

Novel Mechanistic Link between Focal Adhesion Remodeling and Glucose-stimulated Insulin Secretion^{*S}

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Actin cytoskeleton remodeling is well known to be positively involved in glucose-stimulated pancreatic β cell insulin secretion. We have observed glucose-stimulated focal adhesion remodeling at the β cell surface and have shown this to be crucial for glucose-stimulated insulin secretion. However, the mechanistic link between such remodeling and the insulin secretory machinery remained unknown and was the major aim of this study. MIN6B1 cells, a previously validated model of primary β cell function, were used for all experiments. Total internal reflection fluorescence microscopy revealed the glucose-responsive co-localization of focal adhesion kinase (FAK) and paxillin with integrin $\beta 1$ at the basal cell surface after short term stimulation. In addition, blockade of the interaction between $\beta 1$ integrins and the extracellular matrix with an anti- $\beta 1$ integrin antibody (Ha2/5) inhibited short term glucose-induced phosphorylation of FAK (Tyr-397), paxillin (Tyr-118), and ERK1/2 (Thr-202/Tyr-204). Pharmacological inhibition of FAK activity blocked glucose-induced actin cytoskeleton remodeling and glucose-induced disruption of the F-actin/SNAP-25 association at the plasma membrane as well as the distribution of insulin granules to regions in close proximity to the plasma membrane. Furthermore, FAK inhibition also completely blocked short term glucose-induced activation of the Akt/AS160 signaling pathway. In conclusion, these results indicate 1) that glucose-induced activation of FAK, paxillin, and ERK1/2 is mediated by $\beta 1$ integrin intracellular signaling, 2) a mechanism whereby FAK mediates glucose-induced actin cytoskeleton remodeling, hence allowing docking and fusion of insulin granules to the plasma membrane, and 3) a possible functional role for the Akt/AS160 signaling pathway in the FAK-mediated regulation of glucose-stimulated insulin secretion.

Pancreatic β cells and their secretory product insulin are central to the pathogenesis of both major types of diabetes. Exocytotic release of insulin is triggered by enhanced glucose metabolism by the β cell followed by increased intracellular ATP/ADP ratios, closure of ATP-sensitive K^+ channels, membrane depolarization, opening of voltage-gated Ca^{2+} channels, and increased intracellular Ca^{2+} concentrations (1–5). A second

mechanism serves to amplify glucose-stimulated insulin secretion (GSIS),² acting in synergy with this canonical K_{ATP} -dependent pathway that is seen as the trigger (6). More than 40 years ago Curry *et al.* (7) demonstrated that insulin secretion in response to glucose exhibits a biphasic pattern consisting of a rapidly initiated and transient first phase followed by a gradually developing and sustained second phase. The ability of glucose to elicit first phase insulin secretion is shared by other stimuli such as high KCl stimulation, resulting in membrane depolarization. However, only fuel secretagogues will also initiate second-phase insulin release (6).

Grodsky *et al.* (8, 9) were the first to propose an insulin storage-limited mathematical model with functionally distinct pools of granules to explain the biphasic kinetics of secretion in pancreatic β cells. This model was more recently confirmed using newly developed methods that allow the study of exocytosis and intracellular granule trafficking in individual cells (for review, see Ref. 10). These experiments show that first-phase release is ascribed to Ca^{2+} -dependent exocytosis of primed granules in a small readily releasable pool, whereas the second phase of secretion requires an ATP-dependent recruitment of a reserve pool of secretory granules to the release site (10, 11).

Insulin granule exocytosis requires docking and fusion of secretory vesicles with the release sites at the plasma membrane. This is mediated by core machinery of membrane-associated SNAP receptors (SNAREs) which can be classified into two subfamilies: vesicle-SNAREs (found on the vesicles) and target-SNAREs (t-SNAREs, found on target membranes) (12). In β cells the vesicle-SNARE protein VAMP-2 is shown to interact specifically with the t-SNARE proteins syntaxin1 and synaptosome-associated protein of 25 kDa (SNAP-25) upon trafficking of a vesicle to a target membrane, bringing the two membranes into close proximity to allow fusion (13–15).

Earlier studies demonstrated that isolated insulin-containing granules co-sediment with filamentous actin (F-actin) (16), which is organized as a dense web beneath the plasma membrane, blocking access of secretory vesicles to the cell periphery (16–19). Additionally, in the non-stimulated condition F-actin was found to be associated with the t-SNARE complex, thereby blocking the latter, whereas glucose stimulation has been shown to induce F-actin remodeling (20–22), transient disruption

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²The abbreviations used are: GSIS, glucose-stimulated insulin secretion; TIRF, total internal reflection fluorescence; FAK, focal adhesion kinase; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive fusion attachment protein receptor; t-SNARE, target-SNARE; ECM, extracellular matrix; NPY, neuropeptide Y; pAb, polyclonal antibody; EGFP, enhanced GFP.

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tion of the t-SNARE/F-actin association, and redistribution of insulin-containing granules to more peripheral regions of the β cell (21), hence facilitating insulin secretion.

Actin cytoskeleton remodeling is a well described feature involved in spreading and migration of cells and has been shown to be regulated by focal adhesion molecules such as focal adhesion kinase (FAK) and paxillin (23). These molecules make up focal contact sites, providing not only a structural link between the extracellular matrix (ECM) and cytoskeletal proteins but serving also as initiation points for outside-in signaling leading to changes in cell activity and gene expression (24–26). We recently demonstrated a functional role for focal adhesion remodeling and more specifically for FAK and paxillin in GSIS of β cells (27). We have also shown that ERK1/2 activation is critical for both GSIS and actin remodeling (22). However, the direct mechanistic link between these various facets of the regulation of insulin secretion from β cells has yet to be elucidated.

The aim of this study was to further explore the glucose-activated FAK-paxillin-ERK1/2 signaling pathway in β cells. We demonstrate here the involvement of β 1 integrin and illustrate a link between glucose-induced actin cytoskeleton remodeling and the induced translocation and activation of FAK, paxillin, and ERK1/2. Finally, we focus specifically on FAK and unveil a mechanistic link between FAK activity and the regulation of GSIS involving actin remodeling and the Akt/AS160 signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The selective FAK inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride (Y15) was purchased from Sigma and used at 1 μ M final concentration. Primary antibodies were: rabbit anti-FAK polyclonal antibody (pAb) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-phospho(Tyr-397)FAK pAb and rabbit anti-phospho(Tyr-118)paxillin pAb from Invitrogen, mouse anti-paxillin monoclonal antibody, rat anti-CD29 (β 1 integrin chain, Ha2/5), and control hamster IgM from BD Transduction Laboratories, and rabbit anti-ERK1/2 pAb, rabbit anti-phospho-ERK1/2 pAb, and rabbit anti-myc-tag pAb from Cell Signaling Technology (Beverly, MA). The monoclonal SNAP-25 antibody used for coimmunoprecipitation was obtained from Sternberger Monoclonals, Inc. (Lutherville, MD). Secondary antibodies were as follows: donkey anti-rabbit horseradish peroxidase (HRP) and sheep anti-mouse HRP (Amersham Biosciences) and donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 555 (Invitrogen). To visualize F-actin, Alexa Fluor 647-phalloidin was obtained from Invitrogen. NSC23766, latrunculin B, and jasplakinolide were from Calbiochem.

Cell Lines and Culture Conditions—MIN6B1 cells were cultured in complete medium as previously described (28). For total internal reflection fluorescence (TIRF) microscopy and confocal live imaging, cells were plated on 35-mm glass-bottom microwell dishes (MatTek, Ashland, MA) coated with the extracellular matrix from 804G rat bladder carcinoma cells to favor cell attachment (29).

Expression Vectors—To obtain an extracellularly fluorescently tagged β 1A integrin (pEGFPn β 1A), the EGFP

sequence was cloned into a flexible loop in the β 1 hybrid domain using AgeI/XhoI sites previously inserted by primer overlap extension mutagenesis. The construct was tested by transient overexpression in 3T3 fibroblasts, where it localized to focal and fibrillar adhesions (data not shown). Neuropeptide Y (NPY)-Cherry was a gift from Prof. Guy A. Rutter (Imperial College London). The pEGFPC1-FAK plasmid was a gift from Prof. David D. Schlaepfer (University of California). cDNA encoding paxillin was excised from the paxillin-pEGFP plasmid (AddGene) and cloned into the pDsRed-monomer plasmid. The plasmid expressing Lifeact-mRFP_{rubry} was a gift from Dr. Roland Wedlich-Soldner (Max Planck Institute of Biochemistry). The plasmid encoding myc-tagged wild type Rac1 was kindly provided by Dr. Romano Regazzi (University of Lausanne, Switzerland).

SDS-PAGE and Western Blotting—Protein samples were prepared, and immunoblots were analyzed as described (27). Western blots were quantified by densitometry, and band density of phosphoproteins was normalized to that of the corresponding total protein as indicated in the figures.

