Importance of Cell-Matrix Interactions in Rat Islet β-Cell Secretion In Vitro
Role of α6β1 Integrin

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It has long been recognized that islet cell function is rapidly altered in vitro, but can be maintained, at least in part, when cells are layered on defined extracellular matrices. The present work addresses the influence of short-term cell-matrix interactions on islet β-cell function and provides first insight into the molecular basis of these interactions. When primary rat β-cells were allowed to attach to a matrix produced by a rat carcinoma cell line (804G), there was an increased insulin secretory response to secretagogues. This change was the result of an increase in the proportion of actively secreting β-cells and in the amount of insulin secreted per active cell, as shown using the reverse hemolytic plaque assay. In turn, the spreading or flattening of β-cells on this matrix was enhanced by secretagogues, and flattened cells secreted more insulin than rounded cells. Using indirect immunofluorescence, it was found that 1) α6β1 integrins are present at the surface of islet cells in situ, 2) α6β1 expression is heterogeneous among purified β-cells and is upregulated by insulin secretagogues, 3) α6β1 expression is higher in spreading cells, and 4) anti-α6β1–specific antibodies decrease spreading. These observations demonstrate that islet cell–matrix interactions can improve the sensitivity of insulin cells to glucose and are mediated, at least in part, by α6β1 integrins, suggesting that outside-in signaling through α6β1 integrin plays a major role in the regulation of β-cell function. Diabetes 49:233–243, 2000

P rimary cells of rat islets of Langerhans lose glucose responsiveness and eventually die when maintained in culture for a long period of time. By contrast, the secretory capability of islet cells remains stable when dishes are first coated with components of extracellular matrices (ECMs) (1). Thus, adult rat islets maintained over several weeks on ECM respond to an acute glucose stimulation by a five- to eightfold increase in insulin secretion (2). In human β-cells cultured 5–11 days on bovine corneal endothelial cell matrix, both basal and stimulated insulin release were also increased, compared with cells layered on gelatin, collagen, or Matrigel (3). Furthermore, when overlaid with collagen, monolayers of human islet cells undergo a gradual and complete reorganization into a three-dimensional islet-like structure with a striking reinforcement of their secretory activity. Under these conditions, cells were able to survive more than 8 weeks (4). By contrast, standard cultures on uncoated plastic petri dishes exhibit a rapid and definitive decline in insulin secretion with a survival time not exceeding 14 days (4). Using a reverse hemolytic plaque assay, Perfetti et al. (5) determined that β-cells cultured for 6 weeks on Matrigel showed an equal number of insulin-secreting cells compared with freshly isolated islets cultured for only 3 days in the absence of Matrigel. The amount of insulin released per single β-cell was nevertheless reduced by as much as 60%. Despite this evidence for a crucial role of islet cell–matrix interactions for normal islet function, the molecular basis of these interactions remains largely unknown.

Cell receptors involved in epithelial cell–matrix interactions mainly belong to the integrin family (6). Integrins are heterodimeric integral membrane glycoproteins composed of an α-subunit that is noncovalently linked to a β-subunit. Certain α-subunits (e.g., α3, α6, and αv) can be further split into two fragments under reducing conditions. Integrins are classified in subfamilies named after the β-subunit to which a number of different α-subunits may be associated (6). β1 integrins are expressed on most epithelial cells, where they bind collagen, fibronectin, laminin-1 (7), and laminin-5 (8). They have been suggested to play important roles in morphogenesis (9,10), secretory cell differentiation (11), establishment of polarity, regulation of proliferation (12), and cell survival (13,14). Within islets, we previously showed (15) that β1-integrins are expressed on endocrine cells and that α3β1, the laminin-5 receptor, accounts for ~50% of them. The other α-subunits associating with β1 were not identified, but based on apparent molecular weights, could have been either α6 or αv. It was the purpose of the present study to appreciate better the role of β-cell–matrix interactions in islet function, confirm the presence of α6β1 integrins on β-cells, and provide insight into the role of α6β1 integrins in these interactions.

