Focal Adhesion Remodeling Is Crucial for Glucose-Stimulated Insulin Secretion and Involves Activation of Focal Adhesion Kinase and Paxillin

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OBJECTIVE—Actin cytoskeleton remodeling is known to be involved in glucose-stimulated insulin secretion (GSIS). We have observed glucose-stimulated changes at the β-cell basal membrane similar to focal adhesion remodeling in cell migration. This led us to study the role of two key focal adhesion proteins, focal adhesion kinase (FAK) and paxillin, in GSIS.

RESEARCH DESIGN AND METHODS—All studies were performed using rat primary β-cells or isolated islets. Protein phosphorylation and subcellular localization were determined by Western blotting and confocal immunofluorescence, respectively. Insulin was measured by radioimmunoassay. Both siRNA and pharmacological approaches were used to assess the role of FAK and paxillin in glucose-stimulated focal adhesion remodeling and insulin secretion.

RESULTS—Glucose stimulation of β-cells in monolayer significantly increased phosphorylation of FAK and paxillin as well as cell surface area. This coincided with the appearance at the basal membrane of numerous shorter actin filopodial extensions, containing not only phosphorylated paxillin, FAK, and extracellular signal–related kinase 1/2 but also two SNARE proteins, synaptosomal-associated protein 25 and syntaxin 1, indicating involvement in exocytosis. SR7037 completely inhibited this sequence of events, indicating the requirement of increased cytosolic Ca^{2+}. Furthermore, knockdown of paxillin significantly decreased GSIS, as did inhibition of glucose-induced FAK phosphorylation by compound Y15. Key findings were confirmed in β-cells within the natural setting of islets.

CONCLUSIONS—Glucose-stimulated remodeling of focal adhesions and phosphorylation of FAK and paxillin are involved in full development of GSIS, indicating a previously unknown role for focal adhesion remodeling in pancreatic β-cell function. ***Diabetes* 60:1146–1157, 2011**

Nearly 40 years ago, studies first indicated the presence of microfilamentous structures that impacted stimulated secretion in pancreatic β-cells (1,2). Filamentous actin (F-actin) in β-cells was reported to be organized as a dense web beneath the plasma membrane (2) and was later shown to undergo remodeling upon glucose stimulation. In addition, others reported enhanced secretagogue-induced insulin secretion in the presence of F-actin disrupting agents (3–7). Taken together, these reports suggest F-actin remodeling as a key factor in insulin granule priming and mobilization through the F-actin web, but the underlying molecular mechanism and key signaling pathways involved in this process remain largely unknown.

Two-way signaling between a cell and its surrounding extracellular matrix (ECM) is highly important for actin cytoskeleton organization and thereby also for β-cell viability and function (8,9). The majority of the cellular receptors involved in cell-matrix interactions belong to the integrin family (10). Studies on rat pancreatic β-cells revealed that α3β1 and α6β1 integrins are highly expressed on the cell surface (8,11). In addition, both integrins are receptors for laminin-5, a component of ECM known to promote rat β-cell function and survival (8,9). The integrin-mediated physical connection between cell and ECM is not simply mechanical but also results in the induction of outside-in integrin-dependent signaling pathways, beginning with tyrosine phosphorylation of the key cytoskeletal protein focal adhesion kinase (FAK) (12).

FAK is a nonreceptor tyrosine kinase considered to be a central molecule in integrin-mediated signaling, involved in cell cycle progression, cell survival, and migration (13). While the NH_2-terminal domain of FAK is important for interaction with integrins (14), the carboxyl-terminal tyrosine (Y) 397 residue constitutes a major phoshorylation site located in a linker region connecting the regulatory and central kinase domain. In the inactivated state, this site and the Src recruitment site, located in the activation loop, are blocked by the regulatory domain, preventing autophosphorylation of Y397 and the ensuing Src-mediated phosphorylation of the activation loop (15). While the precise mechanisms that allow Y397 autophosphorylation and subsequent steps in FAK activation are not clear, interaction with Src results in phosphorylation of multiple other FAK tyrosine residues, as well as other focal complex-associated proteins including $p130^{CAS}$ and paxillin (16–20). These and several other proteins are, upon integrin engagement with the ECM, constantly recruited to or removed from and activated or deactivated in dynamic signaling structures named focal adhesions. This eventually results in cytoskeletal changes and activation of other downstream signaling cascades such as the phosphatidylinositol 3-kinase, Akt, extracellular signal–regulated kinase (ERK)1/2, and mitogen-activated protein kinase (21) pathways.

In the current study, we show that activated FAK-paxillin complexes are incorporated into nascent focal adhesions upon glucose stimulation of primary rat β-cells. Focal adhesion remodeling in response to glucose is Ca^{2+}-dependent, rapid, reversible, and linked to the short-term glucose-induced activation of the ERK1/2 signaling pathway. Finally, we show here for the first time that these glucose-mediated events are essential for regulated insulin secretion from β-cells whether in monolayer cultures or within whole islets.

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RESEARCH DESIGN AND METHODS

Reagents and antibodies. The FAK inhibitor 1,2,4,5-benzenetetraamine tetrahydrochloride (Y15) was purchased from Sigma-Aldrich (St. Louis, MO). To visualize F-actin, Alexa Fluor 647-phalloidin was obtained from Invitrogen (Carlsbad, CA). Primary antibodies (Abs) were as follows: rabbit anti-FAK polyclonal (p)Ab (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–phospho-(Y397)FAK pAb and rabbit anti–phospho-(Y118)paxillin pAb (Invitrogen), mouse anti-paxillin monoclonal (m)Ab (BD Transduction Laboratories, San Jose, CA), rabbit anti-ERK1/2 pAb and rabbit anti–phospho-(T202/Y204) ERK1/2 pAb (Cell Signaling Technology, Beverly, MA), mouse antiactin mAb (Millipore, Temecula, CA). Secondary Abs were as follows: donkey anti-rabbit horseradish peroxidase and sheep anti-mouse horseradish peroxidase (Amersham Biosciences, Uppsala, Sweden), donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 555 (Invitrogen), and 7-amino-4-methylcoumarin-4-acetate (AMCA)-conjugated donkey anti–guinea pig (Jackson ImmunoResearch Laboratories, West Grove, PA).

Primary rat β-cell purification and culture. Rat islet isolation, β-cell sorting, and monolayer culture on ECM from 804G cells (804G-ECM) were performed as previously described (22). Intact islets were first maintained for 24 h and then studied in suspension culture.

RNAi-mediated silencing of paxillin. Paxillin expression was depleted by transfecting primary rat β-cells twice with a pool of three different siRNAs (5′-UGGCACAGUCCUGGACCCCTT-3′, 5′-CCUCUCUGAGCUGGACCGGTT-3′, and 5′-GACAUGGCACCCCGAGCACTT-3′; Microsynth, Balgach, Switzerland) directed against rat paxillin mRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated for 72 h to allow for siRNA expression before analysis.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were performed as previously described (5).Western blots were incubated with the ECL Plus Western Blotting detection system (Amersham Biosciences) and exposed with the Fujifilm LAS-4000 camera system. Protein band intensities were quantified by densitometry using Multi Gauge software (version 3.0).

Immunofluorescence and confocal microscopy. Immunofluorescence and confocal microscopy on sorted β-cells in monolayer was performed as previously described (5). Isolated rat islets were washed in PBS and fixed in 2% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at 5 μm. Before immunofluorescence, sections were deparaffinized and rehydrated with a series of alcohol solutions of decreasing concentration. Primary antibodies (guinea pig anti-insulin, rabbit anti-phospho-paxillin, and mouse anti-actin) and secondary antibodies (AMCA-conjugated anti–guinea pig, Alexa 488–conjugated anti-rabbit, and Alexa 555–conjugated anti-mouse) were diluted in PBS and 0.5% BSA, and incubations were performed at room temperature.

Insulin secretion and transferase-mediated dUTP nick-end labeling assays. Insulin secretion assays were performed as previously described (5). With transferase-mediated dUTP nick-end labeling assay, cell death was measured as previously described (23).