Ha2/5-mediated Blocking of β 1 Integrin—MIN6B1 cells were resuspended at a density of 10^6 cells/ml in complete culture medium and preincubated for 1 h with 4 μ g/ml anti- β 1 integrin blocking antibody (Ha2/5) or purified hamster IgM. Then cells were plated as droplets on dishes coated with 804G matrix and incubated for 5 h at 37 °C in the presence of the respective antibodies. Finally, cells were treated as described, and lysates were analyzed by SDS-PAGE and Western blotting.

Coimmunoprecipitation—Coimmunoprecipitations were performed as described by Thurmond *et al.* (21). After the indicated treatments, cells were solubilized in Nonidet P-40 detergent lysis buffer (25 mM Tris, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 10 mM Na₄P₂O₇, 137 mM NaCl, 1 mM Na₃VO₄, 1 mM PMSE) supplemented with Complete Mini protease inhibitor mixture. Cell lysates were first preincubated with protein G Plus Agarose (Santa Cruz Biotechnology) for 30 min at 4 °C. Cleared lysates were then combined with 1 μ g of SNAP-25 monoclonal antibody for 2 h at 4 °C followed by an incubation with protein G Plus-agarose for 2 h at 4 °C. After centrifugation, removal of the supernatant, and five washing steps in lysis buffer, the immunoprecipitates were resuspended in SDS-PAGE sample buffer, boiled for 5 min, and subjected to SDS-PAGE and Western blotting.

Immunofluorescence and Confocal Microscopy—Immunofluorescence was performed as previously described (22). Cells were observed under a Zeiss LSM510 Meta confocal microscope using a 63 \times oil immersion lens. Images were acquired and processed using the LSM510 software (Carl Zeiss AG). Confocal live imaging was performed with a Nikon A1r microscope (Nikon, Tokyo, Japan) by using a 60 \times CFI plan Apo objective and a filter optimized for mCherry fluorescence. Cells posed on 804G-coated glass-bottom culture dishes were placed on the microscope stage and maintained at 37 °C and 5% CO₂. Transfected cells were chosen at random for analysis and images were captured every 60 s.

TIRF Microscopy—Cells were transfected using LipofectamineTM 2000 reagent (Invitrogen) using the manufacturer's instructions. Transfected cells were incubated for 48 h to

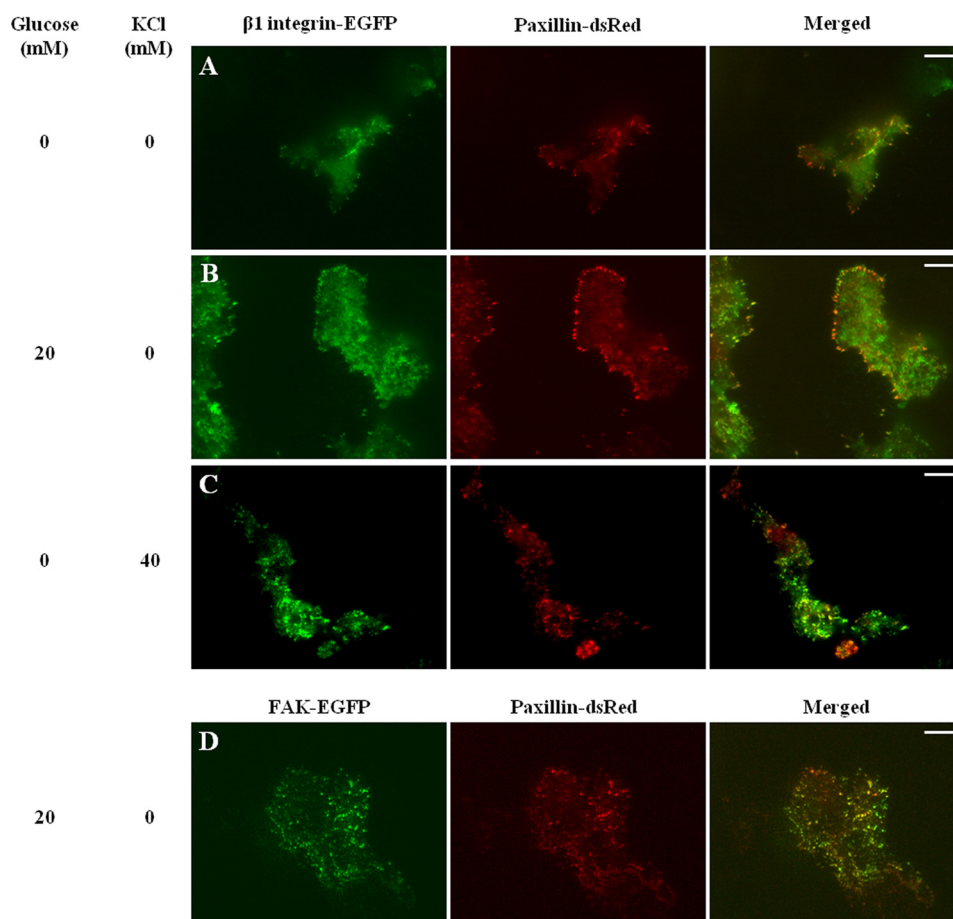


FIGURE 1. $\beta 1$ integrin co-localizes with FAK and paxillin at the basal membrane of MIN6B1 cells. TIRF analysis of the subcellular localization of $\beta 1$ integrin-EGFP (green, top and middle panels (A–C)) and paxillin-dsRed (red) and FAK-EGFP (green, bottom panels (D)) in MIN6B1 cells with or without stimulation for 10 min with 20 mM glucose or 40 mM KCl is shown. The merged panels show $\beta 1$ integrin or FAK co-localization with paxillin. Scale bars, 10 μ m. All pictures are fully representative of multiple images of two independent experiments.

allow for DNA expression and were then treated (as indicated) and fixed. TIRF images of NPY-Cherry fluorescence were obtained with a Plan Neofluar $\times 100$ (NA 1.45) objective mounted on an Axiovert 100M (both from Carl Zeiss Micro-Imaging) equipped with a 12-bit digital charge-coupled device camera (model Orca 4742–95; Hamamatsu Photonics) and controlled by the Openlab software (Improvision). Intraobjective TIRF was obtained with a 535-nm laser through a TIRF adaptor (TILL Photonics).

Insulin Secretion Assays—Insulin secretion assays were performed as previously described (22).

Statistical Analyses—The statistical significance of the differences between the experimental conditions was determined by Student's *t* test for unpaired groups. *p* values less than 0.05 were considered significant.

RESULTS

$\beta 1$ Integrin Co-localizes with FAK and Paxillin at Basal Cell Membrane of MIN6B1 Cells after Glucose Stimulation—We have shown previously that both focal adhesion proteins FAK and paxillin are important players in the regulation of GSIS in primary rat β cells (27). We have also demonstrated that the highly differentiated MIN6B1 mouse β cell line subcloned in our laboratory responds to glucose with a robust increase in

insulin secretion (28). Furthermore, and of particular importance for this study, our earlier work indicates that MIN6B1 cells are a useful model to study β cell exocytosis, with a very similar pattern of biochemical and morphological changes observed in response to glucose when compared with primary β cells (20–22, 27). However, note that MIN6B1 cells exhibit higher and, depending on the batch and passage, more variable basal insulin secretion (as the percentage of cellular insulin content) than primary β cells. Given the technical challenge in obtaining pure primary β cells in sufficient quantity for the detailed biochemical studies performed here, all experiments were done using MIN6B1 cells as a validated model of primary β cells.

To further explore the signaling pathway(s) involved in glucose-induced phosphorylation and activation of FAK, paxillin, and ERK1/2 in β cells posed on a matrix produced by the rat bladder carcinoma cell line 804G (27), we determined whether integrins might be involved in this process. It has been demonstrated previously that integrins of the $\beta 1$ family impact β cell function and survival in a beneficial way (30–32). In view of these findings, we co-transfected MIN6B1 cells with plasmids expressing $\beta 1$ integrin-EGFP or FAK-EGFP and paxillin-dsRed and analyzed the subcellular localization of these fusion proteins at the basal cell surface (defined as the cell membrane in