RESEARCH DESIGN AND METHODS

Islet isolation and β-cell purification. Islets of Langerhans were isolated by collagenase digestion of pancreases from male Sprague-Dawley rats (weighing 180–200 g), followed by Ficoll purification using a modification of previously

described procedures (16,17). For cell preparation, the isolated islets were rinsed three times with Mg^{2+}-, Ca^{2+}-free phosphate-buffered saline (PBS) and resuspended in 1.5 ml of the same buffer containing 0.016% of trypsin (activity against casein, 1:250), and 0.0066% EDTA (Gibco, Life Technologies, Paisley, Scotland). Digestion (with occasional pipetting) was for 6 min at 37°C and was stopped by the addition of 10 ml ice-cold Krebs-Ringer bicarbonate buffer (KRBB), pH 7.4, containing 0.5% bovine serum albumin (BSA), 2.8 mmol/l glucose, and 10 mmol/l HEPES. β-Cells were then separated from non–β-cells by autofluorescence-activated sorting using a fluorescence-activated cell sorter (FACS), FACStar-Plus cell sorter (Becton Dickinson, Sunnyvale, CA), as previously described (17,18). When purity of the β-cell population was tested by immunofluorescence for insulin, in all cases >95% of cells were positive.

Cell culture. Sorted β-cells were washed twice in 10–15 ml sterile Dulbecco's minimum essential medium (DMEM) (Gibco, Life Technologies) containing 10% heat inactivated fetal calf serum (FCS) and supplemented with 110 U/ml penicillin, 110 µg/ml streptomycin, and 50 µg/ml gentamicin, followed by centrifugation for 10 min at 130g. Aliquots of 10^5 cells were seeded in nonadherent 60-mm diameter petri dishes containing 3 ml medium. Glucose and/or isobutylmethylxanthine (IBMX) was added as indicated in RESULTS. Cells were then incubated for 20 h at 37°C to allow full recovery of any cell surface molecules that may have been lost or damaged during islet isolation or cell purification. For immunoflow cytometry, cell reaggregation was prevented by dispersing 10^5 β-cells in 100-mm diameter dishes containing 8 ml DMEM.

The 804G matrix preparation. The 804G cells were obtained from Desmos (San Diego, CA). They were grown in DMEM containing 10% FCS and 5.6 mmol/l glucose. At confluence, cells were rinsed and maintained for a further 3 days in the same medium in the presence or absence of FCS. Conditioned medium (referred to hereafter as 804G matrix) was collected, centrifuged at 120g for 10 min to remove any detached cells and debris, filtered through a 0.22 µm Millipore filter, and frozen at –20°C for later use.

Coating of plastic and glass with ECM. Aliquots (60 µl) of crude 804G matrix or laminin-5 purified from 804G (provided by N. Koshikawal and V. Quaranta, San Diego, CA), at a concentration of 4 µg/ml in PBS, were layered at the center of 35-mm culture petri dishes. Dishes were kept in a damp box at 37°C for 18–20 h before being rinsed three times with sterile H_2O and air-dried. Uncoated petri dishes were used as a control. The glass microscope slides used to build Cunningham's chambers (19) were coated with 300 µl of 804G matrix and incubated for 1 or 18 h in a damp box at room temperature. They were then rinsed 3 times with H_2O and air-dried. Uncoated or poly-L-lysine-coated (molecular weight: 150,000–300,000, 0.1 mg/ml) slides were used as a control.

Analysis of cell adhesion to ECMs. β-Cells cultured for 20 h in nonadherent petri dishes were centrifuged for 10 min at 130g and resuspended at a density of 10^6 cells/ml in DMEM containing 10% FCS. Depending of the experiment, the medium was supplemented with glucose (at various concentrations), glucagon (1 µmol/l), or IBMX (0.5 mmol/l). Aliquots (60 µl) of this suspension were plated as droplets at the center of petri dishes coated or not with 804G matrix and were incubated at 37°C. After 3 h, 2 ml of medium was added, and cells were analyzed for attachment and spreading under an inverted microscope. They were then incubated for 20 h before final analysis and fixation for immunofluorescence.

In perturbation studies, 60-µl aliquots of β-cell suspension were incubated for 15 min at room temperature in the presence of either an anti-rat α6β1 integrin antibody (mouse monoclonal, 1:30 dilution; Immunokontact, Frankfurt, Germany) or an anti-rat β1 integrin subunit rabbit antiserum (1:200 dilution; gift from Dr. C.A. Buck, Philadelphia). Mouse ascites or nonimmune rabbit serum were used accordingly as controls. Each aliquot was then layered at the center of a coated 35-mm diameter petri dish and incubated for 3 h at 37°C. Preparations were then rinsed with DMEM and incubated a further 20 h in 2 ml of DMEM containing 22.2 mmol/l glucose.