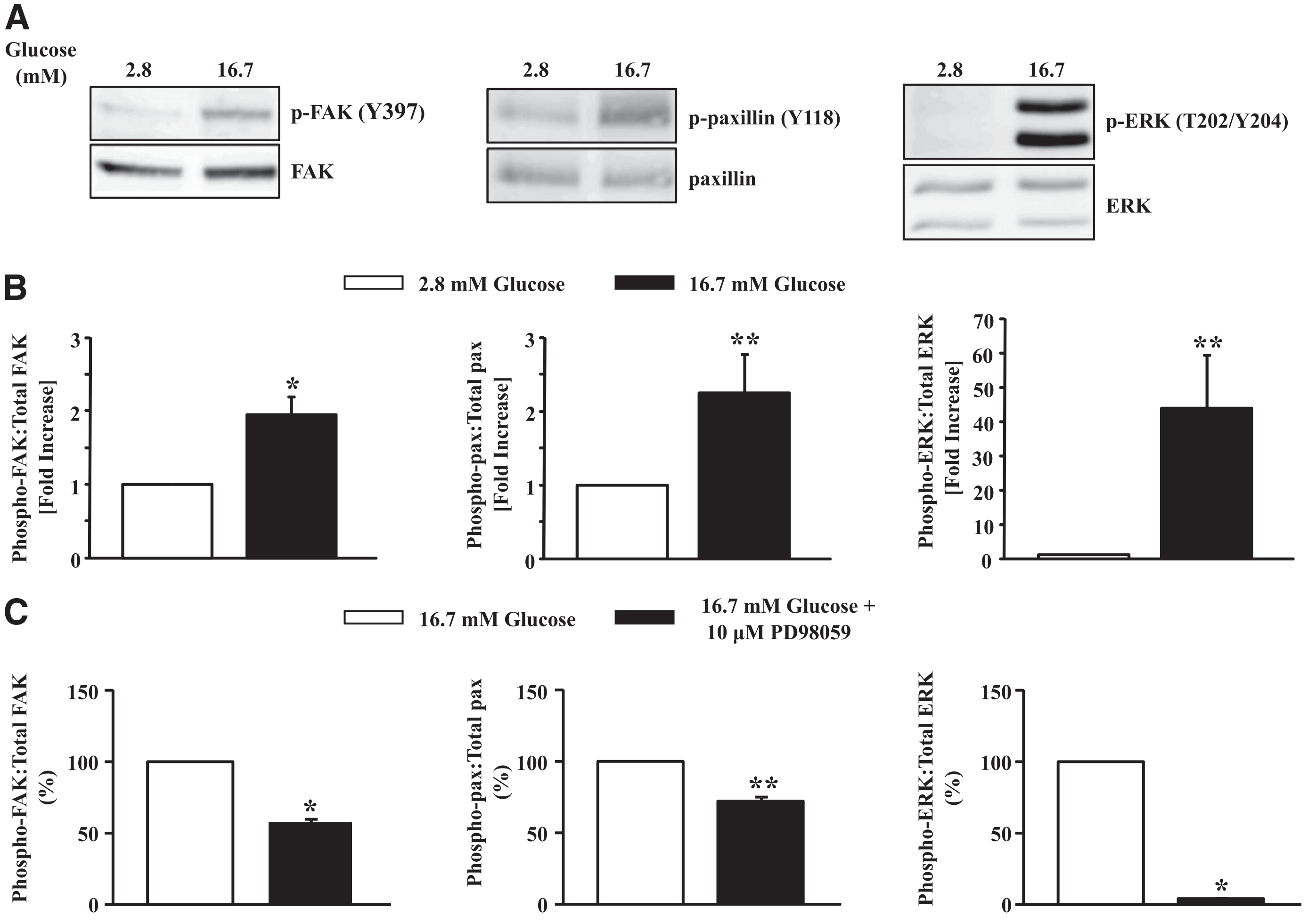

FIG. 1. High glucose stimulation potentiates FAK, paxillin (pax), and ERK1/2 phosphorylation in primary rat β-cells and is inhibited by the ERK1/2 inhibitor PD98059. *A*: Rat β-cells were preincubated for 2 h in the presence of low glucose (2.8 mmol/L) and were further treated for 20 min with low (2.8 mmol/L) or high (16.7 mmol/L) glucose. Cells were then collected and lysates were analyzed by Western blot with the indicated antibodies. A representative blot from six independent experiments is shown. *B*: The relative intensities of the phosphorylated (p) and total protein bands were quantified by densitometry and expressed as a ratio. Data presented as fold increase in this ratio for 16.7 vs. 2.8 mmol/L glucose are means ± SEM from six independent experiments (P* < 0.01 and ***P* < 0.05). *C*: After a 2-h preincubation in the presence of low glucose with 10 μmol/L PD98059 (or DMSO as a negative control), rat β-cells were stimulated for 20 min with high glucose with PD98059 (or DMSO). Cell lysates were analyzed by Western blot, and the relative intensities of the phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio. Ratios were normalized to control high glucose–stimulated cells. Data are means ± SEM from three independent experiments (**P* < 0.0001 and ***P* < 0.01).**