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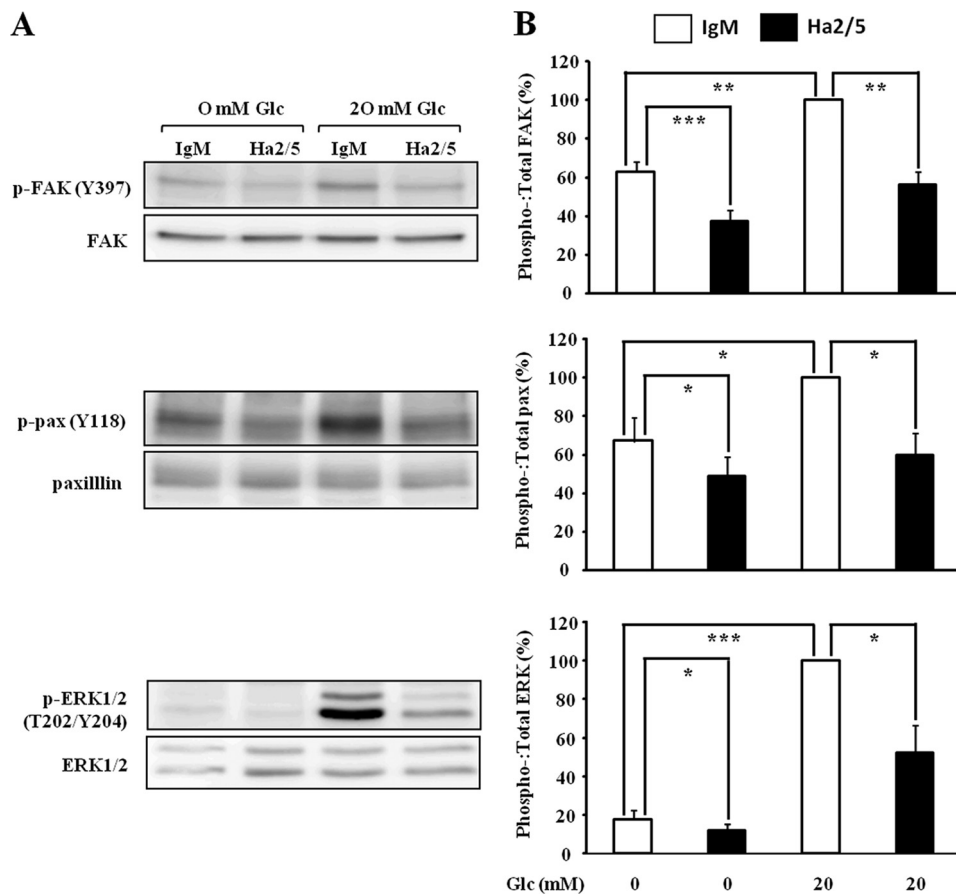


FIGURE 2. Blocking $\beta 1$ integrin inhibits glucose-induced phosphorylation of FAK, paxillin, and ERK1/2 in MIN6B1 cells. *A*, lysates of MIN6B1 cells treated with a control (IgM) or anti- $\beta 1$ integrin (Ha2/5) antibody and kept at 0 mM glucose or stimulated for 10 min with 20 mM glucose were analyzed by Western blot using the indicated antibodies. A representative blot from four independent experiments is shown. *B*, the relative intensities of the phosphorylated and total protein bands of the basal and stimulated conditions were quantified by densitometry and expressed as a ratio. Ratios were normalized to control (IgM) glucose-stimulated cells. Data are the means \pm S.E. from four independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

direct contact with the ECM) by TIRF microscopy. This technique enables examination of specifically the bottom 100 nm of a spreading cell (33). For this purpose MIN6B1 cells were taken 48 h after co-transfection, preincubated for 2 h at 0 mM glucose, and further incubated for 10 min at 0 mM glucose, 20 mM glucose, or 40 mM KCl. In the basal condition (0 mM glucose), $\beta 1$ integrin co-localizes with paxillin at few specific sites at the basal cell membrane (Fig. 1*A*, upper panels). However, upon short term glucose and KCl stimulation, we observed a striking increase in the number of sites at the basal cell membrane where $\beta 1$ integrin and paxillin were co-localized (Fig. 1, *B* and *C*, middle panels). Further analysis also indicated the presence of FAK along with paxillin at these specific sites (Fig. 1*D*, lower panels), suggesting a functional interaction between $\beta 1$ integrin, FAK, and paxillin at these previously defined focal adhesion sites that develop rapidly in response to glucose (27).

Blocking $\beta 1$ Integrin Inhibits Glucose-induced Phosphorylation of FAK, Paxillin, and ERK1/2—The fact that $\beta 1$ integrin co-localizes with FAK and paxillin at specific focal contact sites led us to investigate the effect of blocking $\beta 1$ integrin on glucose-induced phosphorylation and activation of FAK and paxillin and their downstream signaling effector, ERK1/2. We, therefore, used a specific anti- $\beta 1$ integrin blocking antibody

(Ha2/5) previously shown to significantly inhibit GSIS and β cell spreading (30). We then analyzed the phosphorylation status of FAK, paxillin, and ERK1/2 in MIN6B1 cells preincubated with Ha2/5 antibody (or control IgM) in the basal (0 mM glucose) and stimulated (10 min at 20 mM glucose) conditions. In the control situation (IgM), glucose stimulation resulted in a clear phosphorylation of FAK, paxillin, and ERK1/2 as compared with the basal condition (Fig. 2, *A* and *B*), which correlates with our previous findings in primary β cells (27). However, blocking $\beta 1$ integrin (Ha2/5) significantly decreased glucose-induced phosphorylation of FAK, paxillin, and ERK1/2 by $43.8 \pm 6.9\%$ ($p < 0.01$), $40.1 \pm 11.5\%$ ($p < 0.05$), and $47.4 \pm 14.3\%$ ($p < 0.05$) respectively as compared with the control stimulated condition (IgM, 20 mM glucose) (Fig. 2, *A* and *B*). These results show that $\beta 1$ integrin signaling is involved in glucose-induced phosphorylation and activation of ERK1/2 and the two focal adhesion proteins, FAK and paxillin. Finally, further analysis also revealed a significant inhibition of the basal phosphorylation of FAK, paxillin, and ERK1/2 by blocking $\beta 1$ integrin (Ha2/5, 0 mM glucose) as compared with the control basal condition (IgM, 0 mM glucose), which, as regards FAK, correlates with earlier data reported by Parnaud *et al.* (30) showing the effect of this same anti- $\beta 1$ integrin antibody on β cell spreading.

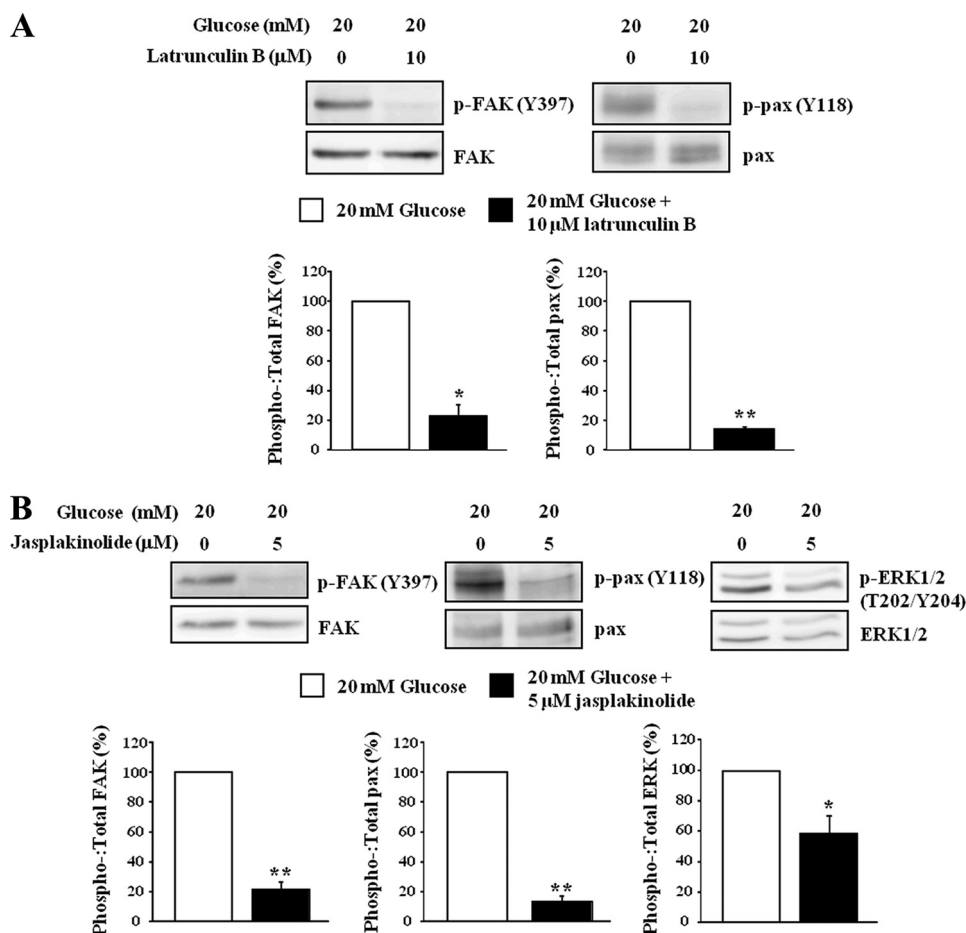


FIGURE 3. The effect of latrunculin B and jasplakinolide on glucose-induced phosphorylation of FAK, paxillin, and ERK1/2. *A*, MIN6B1 cells were preincubated for 2 h in the presence of 0 mM glucose combined with the actin depolymerizing agent latrunculin B (10 μ M, or DMSO as a negative control) and were further stimulated for 10 min with 20 mM glucose containing latrunculin B (or DMSO). Cell lysates were analyzed by Western blot with the indicated antibodies, and a representative blot from three independent experiments is shown. Relative intensities of the phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio. Ratios were then normalized to the control high glucose (20 mM glucose) condition. Data are the means \pm S.E. from three independent experiments (* p < 0.01 and ** p < 0.001). *B*, MIN6B1 cells were incubated and analyzed as above but with the actin stabilizing agent jasplakinolide (5 μ M, or DMSO as a negative control). Data are the means \pm S.E. from three independent experiments (*, p < 0.05; **, p < 0.001).

