, therefore, support insulin secretion via ephrin-A reverse signaling (positive feedback; Figure 7G).

The proposed mechanism therefore explains how β cell communication suppresses basal insulin secretion (Figure 7F) and, at the same time, enhances glucose-stimulated secretion (Figure 7G).

A New Role of EphA and Ephrin-A Coexpression

In many cell types, EphA forward signaling and ephrin-A reverse signaling mediate opposite effects (Marquardt et al., 2005), and cells coexpressing EphAs and ephrin-As exist in many tissues (Carvalho et al., 2006; Marquardt et al., 2005). This raises the fundamental question about the role of their coexistence. Two hypotheses have been recently proposed (Carvalho et al., 2006; Marquardt et al., 2005). According to one hypothesis, EphAs and ephrin-As are segregated in different plasma membrane domains, from which they interact in *trans* with the ephrin-As and

EphAs of adjacent cells, thus resulting in signaling events (Marquardt et al., 2005). According to the other hypothesis, EphAs and ephrin-As are not segregated, but they interact in *cis*, thus preventing *trans*-interactions and signaling (Carvalho et al., 2006).

For pancreatic β cells, we show that EphAs and ephrin-As interact in *trans* and that glucose stimulation modulates EphA forward signaling. Firstly, EphAs and ephrin-As are segregated since EphAs are present on insulin secretory granules, whereas ephrin-As are absent from these exocytotic vesicles. Secondly, EphA5 phosphorylation increases with increasing cell-cell contacts, suggesting that endogenous EphA5 interacts in *trans* with endogenous ephrin-As. Finally, ephrin-A5-Fc significantly increases EphA5 phosphorylation in β cells, showing that EphA5 can interact with ephrin-A5 in *trans*.

Based on our results, we propose that coexpression of EphAs and ephrin-As regulates the physiologic insulin secretory response of β cells to glucose. Interestingly, many synapses express juxtaposed Eph receptors and ephrins (Armstrong et al., 2006; Dalva et al., 2000; Grunwald et al., 2001; Henderson et al., 2001; Murai and Pasquale, 2004), and it is possible that secretory events in the synapse are modulated by EphA-ephrin-A bidirectional signaling in a similar way as insulin secretion in pancreatic islets.

Two Pathways to Stimulate Insulin Secretion

In this study, we showed that either inhibition of EphA forward signaling or activation of ephrin-A reverse signaling increases glucose-stimulated insulin secretion. The EphA forward-signaling pathway involves the transmembrane EphA5 RTK. In contrast, the ephrin-A reverse signaling pathway involves the GPI-linked ephrin-A5. Since ephrin-A5 is localized on the outer leaflet of the plasma membrane, it cannot transmit signals on its own and probably depends on transmembrane proteins for signal transmission. Thus, the ephrin-A reverse-signaling pathway may be similar to the signaling pathway of other GPI-linked proteins, such as GDNF receptors (GFRα) that transmit signals into the cell by modulating the activity of associated transmembrane proteins (Paratcha et al., 2003).

In the future, it will be necessary to identify the properties of all Ephs and ephrins expressed in β cells in order to design strategies for improving glucose-stimulated insulin secretion in humans. For example, EphA7-Fc moderately, but selectively, increases glucose-stimulated insulin secretion rather than basal insulin secretion, possibly due to its special affinity for the ephrin-As expressed in β cells. Thus, drugs may be developed that target the Eph-ephrin-signaling pathways in β cells and that may circumvent the hypoglycemic side effects of most drugs currently used for diabetes treatment. This study therefore uncovered two new pathways in β cells that can now be exploited for developing novel treatments of type II diabetes, a disease currently affecting 200 million people worldwide.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Procedure

MIN6 cells (Miyazaki et al., 1990) were electroporated via nucleofection (Amaxa) with *pEGFP*, *pEGFP-wtEphA5* (Gao et al., 1998), *pEGFP-DN-EphA5* (Gao et al., 1998), *pEGFP-ephrin-A5* (Wimmer-Kleikamp et al., 2004), *pEGFP-wtRac1*, *pEGFP-DN-Rac1* (Rac1N17), and *pcDNA-Cx36* constructs as well as siRNA against *firefly luciferase* (control siRNA), *ephrin-A5*, and *cx36*.

Mouse Models, Pancreatic Islets, and Glucose Tolerance Test

Pancreatic islets were isolated from female NMRI mice. Exceptions were the islets from ephrin-A5$^{-/-}$ and control mice (Knoll et al., 2001) as well as the islets used for TIR-FM, which were C57BL/6. All mice used were 8 to 10 weeks old. Human pancreatic islets were isolated from a perfused human pancreas by using a protocol approved by the Ethical Research Committee of the Technical University Medical School Dresden and according to the law of the State of Saxony. The glucose tolerance test was performed as previously described (Lammert et al., 2003).