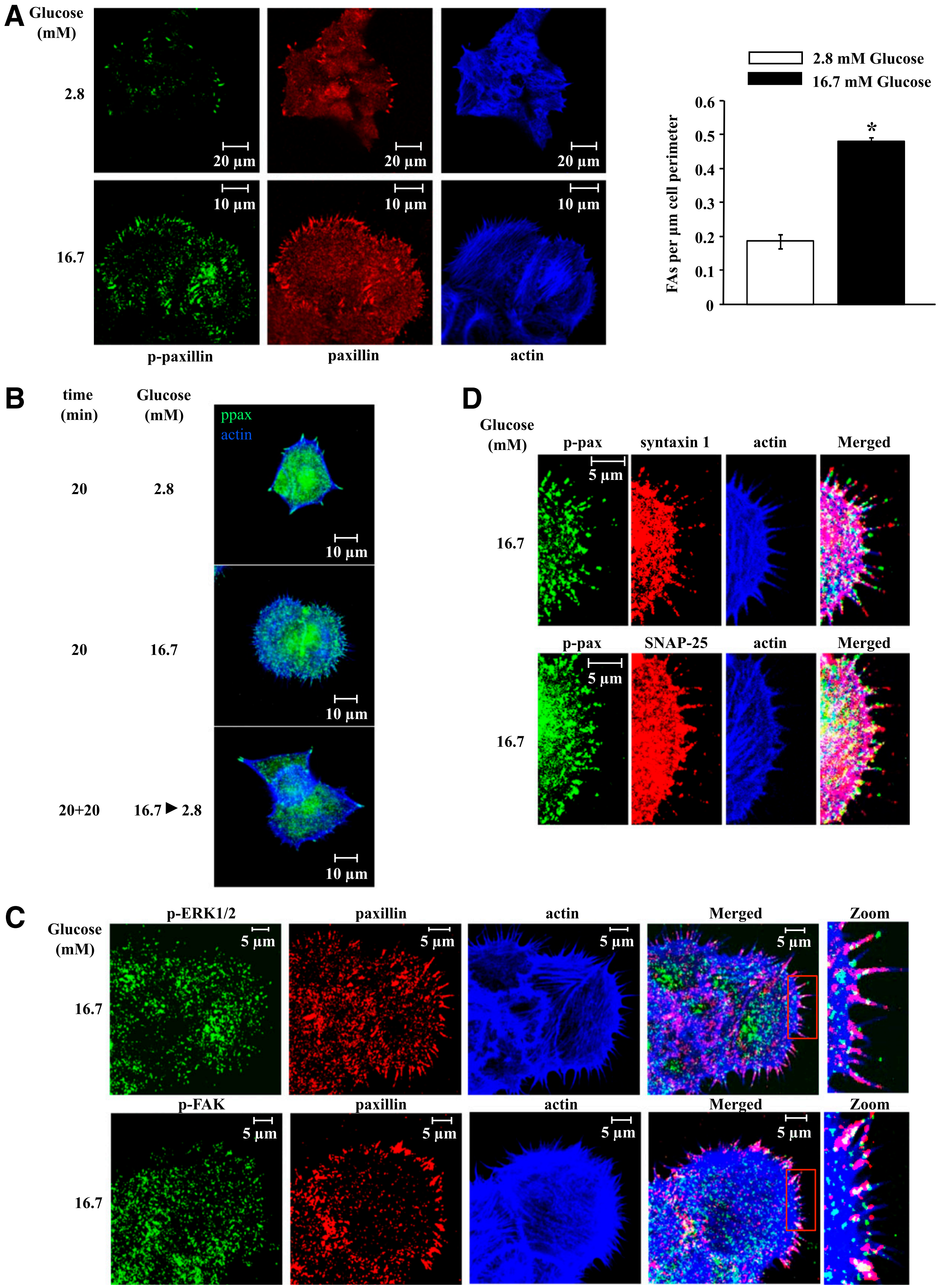

FIG. 2. High glucose results in the accumulation of phospho (p)-FAK, phospho-paxillin, and phospho-ERK1/2 at newly formed focal adhesions in primary rat β-cells. *A*: Rat β-cells were preincubated for 2 h in the presence of low glucose (2.8 mmol/L) and were further treated for 20 min with low (2.8 mmol/L) or high (16.7 mmol/L) glucose. Cells were subsequently fixed and stained for phospho-paxillin (green), total paxillin (red), and actin (with phalloidin, blue). ***Right panel***: Quantification of phospho-paxillin–containing focal adhesions in low- vs. high-glucose conditions. Data are expressed as number of focal adhesions per micrometer of cell perimeter and are means ± SEM from four independent experiments (*$P <$ 0.0001). *B*: After preincubation for 2 h with 2.8 mmol/L glucose, rat β-cells were incubated for 20 min with 2.8 mmol/L glucose (*upper panel*), stimulated for 20 min with 16.7 mmol/L glucose (*middle panel*), or stimulated for 20 min with 16.7 mmol/L glucose and shifted back to 2.8 mmol/L glucose for 20 min (*lower panel*). Cells were fixed and stained for phospho-paxillin (green) and actin (blue). *C*: Rat β-cells were preincubated for

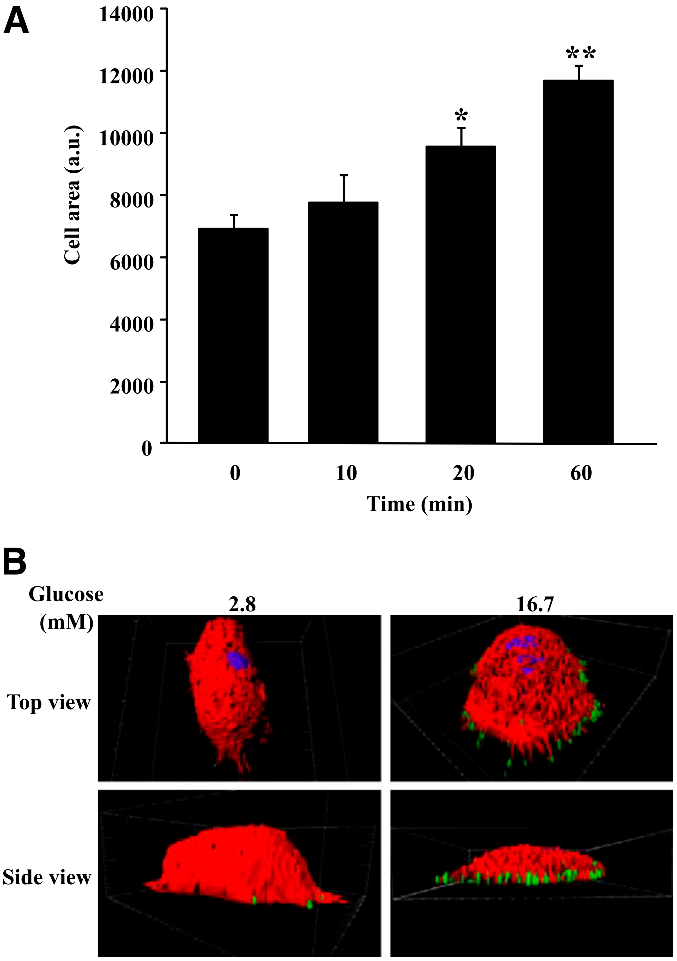

FIG. 3. Glucose-stimulated focal adhesion remodeling is accompanied by progressive β-cell spreading. *A*: Rat primary β-cells were preincubated for 2 h with 2.8 mmol/L glucose and then stimulated with 16.7 mmol/L glucose. Cells were fixed at the indicated times and stained with Evans blue, and cell surface areas were quantified using AxioVision 4.7.2. Data are means ± SEM from four independent experiments; **P* < 0.05 and ***P* < 0.01 vs. control cells kept at 2.8 mmol/L glucose (0 min). a.u., arbitrary units. *B*: Rat primary β-cells were preincubated for 2 h with 2.8 mmol/L glucose and then treated for 20 min with 2.8 mmol/L or 16.7 mmol/L glucose. Cells were then fixed and stained for phospho-paxillin (green) and actin (red). Nuclei (blue) were stained with Hoechst 33342. Three-dimensional reconstructions from confocal image stacks were performed using Imaris (version 4.0). Pictures are representative of multiple images from a single experiment. (A high-quality digital representation of this figure is available in the online issue.)

Statistical analyses. The statistical significance of the differences between the experimental conditions was determined by Student *t* test for unpaired groups. *P* values >0.05 were considered significant.

RESULTS

Aside from experiments using intact islets when indicated, all studies were performed using sorted rat primary β-cells cultured in monolayer on a surface coated with 804G-ECM (22).

FAK, paxillin, and ERK1/2 are activated upon glucose stimulation of rat primary β-cells. To gain insight into a possible role of FAK and two of its downstream targets, paxillin and ERK1/2, in glucose-regulated β-cell function, we studied the phosphorylation status of these proteins in response to glucose. Phosphorylation of both FAK (Y397) and paxillin (Y118) was already detectable under basal conditions but was significantly increased following glucose stimulation (20 min), indicating short-term glucose-mediated activation of these two focal adhesion factors. A similar effect was observed for ERK1/2 (Fig. 1*A* and *B*).

A previous study in the mouse pancreatic β-cell line MIN6 B1 documented the rapid glucose-stimulated activation of ERK1/2 and subcellular localization of active phospho-ERK1/2 at newly formed tips of actin fibers (5). Based on these observations, we used the ERK1/2 inhibitor PD98059 to determine whether ERK1/2 is implicated in the short-term glucose-induced phosphorylation of FAK and paxillin. Figure 1*C* clearly indicates the inhibitory effect of PD98059 on the phosphorylation of both focal adhesion proteins.

Activated FAK, paxillin, and ERK1/2 colocalize in actin filopodial extensions upon glucose stimulation. To determine the main sites of action of phosphorylated FAK and paxillin, we analyzed the subcellular localization of the respective activated proteins in conjunction with the F-actin cytoskeleton by confocal immunofluorescence of the basal cell surface (defined as the cell membrane in direct contact with ECM). In β-cells preincubated for 2 h at 2.8 mmol/L glucose, phosphorylated paxillin localized in a few long filopodial extensions (Fig. 2*A*). Upon short-term glucose stimulation, there was actin cytoskeleton reorganization coinciding with the striking accumulation of phospho-paxillin at the tips of numerous newly formed, shorter filopodia, resembling classical nascent focal adhesions (16.7 mmol/L glucose) (Fig. 2*A*). Similar morphological changes were apparent following stimulation with KCl, phorbol myristic acid (PMA), or glucagon-like peptide (GLP)-1 (data not shown). Quantification of these phospho-paxillin–containing focal adhesions showed a significant increase in focal adhesion density in response to glucose stimulation (Fig. 2*A*). When such glucose-stimulated β-cells were shifted back to low glucose, they regained their basal conformation within 20 min (Fig. 2*B*), indicating the dynamic, reversible character of the glucose-mediated formation of focal adhesions. Shorter stimulations of 5 or 10 min at 16.7 mmol/L glucose resulted in similar changes, indicating the rapid time course of this glucose-induced remodeling process (data not shown).