Induced Actin (De)polymerization Affects Glucose-induced Phosphorylation of FAK, Paxillin, and ERK1/2—In endothelial cells, integrin receptors located in focal adhesion complexes are known to be crucial in regulating cell-ECM interactions (34) and mediating FAK activation (35, 36). Activated FAK together with downstream focal adhesion complex-linked cytoskeletal proteins such as paxillin thereby form a mechanical link between the ECM and the cell actin cytoskeleton (37). To determine a possible role for actin and more specifically its polymerization state in the regulation of glucose-induced activation of FAK, paxillin, and ERK1/2 in β cells, we analyzed the effect of the actin-disrupting agents latrunculin B and jasplakinolide on glucose-stimulated MIN6B1 cells. Previously, both compounds have been reported to potentiate GSIS by disrupting the dynamic equilibrium of the actin polymerization state between monomeric G-actin and filamentous F-actin (20–22).

We show here that exposure of MIN6B1 cells to the actin depolymerizing agent latrunculin B significantly inhibits glucose-induced phosphorylation of FAK and paxillin by $77.1 \pm 7.6\%$ (p < 0.01) and $85.8 \pm 1.6\%$ (p < 0.001), respectively, as compared with the control stimulated condition (Fig. 3*A*). Glucose-induced phosphorylation of ERK1/2 on the other hand

was confirmed to be potentiated by latrunculin B, as described by us previously (22) (data not shown). Similar to latrunculin B, MIN6B1 cells treated with the actin filament stabilizing compound jasplakinolide also showed a significant decrease in glucose-induced phosphorylation of FAK and paxillin by $78.6 \pm 5.1\%$ (p < 0.001) and $86.7 \pm 3.3\%$ (p < 0.001), respectively, as compared with the control stimulated condition (Fig. 3*B*). However, in contrast to latrunculin B, pretreatment with jasplakinolide resulted in a significant decrease ($42.1 \pm 12.6\%$, p < 0.05) in glucose-induced phosphorylation of ERK1/2 as compared with control stimulated cells (Fig. 3*B*, right panels).

We next determined the effect of actin depolymerization induced by latrunculin B on the subcellular localization of the phosphorylated isoforms of FAK, paxillin, and ERK1/2 in MIN6B1 cells. In the control glucose-stimulated condition (20 mM glucose), both phospho-FAK, phospho-paxillin and phospho-ERK1/2, localized, as earlier described in rat primary β cells (27), in numerous focal adhesion sites formed at the tips of actin fibers (Fig. 4, left panels). Treatment with latrunculin B has been previously demonstrated to disrupt the actin cytoskeleton, with F-actin fragmented and scattered throughout the cell (22). We observed that this coincides with a clear loss in well

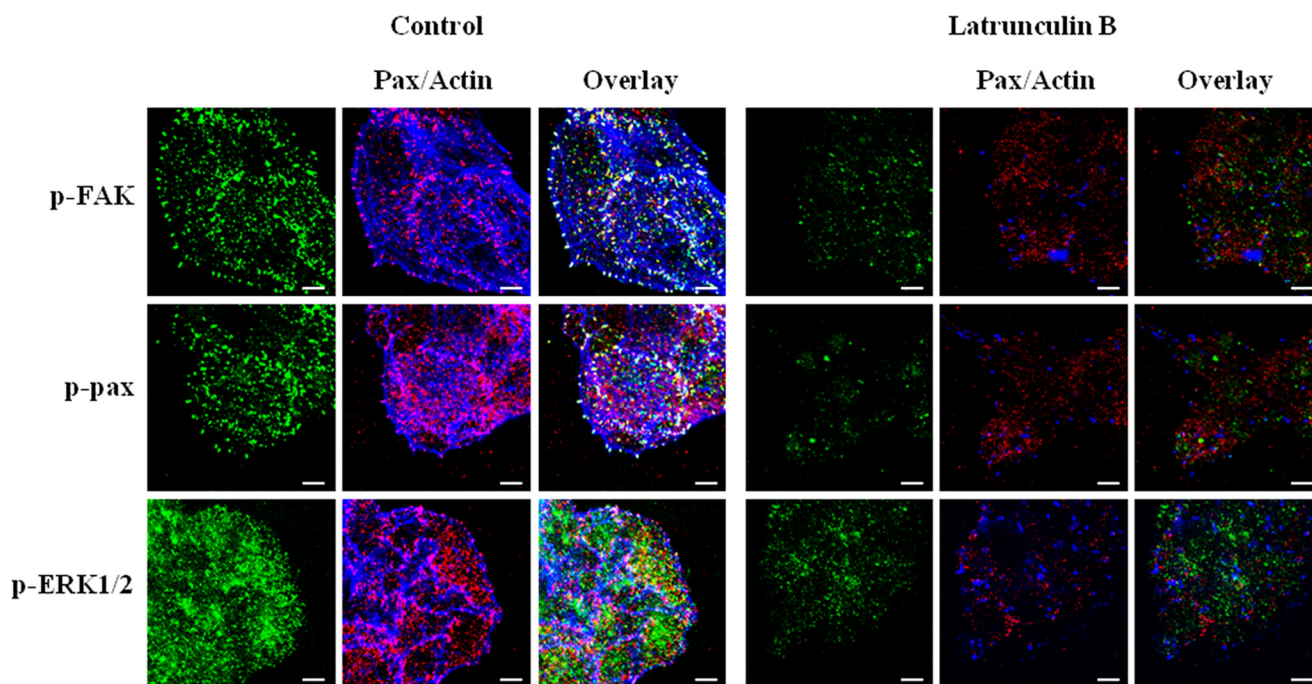


FIGURE 4. **Effect of latrunculin B-induced actin depolymerization on glucose-mediated translocation of FAK, paxillin, and ERK1/2.** MIN6B1 cells treated with or without latrunculin B were stimulated for 10 min with 20 mM glucose, fixed, and triple-stained for F-actin (blue), paxillin (red), and phospho-FAK, phospho-paxillin or phospho-ERK1/2 (green), respectively. All pictures are fully representative of multiple images from three independent experiments. Scale bars, 5 μ m.

defined phospho-FAK-, phospho-paxillin-, and phospho-ERK1/2-containing focal adhesions (Fig. 4, right panels), indicating the absence of glucose-induced FAK, paxillin, and ERK1/2 translocation to focal adhesion sites upon inhibition of dynamic actin remodeling normally mediated by physiological signals that result from glucose stimulation. Increased amounts of phosphorylated ERK1/2 upon latrunculin B treatment, as shown by Western blot by Tomas *et al.* (22), were not observed by immunolocalization. This is probably due to the latrunculin B-induced disorganization of focal adhesions resulting in a diffuse signal for phosphorylated ERK1/2. Furthermore, analysis of the subcellular localization of all three proteins in the presence of jasplakinolide revealed an effect of actin filament stabilization on glucose-induced mobilization of FAK, paxillin, and ERK1/2 that was very similar to that seen with latrunculin B-induced actin depolymerization (data not shown).

These data suggest that by interfering with the dynamic integrity of the actin cytoskeleton, both stabilization and inhibition of actin polymerization can block translocation of FAK and paxillin to focal adhesion sites, thereby also inhibiting their glucose-induced phosphorylation and activation. By contrast, glucose-induced phosphorylation of ERK1/2 is potentiated by actin depolymerization and inhibited by actin filament stabilization, whereas its glucose-induced mobilization to focal adhesion sites is inhibited by both conditions.