Immunohistochemical studies. Rat pancreases were fixed in situ by intracardiac infusion of PBS containing 4% paraformaldehyde. Dissected pancreases were then incubated for 2 h in this same solution. After overnight incubation in a 30% sucrose solution, they were frozen in liquid nitrogen. Cryostat sections (5 µm thick) were then prepared, attached to slides, postfixed with 2% paraformaldehyde, and kept at –20°C until use. Purified islet β-cells, attached in either petri dishes or Cunningham's chambers, were rinsed with PBS, fixed with cold (–20°C) ethanol for 3 min, and again rinsed with PBS. Pancreatic sections or β-cells were then preincubated for 30 min at room temperature in PBS containing 0.5% BSA with (sections) or without (cells) addition of 0.1% Triton X-100. Preparations were then exposed for 2 h at room temperature to either the anti-rat α6β1 integrin monoclonal antibody (1:40 dilution in PBS) or the anti α3-integrin subunit monoclonal antibody (1:40 dilution in PBS). After three washes in PBS, they were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Antibodies Inc., Davis, CA) diluted 1:1,000 in PBS, then rinsed with PBS, and counterstained with Evans blue. Control samples were reacted with the second antibody only. Samples were examined by fluorescence microscopy (Zeiss Axiophot, Oberkochen, Germany).

Islet cell surface biotinylation. Cell surface proteins were biotinylated with N-hydroxysuccinimidobiotin (NHS-biotin) (Pierce, Rockford, IL), as previously described (20). Briefly, cells (10^7/ml) were incubated in a buffer containing 137 mmol/l NaCl, 5.4 mmol/l KCl, 0.8 mmol/l $MgSO_4\cdot7H_2O$, 1.36 mmol/l $CaCl_2\cdot2H_2O$, 6 mmol/l $NaHCO_3$, and 5 mmol/l glucose, pH 7.4. Freshly dissolved NHS-biotin (13 mg/ml in DMSO) (Fluka, Buchs, Switzerland) was added to a final concentration of 300 µg/ml, and cells were incubated for 30 min at room temperature under constant agitation. Unreacted biotin was then removed by washing cells once with culture medium and once with PBS.

Solubilization of biotinylated cells. Solubilization of cells was performed by a 30-min incubation on ice under constant agitation in a lysis buffer containing 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 3 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2\cdot2H_2O$ (pH 7.4), 1% Triton X-100 and protease inhibitors (10 µg/ml leupeptin [Sigma, St. Louis, MO], 4 µg/ml pepstatin [Sigma], 0.1 trypsin inhibitor unit/ml aprotinin [Sigma]), and 1 mmol/l phenylmethylsulfonylfluoride (Fluka). Insoluble material was removed by ultracentrifugation at 30,000 rpm for 1 h at 4°C.

Immunoprecipitation of biotinylated integrins. Supernatants from ultracentrifugation were precleared by a 2-h preincubation at 4°C with protein A Sepharose. The cleared materials were then incubated with appropriate antibodies at a dilution of 1:100. Incubation with the primary antibody was performed overnight at 4°C with mild agitation. A 50% slurry of protein A Sepharose (50 µl) was added to 500 µl of cell extract, and the incubation was continued for 2 h. Sepharose-bound material was centrifuged and washed five times with lysis buffer. Antibody-antigen complexes were dissociated by boiling for 5 min in Laemmli sample buffer (21).

Electrophoresis of immunoprecipitated integrins. Immunoprecipitated proteins were separated by SDS-PAGE (7.5% acrylamide) under reducing (5% mercaptoethanol) or nonreducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) (22). Background staining was reduced by immersing membranes at 37°C for 1 h in Tris-buffered saline (TBS) (25 mmol/l Tris, 150 mmol/l NaCl, pH 7.4) containing 5% nonfat milk, 0.1% Tween-20, and 1% polyvinylpyrrolidone. Membranes were incubated for 1 h at room temperature with 2 µg/ml horseradish peroxidase–conjugated streptavidin (Pierce) in TBS supplemented with 0.1% BSA and 0.1% Tween-20. After three washes with TBS/BSA/Tween-20 at 37°C, streptavidin-bound proteins were detected by the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, U.K.).