Further analysis of these filopodial extensions of glucose-stimulated β-cells revealed the colocalization of activated phospho-FAK (Fig. 2*C*) and phospho-ERK1/2 (Fig. 2*C*) along with paxillin, indicating that the previously described sites of short-term glucose-dependent ERK1/2 activation at the tips of actin fibers (5) correspond to the nascent focal adhesions formed in response to glucose. Moreover, (co)localization of phospho-paxillin with two SNARE proteins, synaptosomal-associated protein (SNAP)-25 and syntaxin 1, at these glucose-induced filopodial extensions (Fig. 2*D*) suggested a functional role for these sites in regulated insulin secretion.

Short-term glucose-stimulated β-cell spreading coincides with focal adhesion remodeling. Rat islet β-cells attached on ECM spread in response to high glucose over 24 h (8). To assess the effect of acute exposure, β-cells

2 h in the presence of low glucose (2.8 mmol/L) and were then stimulated for 20 min with 16.7 mmol/L glucose. Cells were fixed and stained with the indicated antibodies. Parts of the merged images (red boxes) are shown at higher magnification (*right panels*) to clearly visualize the presence of paxillin (red) with phospho-FAK and phospho-ERK1/2, respectively (green), in actin-containing filopodia (blue). White regions indicate colocalization. *D*: Cells were treated as described above and were stained with the indicated antibodies. All images are fully representative of three to four independent experiments. (A high-quality digital representation of this figure is available in the online issue.)

were stimulated for 10, 20, 40, and 60 min with high glucose and cell surface was measured. As shown in Fig. 3*A*, there was already a significant increase in spreading after 20 min, with a further increase at 60 min. Furthermore, three-dimensional reconstruction of confocal stack images clearly illustrates that glucose-stimulated cell spreading is accompanied by the appearance of the phospho-paxillin–containing filopodial extensions previously described (Fig. 3*B*).

Glucose-stimulated Ca^{2+} influx is required for activation and translocation of FAK and paxillin. A selective blocker of voltage-gated Ca^{2+} channels, SR7037, was used to establish the need for increased cytosolic Ca^{2+} in glucose-induced focal adhesion remodeling and FAK, paxillin, and ERK1/2 activation. Having confirmed the previously described inhibitory effect of SR7037 on glucose-stimulated insulin secretion (GSIS) (22) (data not shown), we demonstrated that SR7037 markedly decreased short-term glucose-stimulated formation of phospho-paxillin–containing filopodial extensions (Fig. 4*A*) which was validated by quantification of these structures (Fig. 4*A*). Furthermore, Western blot analysis revealed that glucose-induced phosphorylation of FAK, paxillin, and ERK1/2 was inhibited by SR7037 (Fig. 4*B*).

FAK inhibition reduces glucose-stimulated focal adhesion remodeling and insulin secretion. In view of the important role for proteins such as FAK and paxillin in ECM-integrin-actin cytoskeleton interactions and the data presented above, we hypothesized that the activated FAK-paxillin-ERK1/2 complexes located at the newly formed filopodia might mediate GSIS. To test this hypothesis, we first examined whether FAK activation represents a regulatory step in glucose-stimulated phosphorylation of paxillin and ERK1/2, using compound Y15, which specifically inhibits FAK-Y397 autophosphorylation (24,25). As shown in Fig. 5*A*, glucose-induced phosphorylation of FAK, paxillin, and ERK1/2 is significantly inhibited by Y15. This indicates that glucose-induced activation of ERK1/2 is at least partially dependent on FAK activation, whereas we showed (described above) that the opposite is also true, with FAK activation being inhibited by the ERK1/2 inhibitor PD98059. These two observations are compatible with earlier evidence

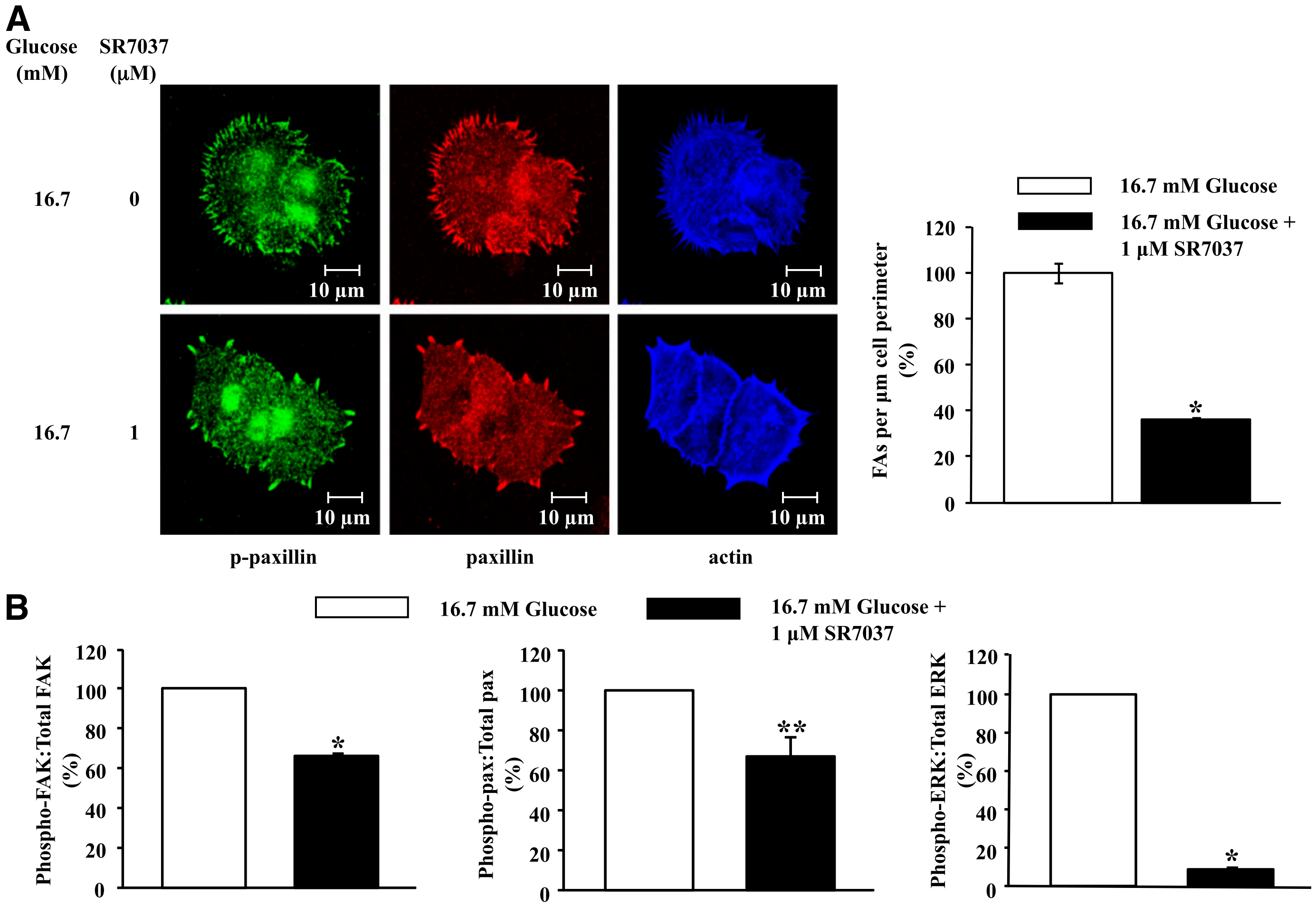

FIG. 4. SR7037, a selective L-type Ca^{2+} channel blocker, decreases glucose-induced focal adhesion remodeling and glucose-stimulated phosphorylation of FAK, paxillin, and ERK1/2 in primary rat β-cells. *A*, *left panel*: Rat β-cells were preincubated for 2 h in the presence of low glucose (2.8 mmol/L) and were further stimulated for 20 min with high (16.7 mmol/L) glucose with 1 µmol/L SR7037 (or DMSO as a negative control). Cells were fixed and stained for phospho-paxillin (green), total paxillin (red), and actin (with phalloidin, blue). All images are fully representative of three independent experiments. *Right panel*: Quantification of phospho-paxillin–containing focal adhesions (FAs). Data are expressed as number of focal adhesions per micrometer of cell perimeter normalized to control high glucose–stimulated cells and are means ± SEM from three independent experiments. $^*P < 0.001$ vs. control-stimulated cells. *B*: Rat β-cells were treated as described above. Cell lysates were analyzed by Western blot with the indicated antibodies, and relative intensities of the phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio. Ratios were normalized to control high glucose–stimulated cells. Data are means ± SEM from three independent experiments ($^*P < 0.0001$ and $^{**}P < 0.05$). (A high-quality digital representation of this figure is available in the online issue.)