FAK Inhibition Blocks Glucose-induced Actin Cytoskeleton Remodeling—To establish to which extent FAK activity is required for F-actin cytoskeleton organization and remodeling in glucose-stimulated β cells, we analyzed the F-actin fiber structure (stained with phalloidin) at the basal plane and the midplane of MIN6B1 cells stimulated with 20 mM glucose in the presence of the selective FAK inhibitor, compound Y15 (1 μ M,

or DMSO as a negative control), which specifically inhibits FAK-Tyr-397 autophosphorylation (38, 39) (Fig. 5A). As previously reported (21, 22) and as shown in the lower left panel of Fig. 5A, MIN6B1 cells in the basal condition (0 mM glucose) display shorter, more dense actin filaments at the basal plane, whereas short term stimulation with 20 mM glucose resulted in a partial depolymerization of the β cell actin cytoskeleton as compared with the basal condition (0 mM glucose). However, in the presence of compound Y15, the actin cytoskeleton remained largely unchanged upon glucose stimulation (Fig. 5A, lower right panel), suggesting that FAK activity is required for glucose-induced F-actin remodeling. Analysis of the midplane of the cells revealed that in the basal condition (0 mM glucose, upper left panel of Fig. 5A) MIN6B1 cells display an intense and nearly continuous band of F-actin staining that was locally disrupted upon glucose stimulation (20 mM glucose). Similar to the basal plane, this glucose-induced remodeling process was largely inhibited by treatment of the cells with compound Y15 (Fig. 5A, upper right panel), indicating that F-actin at the midplane is equally responsive to glucose stimulation and FAK inhibition (Fig. 5A, upper panels) as compared with actin remodeling at the basal membrane.

This was further confirmed in MIN6B1 cells transfected with a construct expressing Lifeact-mRFP. Lifeact is a 17-amino acid peptide that stains F-actin structures without interfering with actin dynamics *in vitro* and *in vivo*, thereby allowing live imaging of actin dynamics (40). Two days after transfection, cells were preincubated for 2 h at 0 mM glucose combined with compound Y15 (1 μ M, or DMSO as a negative control) and were further stimulated for 10 min with 20 mM glucose in the presence of Y15 (or DMSO). In control MIN6B1 cells (Fig. 5B, upper panels; see also supplemental Movie 1), we observed clear actin

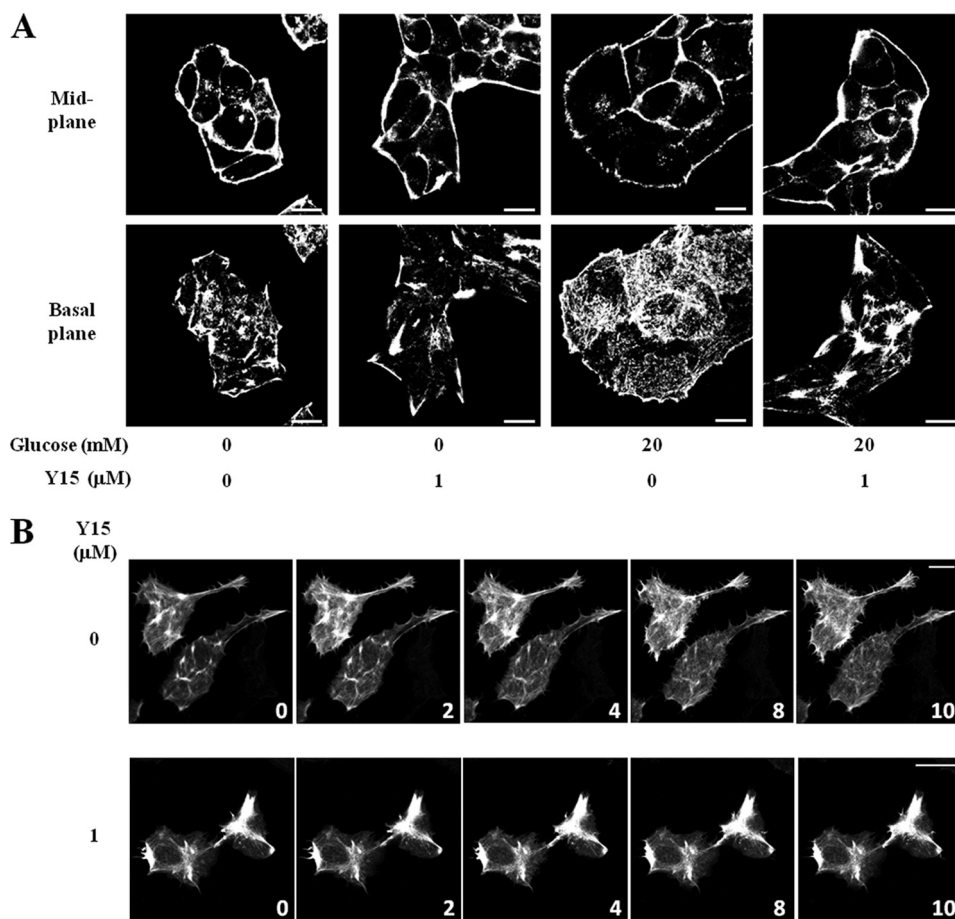


FIGURE 5. Specific FAK inhibition with compound Y15 blocks glucose-induced actin cytoskeleton remodeling. *A*, MIN6B1 cells were preincubated for 2 h in the presence of 0 mM glucose with 1 μ M Y15 (or DMSO, as a negative control) and were further treated for 10 min with 0 or 20 mM glucose combined with Y15 (or DMSO). Cells were subsequently fixed and stained for actin (with phalloidin). All images are fully representative of four independent experiments. *B*, MIN6B1 cells expressing Lifeact-mRFP_{rub} were stimulated with 20 mM glucose in combination with 1 μ M Y15 (or DMSO, as a negative control) and analyzed by live microscopy. Images were captured every 60 s. Numbers indicate time (minutes) after stimulation. Scale bars, 10 μ m. See supplemental Movies 1 and 2; time 0 indicates the stimulation with 20 mM glucose in combination with DMSO (supplemental Movie 1) or Y15 (supplemental Movie 2).

fiber remodeling shortly after glucose stimulation, whereas this process was markedly inhibited in MIN6B1 cells treated with compound Y15 (Fig. 5*B*, lower panels, see also supplemental Movie 2). Taken together, these results indicate that FAK activity has an important regulatory role in glucose-induced actin cytoskeleton remodeling in MIN6B1 cells, comparable with the role of FAK in insulin-induced actin reorganization in muscle cells (41).

FAK Inhibition Stabilizes Interaction between F-actin and SNAP-25—F-actin has been shown to associate in a glucose-responsive manner with the t-SNARE proteins syntaxin 1A and SNAP-25, limiting the rate and extent of insulin release in β cells (21). To determine whether FAK activation and its role in actin cytoskeleton remodeling represent a regulatory step in F-actin/t-SNARE complex association, we immunoprecipitated SNAP-25, reflective of this complex (21), from MIN6B1 cells extracted after glucose stimulation (10 min at 20 mM glucose) in the presence or absence of the FAK inhibitor compound Y15 (1 μ M) (Fig. 6*A*). Quantification of the amount of coimmunoprecipitated actin indicated as previously shown (21) a significant decrease ($36.2 \pm 10.8\%$, $p < 0.05$) in F-actin/SNAP-25 association upon glucose stimulation, as compared with the control condition (0 mM glucose) (Fig. 6*B*). However,

treatment of MIN6B1 cells with compound Y15 (1 μ M) resulted in a $74.5 \pm 17.2\%$ ($p < 0.05$) increase in F-actin/SNAP-25 association as compared with the control stimulated condition (20 mM glucose) (Fig. 6*B*). These data suggest that FAK inhibition might affect the functionality of t-SNARE complexes and, thereby, GSIS through a decrease in glucose-induced F-actin depolymerization in β cells.

Inhibition of FAK Decreases the Number of Morphologically Docked Insulin Granules at Plasma Membrane—We recently demonstrated that FAK inhibition in rat primary β cells decreases insulin secretion throughout both early and late phases of the glucose stimulatory period (27), which was also confirmed in MIN6B1 cells (Fig. 7, *A* and *B*). In view of these results and previous reports indicating the involvement of F-actin remodeling in stimulated insulin secretion (17, 20, 22, 42) and more specifically in dense-core vesicle transport and docking at the plasma membrane (21, 43, 44), we investigated whether FAK activity affects docking of insulin granules to the plasma membrane. For this purpose we transfected MIN6B1 cells with a construct expressing the fluorescent fusion protein NPY-Cherry to label dense-core vesicles (45, 46). Two days after transfection, cells were incubated for 2 h at 0 mM glucose in the presence of compound Y15 (1 μ M, or DMSO as a negative