Immunoflow cytometry. After an overnight culture in 100-mm dishes, sorted primary β-cells were first incubated on ice for 45 min with the antibody against α6β1 integrin diluted 1:50 in Ca^{2+}-free KRBB supplemented with 0.5 mmol/l EDTA and 1% BSA. Cells were then washed twice with this same buffer and incubated with the FITC-conjugated goat anti-mouse antibody (1:1,000) for another 45 min on ice. After washing, cells were analyzed in a FACScan flow cytometer (Becton Dickinson).

Reverse hemolytic plaque assay. Insulin secretion of single and reaggregated β-cells was assessed by a reverse hemolytic plaque assay (RHPA), as previously described (19,23,24). Briefly, β-cells were diluted in either DMEM (without serum) or KRBB (pH 7.4) supplemented with 0.1% BSA, 2.8 mmol/l glucose, and when mentioned, 0.5 mmol/l IBMX. Packed sheep red blood cells (5% vol/vol) (Behring Institute, Marburg, Germany) previously coated with protein A Sepharose were then mixed with β-cells, and 50–60 µl of this preparation was injected in Cunningham's chambers (19,23). After a 45-min or 3-h incubation at 37°C, the chambers were first rinsed with KRBB containing either 2.8, 16.7, or 22.2 mmol/l glucose, and then filled with the same buffer supplemented with a heat-inactivated (45 min at 56°C) anti-insulin guinea pig serum (1:50) (25). After a 1-h incubation at 37°C, chambers were rinsed with KRBB containing 2.8 mmol/l glucose, filled with the same buffer containing guinea pig complement (1:40, Behring Institute), and incubated at 37°C for 1 h. Chambers were then filled with a 0.04% (wt/vol) solution of trypan blue in KRBB, rinsed with KRBB, and filled with either Bouin's fixative or ethanol for insulin or α6β1 immunostaining, respectively. Immunofluorescence staining for insulin was performed to identify β-cells in the red blood cell layer. To this end, slides were passed through graded concentrations of ethanol and incubated for 2 h at room temperature with the anti-insulin antibody now diluted 1:400 in PBS. After rinsing, slides were incubated for 1 h at room temperature with a fluorescein-labeled goat anti–guinea pig second antibody (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA). After rinsing in PBS, slides were covered with 0.02% p-phenylenediamine in PBS-glycerol (1:2, vol/vol) and screened by fluorescence microscopy.

Analysis was restricted to single or paired cells that excluded trypan blue at the end of the plaque assay and were positive for insulin by immunofluorescence. For each experiment, the proportion of cells or pairs surrounded by hemolytic plaques was established, and the area of hemolytic plaques was measured using a calibrated microscope eyepiece. Total plaque development was calculated by multiplying the average plaque area by the proportion of plaque-form-
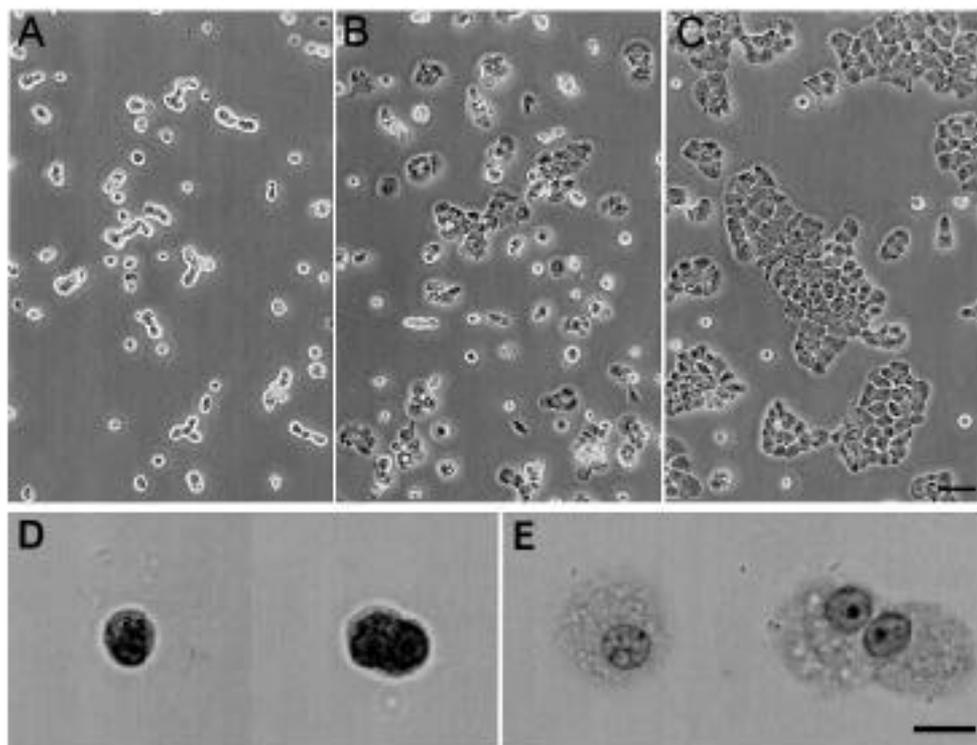
FIG. 1. Spreading of rat islet β-cells on 804G matrix. FACS-purified β-cells from isolated rat islets were plated on either untreated petri dishes (A and D) or petri dishes coated with 804G matrix (B, C, and E). After 48 h in control dishes (A and D), β-cells remained round. On 804G matrix, flattening and spreading was apparent at 3 h (B) and extensive by 48 h (C and E). A–C: Low magnification views (bar = 60 μm) of single and aggregated β-cells. D and E: Higher magnifications (bar = 15 μm) of single and doublet β-cells.