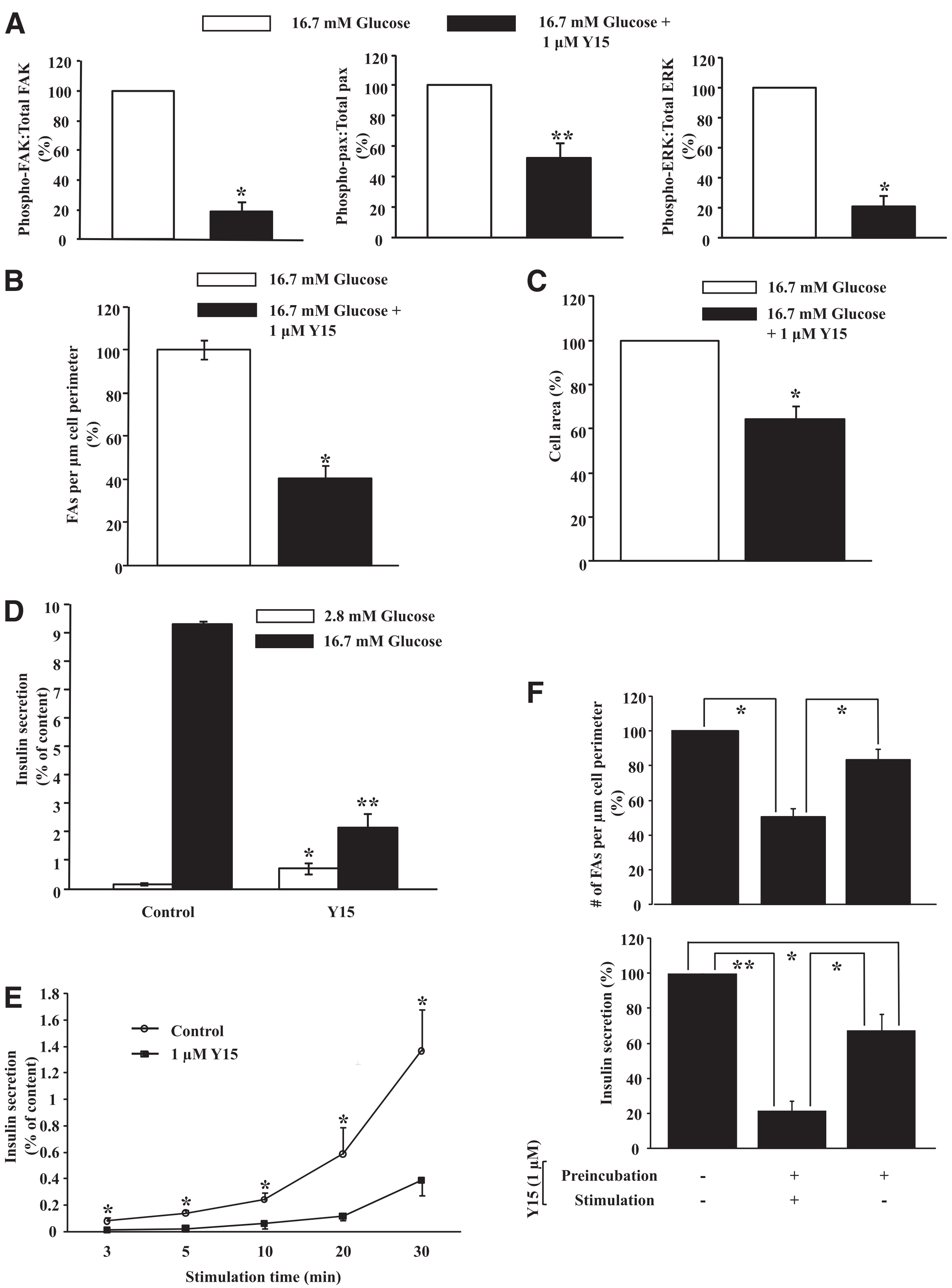

FIG. 5. Effect of FAK inhibition on focal adhesion remodeling and insulin secretion in primary β-cells. *A*: Rat β-cells were preincubated for 2 h in the presence of low glucose (2.8 mmol/L) with Y15 (1 µmol/L or DMSO) and were further stimulated for 20 min with high (16.7 mmol/L) glucose in the presence of Y15 (1 µmol/L or DMSO). Cell lysates were analyzed by Western blot with the indicated antibodies, and the relative intensities of the phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio. Ratios were normalized to control high glucose–stimulated cells. Data are means ± SEM from three independent experiments (*$P < 0.001$ and **$P < 0.01$). pax, paxillin. *B*: Quantification of phospho-paxillin–containing focal adhesions (FAs) in primary β-cells after 20 min stimulation with 16.7 mmol/L glucose with or without 1 µmol/L compound Y15. Data are expressed as number of focal adhesions per micrometer of cell perimeter normalized to control high glucose–stimulated cells and are means ± SEM from three independent experiments. *$P < 0.01$ vs. control-stimulated cells. *C*: β-Cells were incubated for 2 h with 2.8 mmol/L glucose with compound Y15 (or DMSO) and then stimulated for 20 min with 16.7 mmol/L glucose with or without compound Y15. Cells were

of ERK1/2 acting both up- and downstream of FAK in focal adhesion remodeling (26,27).

Immunofluorescence experiments revealed that treatment with Y15 resulted in a marked reduction in glucose-induced translocation and incorporation of phospho-paxillin in newly formed short focal adhesions (data not shown), which was confirmed by quantification of the latter (Fig. 5*B*). Furthermore, Y15 significantly decreased short-term glucose-induced β-cell spreading (Fig. 5*C*).

Finally, we investigated the effect of inhibition of FAK phosphorylation on GSIS. In the presence of compound Y15, insulin secretion in response to glucose was decreased by 76.9 ± 5.4% ($P < 0.001$), whereas basal secretion was 4.4-fold increased (from 0.16 ± 0.02 to 0.72 ± 0.20%; $P < 0.05$) (Fig. 5*D*). This decrease was sustained throughout both early (corresponding to first phase) and late (second phase) periods of the stimulatory period (Fig. 5*E*). There was also a 27.3 ± 8.4% ($P < 0.05$) decrease in the total (secreted plus intracellular) insulin content after compound Y15 treatment (data not shown), which might explain the increase in basal insulin secretion (as a fraction of cell content). The Y15-mediated inhibition of glucose-induced focal adhesion formation was reversible (Fig. 5*F*), and this was attended by the partial recovery of GSIS (Fig. 5*F*).

RNAi-mediated knockdown of FAK and paxillin inhibits GSIS. Although inhibition of FAK activation by Y15 indicated its involvement in focal adhesion remodeling and insulin secretion in response to glucose, there is always the risk of unexpected nonspecific or off-target effects when pharmacological agents are used. We used two different strategies to overcome this. For the first approach, RNAi-mediated silencing of FAK was performed in MIN6 B1 cells, resulting in an ~40% reduction in the level of FAK, which was associated with a 40.2 ± 2.3% ($P < 0.001$) decrease in GSIS (data not shown).

The second series of experiments involved knockdown of paxillin in primary rat β-cells using siRNA duplexes. We have shown previously that siRNA duplexes can be successfully introduced into the vast majority of primary β-cells using standard transfection procedures (28). Paxillin knockdown (Fig. 6*A*) did not affect β-cell death (Fig. 6*B*) but did, similar to Y15, result in a significant inhibition of glucose-induced ERK1/2 phosphorylation (Fig. 6*C*), focal adhesion formation (Fig. 6*D*), and β-cell spreading (Fig. 6*E*). Furthermore, silencing of paxillin also decreased GSIS by 60.0 ± 5.7% ($P < 0.05$) (Fig. 6*F*). However, in contrast to Y15, paxillin knockdown did not affect total insulin content (data not shown) or basal secretion. Finally, Table 1 illustrates that silencing of paxillin also significantly inhibited KCl-, PMA, and GLP-1–stimulated insulin secretion.