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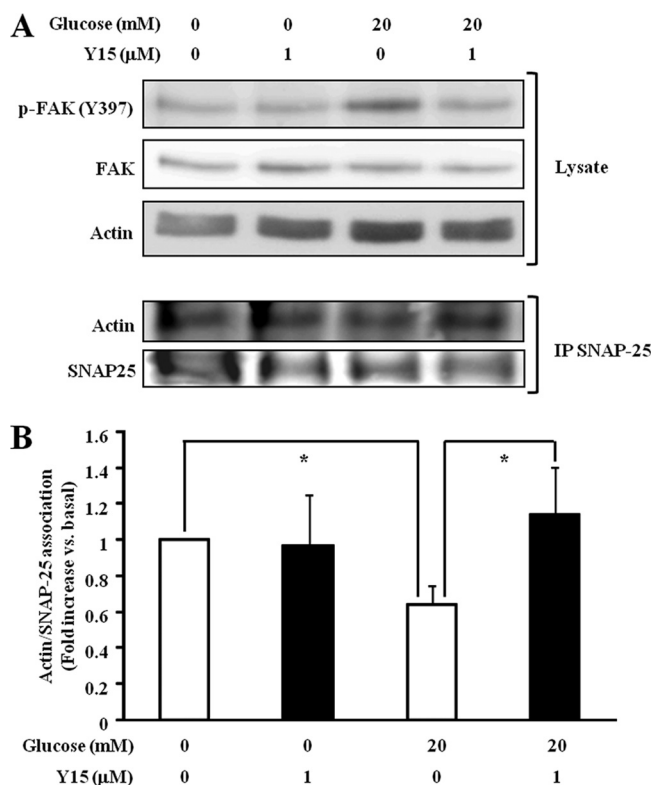


FIGURE 6. Compound Y15-mediated FAK inhibition stabilizes the association of SNAP-25 with F-actin. *A*, MIN6B1 cells were preincubated for 2 h in the presence of 0 mM glucose with 1 μ M Y15 (or DMSO, as a negative control) and were further treated for 10 min with 0 or 20 mM glucose combined with Y15 (or DMSO). Precleared whole-cell detergent extracts were prepared and immunoprecipitated (IP) with an anti-SNAP-25 monoclonal antibody (1–2 μ g/mg lysate protein). Lysates and immunoprecipitates were immunoblotted with the indicated antibodies. A representative blot from four independent experiments is shown. Identical amounts of proteins were analyzed, and as a control, relative quantities of actin in cell lysates are shown. *B*, the relative intensities of the immunoprecipitated actin bands (IP SNAP-25) were quantified by densitometry and were then normalized to the respective actin bands in the cell lysates. Data are the means \pm S.E. from four independent experiments (*, $p < 0.05$).

control), stimulated for 10 min with 20 mM glucose combined with Y15 (or DMSO), fixed, and observed by TIRF microscopy. In both basal and stimulated (10 min stimulation) conditions we counted a significant reduction in the number of plasma membrane-docked vesicles (Fig. 7, *C* and *D*) by FAK inhibition (Y15) as compared with the control condition (DMSO), suggesting that active FAK allows docking of insulin-containing granules to the plasma membrane, presumably through actin cytoskeleton remodeling.

Rac1 Is Involved in Glucose-induced Focal Adhesion and Actin Cytoskeleton Remodeling in MIN6B1 Cells—In muscle and adipose cells, insulin signaling downstream of PI3K, a known substrate of FAK, bifurcates into at least two separate arms, both involved in glucose transporter-4 translocation to the plasma membrane. The first arm involves the small GTPase Rac1 leading to actin remodeling (47), whereas the second one involves activation of Akt and its substrate AS160 (48, 49). To study the possible involvement of Rac1 in glucose-induced focal adhesion and actin remodeling in β cells, we used the chemical compound NSC23766 that specifically binds to Rac1 at the Tiam binding pocket, thereby preventing Rac1 activation.

Due to the relatively slow rates of uptake of this compound as reported in β cells (50) and other cell types (51, 52), MIN6B1 cells were cultured overnight at 10 mM glucose with either vehicle alone or NSC23766 (50 μ M). This Rac1 inhibitor has been shown to significantly attenuate GSIS in INS832/13 β cells and isolated islets (50), which was confirmed by us in MIN6B1 cells (data not shown). Analysis of the phosphorylation status of FAK, paxillin, and ERK1/2 in glucose-stimulated β cells revealed a 28.1 ± 11.8 , 20.3 ± 4.1 , and $24.0 \pm 8.4\%$ reduction, respectively, in glucose-induced phosphorylation of these three proteins in the presence of NSC23766 (Fig. 8*A*). This coincided with a decreased glucose-induced formation of phospho-FAK- and paxillin-containing focal adhesions as illustrated by the confocal images in Fig. 8*B*, confirming the earlier described role of Rac1 in the maintenance of focal adhesion complexes in other cell types (53, 54). As shown in the actin panels of Fig. 8*B*, treatment with NSC23766 also altered glucose-induced actin cytoskeleton remodeling but not as drastically as observed in case of FAK inhibition with compound Y15 (Fig. 5), suggesting the involvement of other players in addition to Rac1 in the regulation of glucose-induced actin remodeling downstream of FAK. Finally, immunofluorescence experiments performed on MIN6B1 cells expressing wild type Rac1 illustrate that treatment with compound Y15 substantially altered the basal membrane-associated localization of Rac1 in glucose-stimulated cells (Fig. 8*C*). In conclusion, these results indicate the importance of Rac1 activity in glucose-induced focal adhesion and actin remodeling in β cells but also suggest the involvement of FAK in glucose-mediated Rac1 function in β cells.

Glucose-induced FAK Activation Acts Upstream of the Akt/AS160 Signaling Pathway in MIN6B1 Cells—We have shown previously that AS160 phosphorylation plays an important role in GSIS (55). Accordingly, we investigated whether FAK activity, aside from regulating glucose-induced actin remodeling, also affected the Akt/AS160 signaling pathway downstream of PI3K in glucose-stimulated β cells. MIN6B1 cells were preincubated for 2 h at 0 mM glucose in the presence of compound Y15 (1 μ M, or DMSO as a negative control) and then stimulated for 10 min with 20 mM glucose containing compound Y15 (or DMSO). Analysis of the phosphorylation status of Akt (Ser-473) and AS160 (Ser/Thr) by Western blot revealed a 50.6 ± 9.8 and $75.2 \pm 8.0\%$ ($p < 0.01$) reduction, respectively, in glucose-induced phosphorylation of both factors by FAK inhibition as compared with the control stimulated condition (Fig. 9, *A* and *B*), indicating that FAK-mediated PI3K was effectively inhibited and could not exert its effect on its downstream substrates. These data suggest that FAK activity might indeed not only regulate the insulin secretory machinery through actin cytoskeleton remodeling but also through the Akt/AS160 signaling pathway.

DISCUSSION

The ECM is well known to be a critical regulator of β cell function and survival (56, 57). Cell-matrix contacts are enabled by specific focal adhesion sites providing both initiation points for outside-in signaling and a structural link between ECM components and the actin cytoskeleton (25, 26). Both FAK and