RT-PCR, siRNA Syntheses, and Real-Time RT-PCR

Total RNA was extracted from MIN6 cells and mouse pancreatic islets, transcribed into cDNA, and used for RT-PCR. All *Ephs* and *ephrins* primers were initially tested on cDNA isolated from different embryonic and adult mouse organs as positive controls.

Small interfering RNA against *3′-UTR* of *ephrin-A5* and *cx36* was prepared as previously described (Nikolova et al., 2006), and knockdown efficiencies were tested by using real-time RT-PCR. All primer sequences are listed in Table S1.

Insulin Secretion from Pancreatic Islets and MIN6 Cells

For insulin secretion measurements, islets or MIN6 cells were starved for 1 hr in Krebs Ringer Buffer (KRB) containing 2 mM glucose. After starvation, medium was exchanged for the same buffer ± Fc-fusion proteins to measure basal secretion or for KRB containing 25 mM glucose ± Fc-fusion proteins to measure glucose-stimulated insulin secretion during 1 hr incubation. Islets were continuously shaken (300–500 rpm) to facilitate access of Fc-fusion proteins to islet β cells. The amount of secreted insulin was measured in the medium, and islets or MIN6 cells were subsequently dissolved in RIPA buffer to measure insulin content and total protein content. Secreted insulin was normalized to total insulin content and total protein content and presented as percent of basal control insulin secretion. In all histograms, the first column represents the basal control (=100%). Secreted insulin and insulin content were measured by using ultrasensitive rat insulin ELISA (Crystal Chem.). The total protein content was measured by using BCA kit (Molecular Probes). Fc-fusion proteins (R&D systems) and sodium vanadate (Sigma) were used at concentrations of 4 μg/ml and 10 μM, respectively. To control for cell viability and proliferation during 1 hr insulin secretion assay, water-soluble tetrazolium (WST-1) reagent (Roche) and BrdU ELISA (Roche) were used, respectively (Figure S4).

Confocal Light Microscopy and TIR-FM

1:100-diluted rabbit anti-EphA5, rabbit anti-EphA7, rabbit anti-ephrin-A1 (Santa Cruz), goat anti-ephrin-A5 (R&D systems), guinea pig anti-insulin (DAKO), and rabbit anti-connexin-36 antibodies (Invitrogen) as well as 4 μg/ml Fc-fusion proteins (R&D systems) were used for staining of cells and fixed pancreas sections. Secondary antibodies were conjugated with AlexaFluor488 (Molecular Probes) and with Cy5 (Dianova). DAPI (Sigma) was used to stain cell nuclei. MIN6 cells were treated with Fc-fusion proteins for 10 min, fixed, and stained with 1:500-diluted phalloidin-rhodamine (Molecular Probes). Confocal images were acquired by using a Zeiss confocal microscope. Intensities were quantified by using ImageJ software (NIH).

The Olympus TIR-FM was used with a high-aperture objective lens (Apo 100× OHR; NA 1.65, Olympus). To monitor single insulin granules, adenovirus (insulin-GFP)-infected primary mouse β cells on high-refractive-index glass were mounted in an open chamber and incubated for 60 min at 37°C in KRB containing 2.2 mM glucose (starvation). Cells were preincubated for 15 min ± Fc-fusion proteins under basal conditions, transferred to a thermostat-controlled stage (37°C), and stimulated by addition of 22 mM glucose-KRB ± 4 μg/ml EphA5-Fc or ephrin-A5-Fc. Images were acquired every 300 ms and analyzed by using Metamorph software (Molecular Devices).

Immunoprecipitation and Western Blot

MIN6 cell lysates and islet cell lysates containing 1 μg or 500 ng total protein, respectively, were used for immunoprecipitation with rabbit anti-EphA5 antibody (Santa Cruz) and protein-A beads (Amersham). For pulldown experiments, a Rac1-activation StressXPress Kit (Biomol) was used. For western blots, rabbit anti-EphA5 antibody (Santa Cruz), mouse anti-PY antibody (Biomol) and rabbit anti-Rac1 antibody (Santa Cruz) were used in combination with HRP-conjugated secondary antibodies (Dianova). Western blots were developed by using an ECL system (Amersham). The intensities of EphA5 bands and Rac1 bands were normalized to the intensities of total EphA5 and total Rac1, respectively, and presented as percent of basal control. The intensities of the bands were quantified by using TotalLab software (Stratagene).

Statistical Analyses

Statistical significance was determined by using Student's t test with two-tailed distribution and two-sample unequal variance. In all tests, two groups with only one changed parameter were compared. p values for all relevant comparisons are given in Table S2.

Supplemental Data

Supplemental Data include six figures, two tables, and four movies and can be found with this article online at http://www.cell.com/cgi/content/full/129/2/359/DC1/.

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