Focal adhesion remodeling in intact rat islets. Because all the former experiments were performed using sorted β-cells in two-dimensional monolayer culture on 804G-ECM, a less physiological setting than that of the three-dimensional micro-organ, we also verified β-cell behavior within intact islets. A single preliminary experiment confirmed glucose-induced phosphorylation of FAK at Y397 that was inhibited by treatment of the islets with compound Y15. However, short-term glucose stimulation did not result in increased phosphorylation of paxillin or ERK1/2 (data not shown). Furthermore, and most significantly, three dimensional reconstruction of immunofluorescence confocal stack images of semithin sections of differently treated intact islets (Fig. 7*A*) revealed the glucose-induced concentration of phospho-paxillin in well-defined patches. This glucose-induced effect was inhibited by treatment of the islets with compound Y15 (Fig. 7*A*). The focal adhesion–like filopodial extensions and actin stress fibers observed in two-dimensional β-cell monolayers in response to glucose were not seen in intact islets, but this was anticipated for cells in such a three-dimensional configuration (discussed further below). Finally, we observed that FAK inhibition resulted in a significant decrease (64.5 ± 16.4%; $P < 0.05$) in GSIS from whole islets (Fig. 7*B*). Taken together, these results suggest that, notwithstanding the anticipated differences in glucose-induced physical alterations in β-cells in two-dimensional monolayers versus in whole islets, FAK signaling also appears to play an important role in regulated insulin secretion from β-cells present in the more physiological setting of the three-dimensional micro-organ.

DISCUSSION

Cellular attachment, spreading, and migration involve focal adhesion remodeling (29) that is associated with reorganization of cytoskeletal structures and activation of intracellular signaling pathways. Focal contact sites thereby provide both a structural link between the ECM and cytoskeletal proteins as well as initiation points for outside-in signaling leading to changes in cell activity and gene expression (30–32). In the current study, we describe short-term glucose-stimulated changes at the β-cell basal membrane that are reminiscent of focal adhesion remodeling at filopodia during cell migration and show these to be involved in GSIS.

Both FAK and paxillin have been identified as focal adhesion proteins that become phosphorylated in response to ECM adhesion (33). The best-characterized FAK phosphorylation event is Y397 autophosphorylation, crucial for its kinase activity (34). Regarding paxillin, Y118 has been shown to be the major tyrosine phosphorylation site involved in focal contact turnover and cell motility (20,35). The observation that short-term glucose stimulation of β-cells induces increased phosphorylation of FAK and paxillin at these particular sites thus indicates a possible role for these proteins in β-cell function.

Translocation of FAK and paxillin into focal adhesions has been found to be critical for cell spreading and migration but not for cell adhesion (36,37). In epithelial cells induced to migrate, immunolabeling with phospho-tyrosine

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then fixed and stained with Evans blue and cell surface areas were quantified using AxioVision 4.7.2. Cell areas were normalized to control high glucose–stimulated cells. Data are expressed as means ± SEM from three independent experiments; *$P < 0.01$ vs. control-stimulated cells. *D*: Rat primary β-cells were preincubated for 2 h with 2.8 mmol/L glucose with compound Y15 (or DMSO). Cells were then incubated with low (2.8 mmol/L, basal) followed by high (16.7 mmol/L, stimulated) glucose with or without Y15 for 1 h. Insulin secretion is expressed as a percentage of total insulin cell content. Data are means ± SEM from three independent experiments. *$P < 0.001$ and **$P < 0.05$ vs. control at the same glucose concentration. *E*: Cells were incubated as described above but were stimulated with high glucose with Y15 (or DMSO) for the indicated times. Data are expressed as means ± SEM from four independent experiments. *$P < 0.05$ vs. Y15-treated cells. *F*: Rat β-cells were preincubated for 2 h with 2.8 mmol/L glucose with or without compound Y15 (preincubation conditions). Cells were then stimulated with 16.7 mmol/L glucose with or without Y15 (stimulation conditions). *Upper panel*: Cells were fixed and phospho-paxillin–containing focal adhesions were quantified and expressed as number per micrometer of cell perimeter normalized to control high glucose–stimulated cells. *Lower panel*: Insulin secretion was normalized to control high glucose–stimulated cells. Data are means ± SEM from three independent experiments. *$P < 0.05$ and **$P < 0.001$.