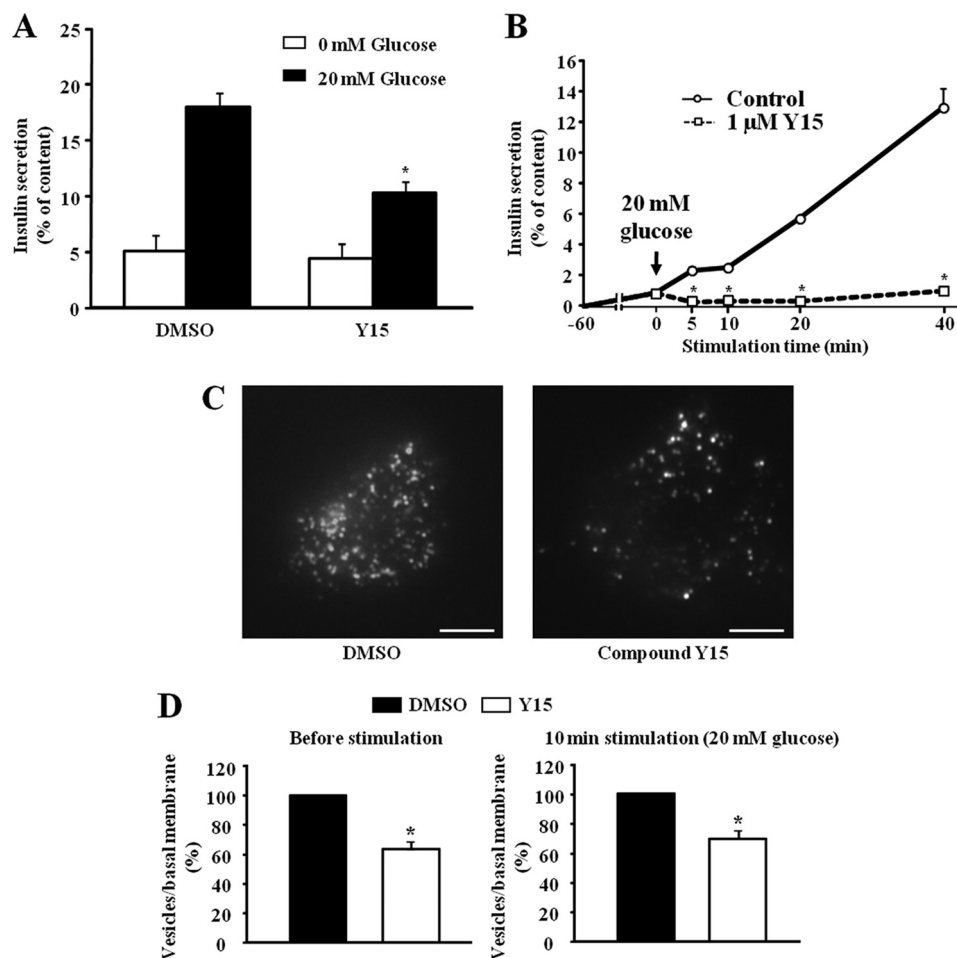


FIGURE 7. FAK inhibition attenuates glucose-stimulated insulin secretion and decreases the number of secretory vesicles at the plasma membrane of MIN6B1 cells. *A*, MIN6B1 cells were preincubated for 2 h with 0 mM glucose with compound Y15 (or DMSO). Cells were then incubated with low (0 mM, basal) followed by high (20 mM, stimulated) glucose with or without Y15 for 1 h. Insulin secretion is expressed as a percentage of total insulin cell content. Data are the mean \pm S.E. from three independent experiments; *, $p < 0.01$ versus control at same glucose concentration. *B*, cells were incubated as described above but were stimulated with high glucose with Y15 (or DMSO) for the indicated times. Data are expressed as the mean \pm S.E. from three independent experiments; *, $p < 0.05$ versus Y15-treated cells. *C*, MIN6B1 cells transfected with a plasmid expressing NPY-Cherry were preincubated for 2 h in the presence of 0 mM glucose with 1 μ M Y15 (or DMSO, as a negative control) and were further stimulated for 10 min with 20 mM glucose combined with Y15 (or DMSO). Cells were subsequently fixed and analyzed by TIRF microscopy. Representative TIRF images of the plasma membrane of MIN6B1 cells transfected with a NPY-Cherry plasmid are shown. Scale bars, 5 μ m. *D*, the number of secretory vesicles detected by TIRF at the plasma membrane and the plasma membrane surface area were quantified by MetaMorph[®] Version 6.0 and expressed as a ratio. Ratios were then normalized to the control condition (DMSO). Data are the means \pm S.E. from three independent experiments (*, $p < 0.01$).

paxillin are focal adhesion proteins shown to be phosphorylated and activated upon ECM adhesion (30, 58) and more specifically to play a central role in the rapid actin remodeling that is seen in β cells upon glucose stimulation (27). We have demonstrated previously that both FAK and paxillin are implicated in GSIS in primary β cells (27). In this study we further explore the FAK/paxillin-ERK signaling pathway in glucose-stimulated β cells and focus on a mechanistic link between FAK activity and the insulin secretory machinery through regulation of actin cytoskeleton remodeling.

Cellular receptors involved in cell-ECM interactions mainly belong to the integrin family (59), of which β 1 integrin is the most extensively studied in islet cell biology. In previous studies both integrins α 3 β 1 and α 6 β 1 have been shown to mediate attachment and spreading of rat pancreatic β cells, improving cell survival and insulin secretion (29, 30, 60). In this study we have demonstrated that β 1 integrin co-localizes in response to glucose and KCl with FAK and paxillin at specific focal adhe-

sion sites at the basal β cell membrane and that β 1 integrin intracellular signaling upon glucose stimulation involves the activation of FAK, paxillin, and ERK1/2 in β cells. These data, in addition to earlier studies on cell survival (31, 61) and GSIS (29, 30, 62), indicate the importance of the β 1 integrin-FAK-ERK signaling pathway in β cells. Furthermore, blocking of this pathway has also been shown to diminish focal contact sites and to induce the formation of aggregated actin filaments, illustrating its function as a molecular scaffold linking integrins with the actin cytoskeleton in islet cells (31). Adding to this and in agreement with previous reports (63–66), both latrunculin B and jasplakinolide, through disruption of the dynamic equilibrium between monomeric G-actin and filamentous F-actin states by unilateral actin depolymerization and polymerization, respectively, blocked glucose-induced mobilization of FAK and paxillin to focal adhesion sites as well as their activation, indicating the importance of the dynamic remodeling of the actin cytoskeleton regarding FAK and paxillin signal transduction. As for

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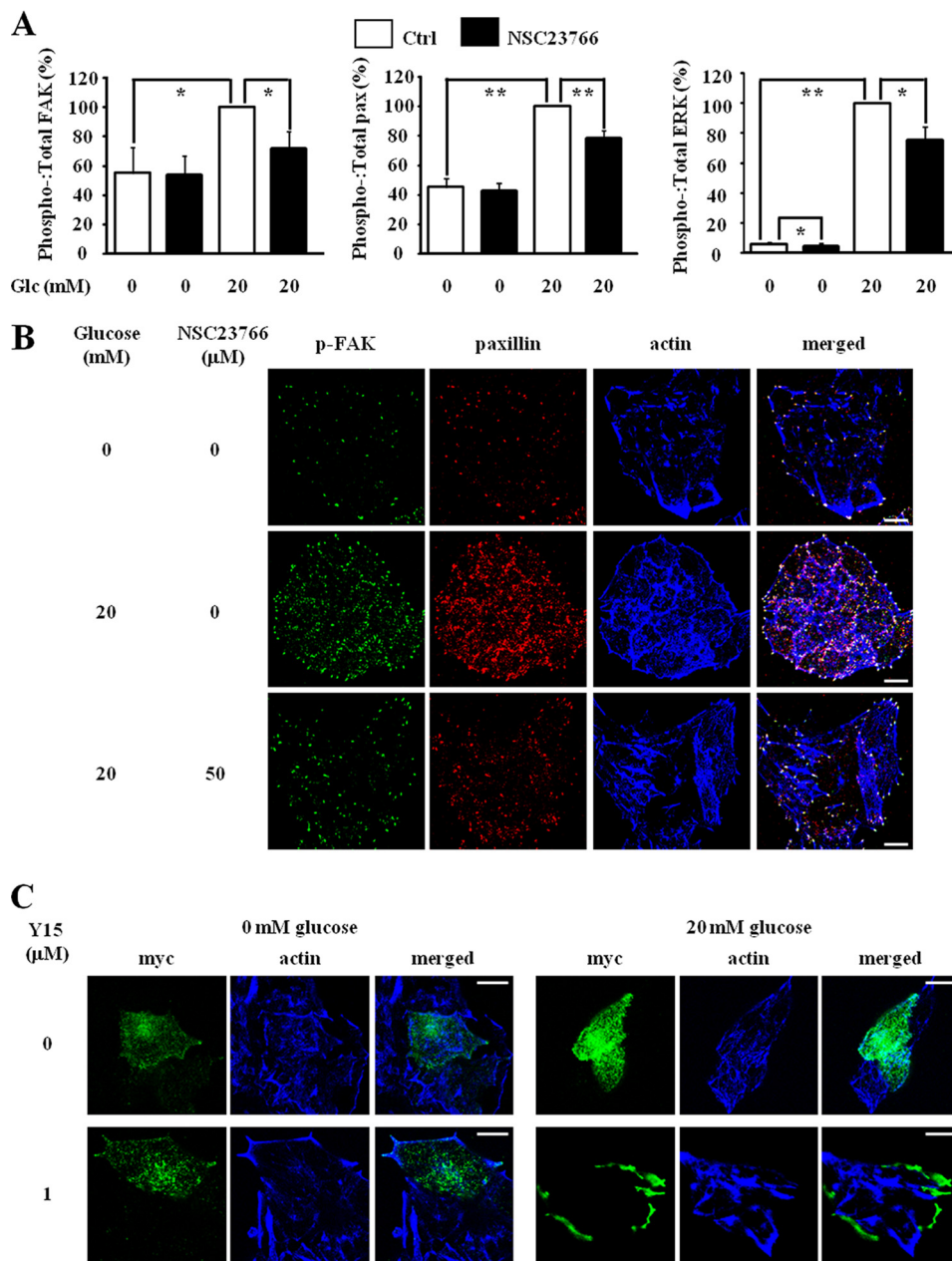


FIGURE 8. Rac1 is involved in glucose-induced focal adhesion and actin remodeling in MIN6B1 cells. *A*, MIN6B1 cells were cultured overnight at 10 mM glucose with the Rac1 inhibitor NSC23766 (50 μ M) or vehicle (H₂O) alone (control). Cells were then preincubated for 2 h with 0 mM glucose combined with NSC23766 (50 μ M) or vehicle as a control and were further stimulated for 10 min with 20 mM glucose with NSC23766 or vehicle. 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 then normalized to control high glucose (20 mM glucose)-stimulated cells. Data are the means \pm S.E. from four independent experiments (*, $p < 0.05$; **, $p < 0.01$). *B*, MIN6B1 cells were treated as described above, fixed, and stained for phospho-FAK (green), paxillin (red), and F-actin (blue). All pictures are fully representative of multiple images from three independent experiments. Scale bars, 10 μ m. *C*, MIN6B1 cells expressing myc-tagged wild type Rac1 were preincubated for 2 h with 0 mM glucose with 1 μ M compound Y15 (or DMSO as a negative control) and were further stimulated for 10 min with 20 mM glucose combined with Y15 (or DMSO). Cells were subsequently fixed and stained for myc-tag (green) and F-actin (blue). All pictures are fully representative of multiple images from three independent experiments. Scale bars, 10 μ m.