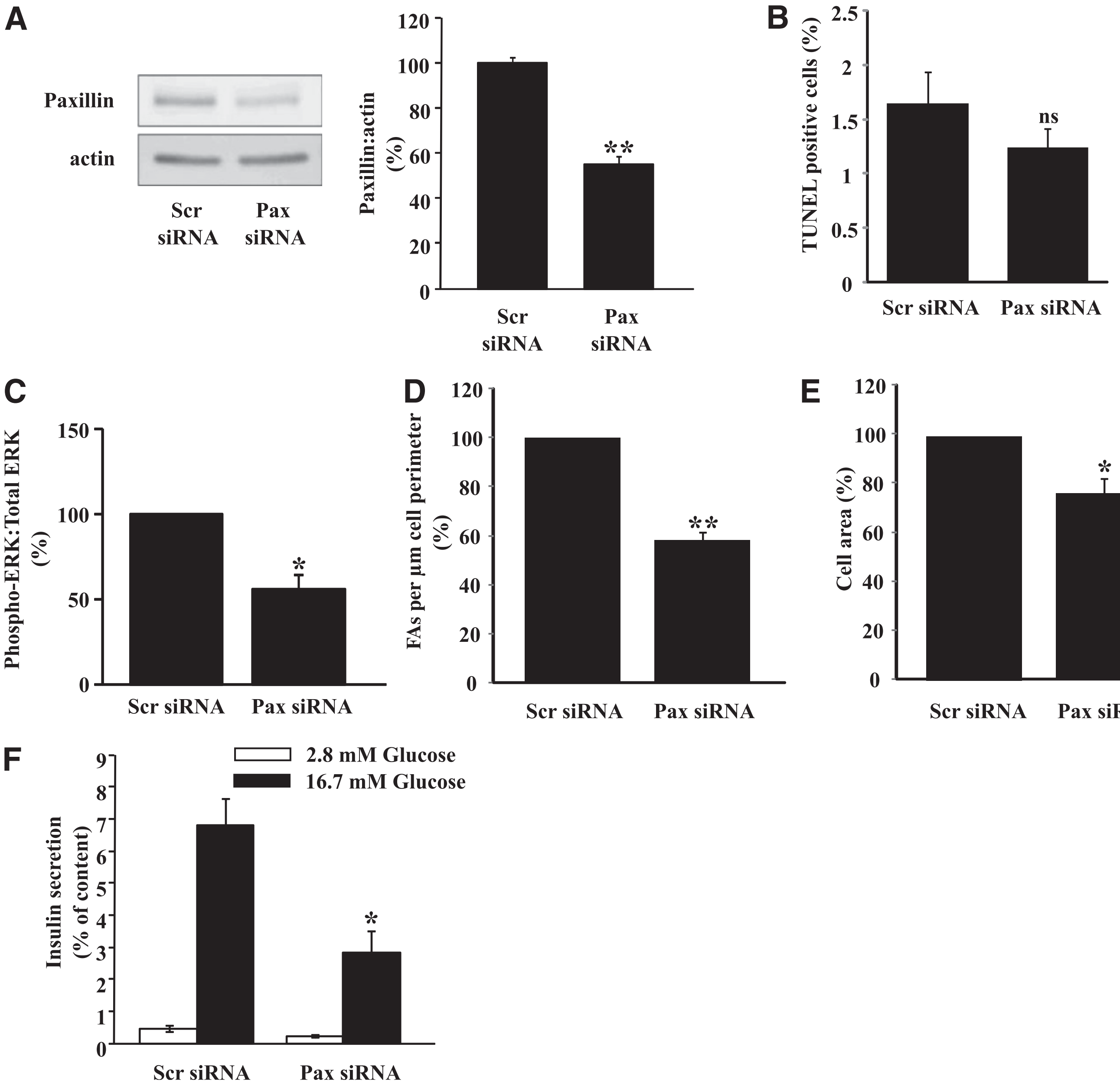

FIG. 6. siRNA-mediated depletion of endogenous paxillin (Pax) significantly attenuates glucose-stimulated focal adhesion remodeling and insulin secretion in primary rat β-cells. β-Cells were transfected with either scrambled (Scr) siRNA or a cocktail of three siRNAs specific for rat paxillin and studied 72 h later. *A*: Paxillin knockdown was verified by Western blot with equal loading confirmed with an antiactin antibody. A representative blot from three independent experiments is depicted (*left panel*), and paxillin-to-actin ratios were quantified by densitometry (*right panel*). *B*: Cell death (apoptosis plus necrosis) measured using the transferase-mediated dUTP nick-end labeling assay. *C*: siRNA-transfected β-cells were incubated for 2 h with 2.8 mmol/L glucose and stimulated for 20 min with 16.7 mmol/L glucose. Lysates were analyzed by Western blot with the indicated antibodies, and the relative intensities of the phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio. Ratios were normalized to scrambled siRNA-transfected cells. *D* and *E*: siRNA-transfected β-cells were incubated as described above. Cells were then fixed and stained with either phospho-paxillin and actin or Evans blue. Both phospho-paxillin–containing focal adhesions and cell surface areas were quantified and normalized to scrambled siRNA-transfected cells. *F*: siRNA-transfected primary β-cells were incubated with low (2.8 mmol/L) followed by high (16.7 mmol/L) glucose for 1 h, and insulin release was monitored and expressed as a percentage of total cell content. Data are means ± SEM from three independent experiments. *$P < 0.05$ and **$P < 0.001$ vs. scrambled siRNA-transfected cells.

antibodies revealed that phospho-paxillin was mainly localized along the cell periphery and connected to actin stress fibers in focal adhesions (38). In agreement with these findings, we observed, prior to cell spreading, the glucose-induced formation of multiple nascent focal adhesions containing both phosphorylated FAK (Y397) and paxillin (Y118) in close proximity. Furthermore, we confirmed the basal membrane–proximal localization of phospho-ERK1/2 at the tips of actin filaments in glucose-stimulated β-cells (5) and now demonstrate the colocalization of phospho-ERK1/2 with paxillin at these particular sites that we define as filopodial focal adhesions.

Glucose stimulation of β-cells results in an increase of intracellular Ca^{2+} that is known to be a key trigger for insulin secretion. SR7037, a selective L-type Ca^{2+} channel blocker, inhibited glucose-stimulated phosphorylation of ERK1/2, FAK, and its substrate paxillin, showing these events to be dependent on Ca^{2+} influx. This is in accordance

TABLE 1
Inhibitory effect of Pax RNAi on insulin secretion from rat primary β-cells in response to various secretagogues

Glucose (mmol/L)	Secretagogues	Time (min)	Stimulated secretion Scr RNAi (%)	Stimulated secretion Pax RNAi (%)	Decrease in stimulated insulin secretion by Pax RNAi (%)	*P*
5	—	60	0.27 ± 0.07	0.17 ± 0.07	40.5 ± 8.8	0.01
5	100 nmol/L GLP-1	60	2.12 ± 0.74	1.32 ± 0.47	36.3 ± 4.8	0.002
2.8	30 mmol/L KCl	20	0.27 ± 0.08	0.12 ± 0.04	55.3 ± 2.3	2.10^{-5}
2.8	100 nmol/L PMA	60	1.45 ± 0.52	1.12 ± 0.53	30.3 ± 9.5	0.02

Data are means ± SEM from three to four independent experiments. siRNA-transfected primary rat β-cells were preincubated under basal conditions (2.8 mmol/L glucose) and subsequently incubated for 1 h with 2.8 mmol/L glucose followed by either 1 h with 5 mmol/L glucose, GLP-1 (plus 5 mmol/L glucose), or PMA (plus 2.8 mmol/L glucose) or 20 min with KCl (plus 2.8 mmol/L glucose). Secretion is expressed as percentage of total insulin (secreted plus cell content) and percent decrease in stimulated insulin secretion compared with scrambled (Scr) siRNA-transfected cells.

with previous studies in other cell types reporting induced FAK autophosphorylation and kinase activity driven by a physiological rise in intracellular Ca^{2+} concentration (39–41). Moreover, earlier reported Ca^{2+}-dependent reversibility of FAK activation and cell spreading (40) coincides with the reversible character of the glucose-induced formation of nascent focal adhesions in β-cells, suggesting an important role for Ca^{2+} in the dynamic regulation of cellular focal adhesions during short-term glucose stimulation. These results, together with the previously described inhibitory effect of SR7037 on GSIS (22), suggest a link thus far unsuspected between focal adhesion remodeling and regulated insulin secretion in β-cells. Note, however, that the observed changes in FAK and paxillin phosphorylation were small compared with the drastic inhibition of GSIS by SR7037, suggesting that the activation of FAK and paxillin is only partially Ca^{2+} dependent and that this in combination with the inhibition of other Ca^{2+}-dependent ERK1/2 activation mechanisms by SR7037 results in a significant inhibition of ERK1/2 and GSIS.

To confirm the possible involvement of FAK in GSIS, we performed both RNAi-mediated FAK knockdown (in MIN6 B1 cells) and pharmacological inhibition of FAK activity by compound Y15 (25). The latter was identified as a specific inhibitor of Y397-FAK phosphorylation by using a structure-based approach combining functional testing and molecular docking of the three-dimensional structure of numerous small molecule compounds into the structural pocket of the Y397 site. Specificity of this inhibitory compound has been further verified using various in vitro kinase assays (24). Both strategies demonstrated a link between the level and activity of FAK, focal adhesion remodeling, ERK1/2 pathway activation, and GSIS in β-cells. We further confirm this by siRNA-mediated silencing of paxillin, resulting in a significant inhibition of glucose-induced ERK1/2 phosphorylation, focal adhesion formation, β-cell spreading, and insulin secretion (without affecting total insulin content or cell survival). These results, in combination with the demonstrated colocalization of activated FAK, paxillin, and ERK1/2 at filopodial focal adhesions at the basal membrane of glucose-stimulated β-cells, indicate that glucose-induced focal adhesion remodeling plays a crucial role in GSIS. Furthermore, the inhibitory effect of paxillin knockdown on KCl-, PMA-, and GLP-1–stimulated insulin secretion indicates that paxillin acts at a distal step in the insulin secretory machinery. A comparable inhibitory effect of compound Y15 on both the early and late phases of insulin secretion corroborates this but could also indicate that both the initial size of the readily releasable pool and the following supply of secretory vesicles to this pool are affected by blocking FAK activation. However, as it cannot be excluded by these experiments that paxillin RNAi also impacts insulin secretion by other, indirect ways (e.g., by modulating expression of key β-cell genes), this is something that will need to be studied in the future.

In view of these observations, we propose that glucose-induced autophosphorylation and thereby activation of FAK is mediated by increased intracellular Ca^{2+} concentrations. Activated FAK then phosphorylates its direct substrate paxillin, which may act as an adaptor protein through FAK to increase linkage between activated integrin complexes and the actin cytoskeleton. We hypothesize that these events and the coinciding actin cytoskeletal reorganization are essential for full development of GSIS, acting at a late stage in exocytosis. This is supported by the (co)localization of phospho-paxillin with SNAP-25 and syntaxin 1 in glucose-induced focal adhesions because these two SNARE proteins are well known to play a pivotal role during vesicle fusion in the process of regulated insulin exocytosis, with documented links to the β-cell actin cytoskeleton (4,42). In addition, as observed in CHO-K1 cells (43), these data might also suggest a role for SNARE-mediated membrane trafficking in glucose-induced focal adhesion signaling in β-cells.