ERK1/2, we observed that latrunculin B and jasplakinolide potentiated and inhibited its glucose-induced activation, respectively, suggesting that glucose-induced ERK1/2 activation is dependent on the actin polymerization state. Note, however, the less pronounced inhibitory effect of jasplakinolide on glucose-induced phosphorylation of ERK1/2 as compared with that on FAK and paxillin. These data indicate the involvement of additional signaling pathways in glucose-induced ERK1/2 activation in β cells.

Numerous reports on β cells (for review, see Ref. 67) support the working model where F-actin, organized as a dense barrier beneath the plasma membrane, undergoes reorganization upon glucose stimulation to allow insulin-containing granules access to the cell periphery. In this study we show that FAK activity plays a crucial role in glucose-induced actin cytoskeleton remodeling and GSIS by using a selective inhibitor of FAK autophosphorylation at Tyr-397 (38, 39). The inhibitory effect of latrunculin B and jasplakinolide on glucose-induced activation

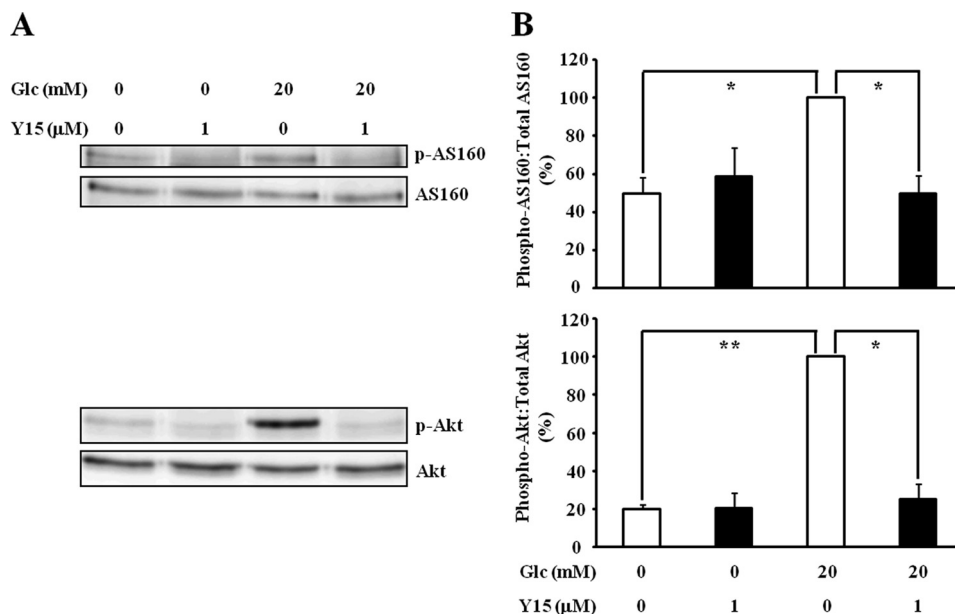


FIGURE 9. Inhibition of FAK attenuates glucose-induced phosphorylation of Akt and AS160 in MIN6B1 cells. MIN6B1 cells were preincubated for 2 h with 0 mM glucose combined with compound Y15 (1 μ M, or DMSO as a negative control) and were further stimulated for 10 min with 20 mM glucose with Y15 (or DMSO). *A*, cell lysates were analyzed by Western blot with the indicated antibodies, and a representative blot from four independent experiments is shown. *B*, the relative intensities of the phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio. Ratios were then normalized to control high glucose (20 mM glucose)-stimulated cells. Data are the means \pm S.E. from four independent experiments (* $p < 0.01$; ** $p < 0.001$).

of FAK that we show here taken together with their previously described stimulatory effect on cortical actin rearrangement and GSIS (20, 21) suggests that both actin-disrupting agents “shortcut” the regulatory role of FAK. More specifically, these data indicate FAK to act upstream of glucose-induced actin cytoskeleton remodeling but also point to a feedback loop from actin remodeling back to focal adhesion remodeling, illustrated by the inhibitory effect of chemical actin disruption on FAK autophosphorylation and focal adhesion assembly. This mechanism thereby allows both FAK inhibition, through blocking of actin remodeling, to attenuate glucose-stimulated insulin secretion and latrunculin B and jasplakinolide treatment, by actin rearrangement and independent from its effect on FAK activity, to potentiate glucose-stimulated insulin secretion.

This regulatory role of FAK on actin organization was further confirmed by our findings that FAK inhibition blocks glucose-induced disruption of the F-actin/SNAP-25 association at the plasma membrane. This interaction model, proposed by Thurmond *et al.* (21), suggests that glucose stimulation of β cells induces the transient remodeling of cortical F-actin, disengaging actin tethering to the SNARE complex, thereby promoting granule docking/fusion and exocytosis. In addition, TIRF-mediated visualization of insulin secretory vesicles revealed a reduction in the number of insulin-containing granules adjacent to the plasma membrane due to FAK inhibition. All together, these data suggest that FAK is crucial for glucose-induced actin cytoskeleton remodeling to allow insulin-containing vesicles to get in close proximity to the plasma membrane, a necessary step for docking and fusion. This correlates with the inhibitory effect of FAK inhibition on both early and late phases of GSIS, suggesting a functional role for FAK both in the regulation of the number of docked insulin granules as well as in mobilization of insulin granules to the plasma membrane.

These observations are consistent with the regulatory function of FAK in insulin-induced actin reorganization and glucose transporter-4 translocation in skeletal muscle cells (41).

Recent studies performed on neuroendocrine chromaffin cells reported the association of SNARE protein patches and clusters of voltage-gated Ca^{2+} channels with the borders of cortical cytoskeletal cages (68, 69). We show here the glucose-responsive colocalization of Ca^{2+} -responsive FAK (27) with β 1 integrin and paxillin in patches at the basal β cell membrane. Considering this and the earlier reported cluster-like distribution of the SNARE proteins syntaxin 1 and SNAP-25 (70), which interact with both L-type Ca^{2+} channels and actin in β cells (71, 72), it will be interesting in the future to explore the presence of similar cortical cytoskeletal cages in β cells and their possible association with FAK. This might clarify the mechanistic link between FAK activity and the organization of the actin cytoskeleton and might thereby further explain its effect on the insulin secretory machinery. In addition, the presence of Ca^{2+} “hotspots” (73), shown to associate with the borders of these cytoskeletal cages (68), would also elucidate the local actin remodeling-mediated activation of ERK1/2 we demonstrate here.

Finally, we show here for the first time the negative effect of FAK inhibition on two signaling pathways downstream of PI3K in glucose-stimulated β cells, namely the Rac1 and the Akt/AS160 signaling pathways. Rac1 is a member of the Rho subfamily of G-proteins known to play a regulatory role in GSIS (50, 74–77). Considering the study of the cellular distribution of Rac1 in glucose-stimulated β cells by Li *et al.* (77), we observed that FAK inhibition clearly altered the basal membrane-associated localization of Rac1, suggesting FAK to be involved in glucose-mediated Rac1 activation. Based on its well known role in cytoskeletal rearrangement and our results pre-

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sented here, Rac1 might thereby act as a factor linking glucose-induced FAK activation and actin cytoskeleton remodeling in the regulation of the insulin secretory machinery in β cells. Furthermore, results obtained with the Rac1 inhibitor also indicated the regulatory role for Rac1 in focal adhesion assembly, which is supported by earlier studies performed in other cell types (53, 54) and which also confirms the observed mutual interaction between glucose-induced focal adhesion and actin cytoskeleton remodeling in β cells as mentioned above.

In agreement with the role of Akt and AS160 in the regulation of glucose transporter-4 vesicle trafficking in muscle cells and adipocytes (49, 78–81) and the functional role of AS160 in GSIS in β cells (55), we hypothesize that FAK might also, aside from its effect on actin remodeling, impact on vesicle provision from the reserve pool or docking/fusion efficiency of the secretory vesicles with the plasma membrane through regulation of the Akt/AS160 signaling pathway. However, further investigation will be necessary to unravel the exact mechanism.

In summary, this study proposes a model of how glucose-induced, β 1 integrin-mediated FAK activation contributes to the regulation of the insulin secretory machinery through actin cytoskeleton remodeling, showing many similarities with the insulin-induced glucose transporter-4 translocation mechanism in muscle cells and adipocytes. Further studies will be necessary to further characterize this signaling pathway, which may facilitate the discovery of new targets for the treatment of diabetes.

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