Our results also point toward the FAK-paxillin complex as the mediator of actin remodeling–induced local phosphorylation of ERK1/2 at focal adhesions after glucose stimulation of β-cells. FAK-Src–mediated tyrosine phosphorylation of paxillin is known to result in association of inactive ERK with its upstream effectors mitogen-activated protein kinase and Raf forming a scaffold and facilitating ERK activation at focal adhesions (27), offering a molecular basis for our own observations. This pathway is self-enhanced; activated ERK can then phosphorylate paxillin itself, which will increase its association to FAK and in turn enhance FAK activation (26,27), consistent with our data using the ERK1/2 inhibitor PD98059. This short-term ERK1/2-triggered phosphorylation of FAK and paxillin correlates with earlier studies showing that ERK1/2 is involved in early-phase GSIS (5). Note, however, the differences in the level of glucose-induced phosphorylation between FAK/paxillin and ERK1/2 that confirm the involvement of additional signaling pathways in glucose-induced ERK1/2 activation. Glucose-activated ERK1/2 has previously been shown to be involved in the regulation of insulin secretion and coincides temporally with transient glucose-induced actin cytoskeleton remodeling (4,5). In addition, activation of FAK and paxillin could also potentiate GSIS in β-cells through ERK1/2-mediated phosphorylation of synapsin 1 (44), a protein believed to function as

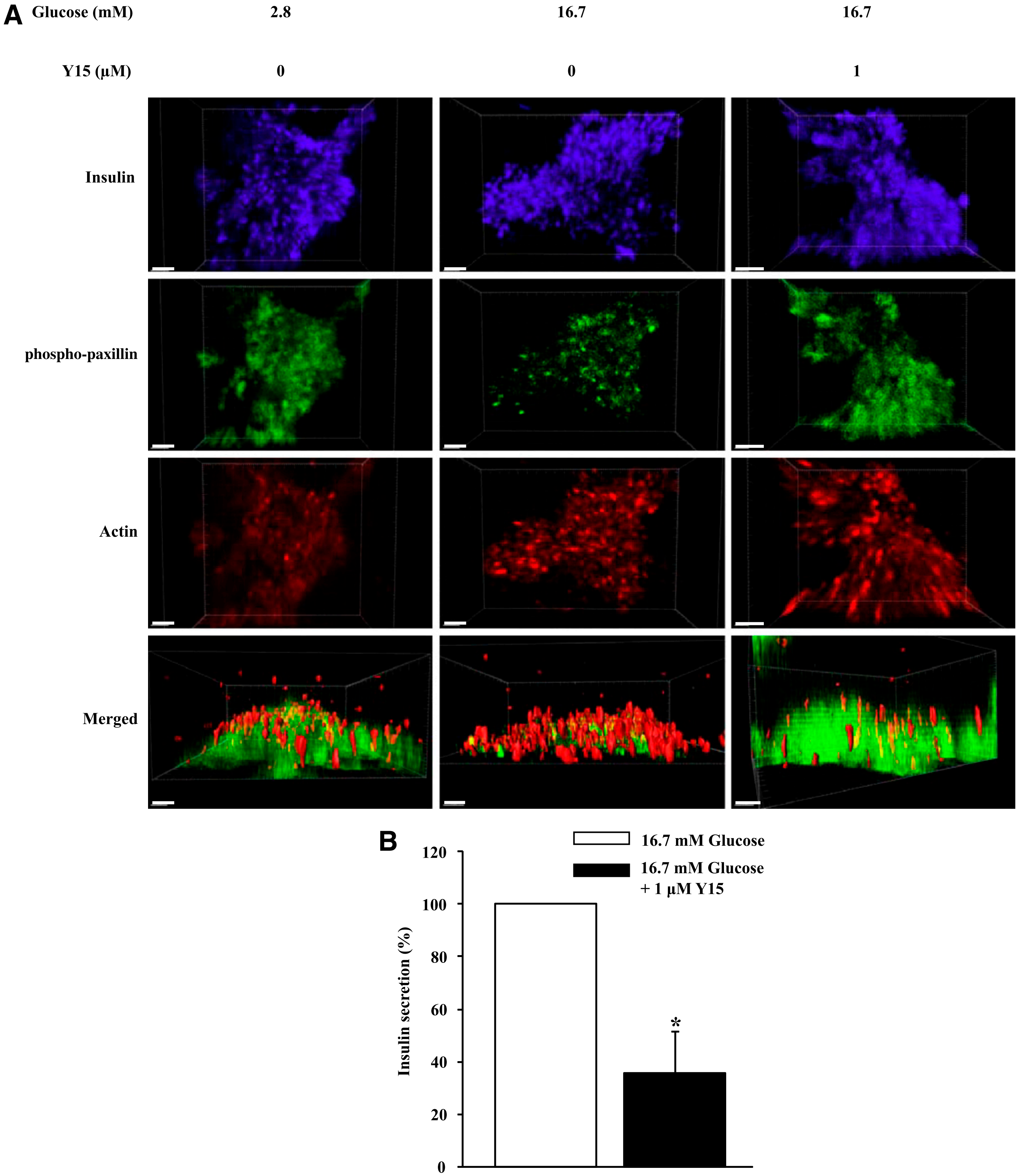

FIG. 7. Effect of FAK inhibition on insulin secretion and subcellular localization of phospho-paxillin in β-cells in intact rat islets. *A*: Isolated islets were incubated for 2 h with 2.8 mmol/L glucose with or without 1 µmol/L Y15 and were then stimulated for 20 min with 2.8 mmol/L glucose, 16.7 mmol/L glucose, or 16.7 mmol/L glucose with Y15. Islet sections (5 µm) were then fixed and stained with the indicated antibodies. Three-dimensional reconstruction from confocal image stacks was performed using Imaris (version 7.2). The *upper three rows* illustrate a top view and the *lower row* a side view of reconstructions of the same set of stacks from a single islet section for each given condition. In the side view images, actin staining (red) is represented by reconstructed isosurfaces. Scale bars = 2 µm. Images are representative of multiple sections from two independent experiments. *B*: Isolated islets were first preincubated for 2 h at 2.8 mmol/L glucose and then incubated for 1 h each at 2.8 mmol/L followed by 16.7 mmol/L glucose in the continued presence of 1 µmol/L compound Y15 (or DMSO). Insulin secretion is expressed as a percentage of total insulin cell content, normalized to control stimulated islets. Data are means ± SEM from three independent experiments. $^*P < 0.05$ vs. control-stimulated islets. (A high-quality digital representation of this figure is available in the online issue.)

a linker between the vesicle membrane and the actin cytoskeleton. Synapsin 1 phosphorylation may result in the release of secretory granules from the cytoskeleton network and mobilization from a passive reserve pool to an active releasable pool (45). Further experiments outside the scope of this study will be required to verify this functional hypothesis.

To confirm our central hypothesis in the more physiological setting of the three-dimensional micro-organ, additional experiments were performed on whole islets. These experiments revealed clear differences in behavior between cells in two-dimensional culture and three-dimensional aggregates. β-Cells within islets did not show clearly evident actin stress fibers, and focal adhesions were not present in filopodial extensions, which was entirely expected based on the comparison of these structures in other cell types in two-dimensional versus three-dimensional assemblies (46). However, glucose induced phosphorylation of FAK and clearly changed phospho-paxillin subcellular localization in β-cells within islets, which was prevented by compound Y15 which also inhibited GSIS from whole islets. In contrast, in a single preliminary experiment paxillin and ERK1/2 phosphorylation in whole islets were not affected by glucose. This could be due to opposite glucose-induced changes in other islet cell types or higher basal phosphorylation levels of both paxillin and ERK1/2 in islets compared with sorted β-cells. The latter could once again be explained by the mixed contribution of all islet cells in Western blots of whole islet proteins but also possibly by the presence of glucagon secreted by α-cells because ERK1/2 is a known substrate of protein kinase A that would be activated by increased cAMP in response to glucagon (47). Finally, we cannot exclude that the three-dimensional organization of cells within islets impacts the phosphorylation of these proteins involved in inside-out and outside-in cell signaling.

In conclusion, the current study shows for the first time the existence of short-term glucose-mediated, Ca^{2+}-dependent activation of the key focal adhesion proteins, FAK and paxillin, in pancreatic β-cells. Most importantly, this focal adhesion remodeling process appears to be an important player in GSIS, providing a novel additional component to the complex molecular machinery underlying this key event in glucose homeostasis. Despite the well-recognized differences in behavior between β-cells in two-dimensional culture and whole islets, it was reassuring to be able to confirm the most important features of this novel component of β-cell–regulated exocytosis using intact islets. Further studies are needed to determine the exact molecular events downstream glucose-induced activation of FAK and paxillin that impact insulin secretion and whether perturbation of this new pathway contributes toward β-cell dysfunction in type 2 diabetes.

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