ing cells (23). Data were expressed as means ± SE and were compared using an unpaired Student's t test.

RESULTS

Attachment and insulin secretion of β-cells on 804G matrix. Rat β-cells were purified by flow cytometry and resuspended in DMEM containing 10% FCS and 11.1 mmol/l glucose. They were maintained in culture in either the presence or absence of 804G matrix, derived from a rat bladder carcinoma cell line. In the absence of matrix, no apparent change in morphology of cells was observed up to 48 h, i.e., cells remained round with refringent borders (Fig. 1A and D). β-Cells cultured on 804G matrix, however, acquired a different morphology, i.e., they rapidly flattened and spread out loosing refringence (Fig. 1B, C, and E), indicating strong adhesion to this matrix.

To test whether adhesion to the 804G matrix influenced insulin secretion, β-cells in DMEM containing 2.8 mmol/l glucose were allowed to attach 3 h to Cunningham's chambers coated or not with 804G matrix. Insulin secretion was measured using an RHPA after 60 min of incubation in the presence of 2.8 mmol/l (basal) or 22.2 mmol/l glucose (stimulated). At the end of the test, qualitative analysis of Cunningham's chambers that contained both single and aggregated β-cells revealed that plaque formation was stimulated in the presence of 22.2 mmol/l glucose (Fig. 2B vs. A). Moreover, it appeared that this increase was higher for β-cells attached on 804G matrix than for β-cells attached on control glass (Fig. 2D vs. B). Plaques formed by single β-cells and β-cell pairs were then quantitatively analyzed (Fig. 3).

Under low glucose, only a few β-cells were able to form hemolytic plaques, whether attached to glass or to 804G matrix. The corresponding mean plaque areas, reflecting the amount of insulin secreted per cell, were similar. Upon stimulation with 22.2 mmol/l glucose, the proportion of plaque-forming cells was increased, as compared with 2.8 mmol/l glucose. The percentage was significantly higher for cells attached to 804G matrix compared with those attached to glass. The mean plaque area was also higher in β-cells attached on 804G matrix than in β-cells attached on glass. Consequently, total plaque development (average plaque area × proportion of plaque-forming cells) was about twofold higher for single β-cells attached to 804G matrix compared with cells on control glass. As already described (23), β-cells within doublets secrete more insulin than do single cells (Fig. 3). The effect of the matrix, however, was similar between doublet and single cells.

To assess whether nonspecific adhesion was sufficient to stimulate secretion, β-cells were attached to slides coated with poly-L-lysine, a polycationic substrate known to provide strong adherence of cells (26). In two independent experiments, total plaque development of β-cells attached to poly-L-lysine was only slightly increased over control (10 and 20%), contrasting with the much larger increase (160 and 180%) of cells attached to the 804G matrix.

Spreading of β-cells is influenced by insulin secretagogues. To investigate the effect of insulin secretagogues on β-cell spreading, sorted β-cells were incubated for 24 h at different concentrations of glucose. On uncoated plastic or glass, virtually no cells spread out at any tested con-
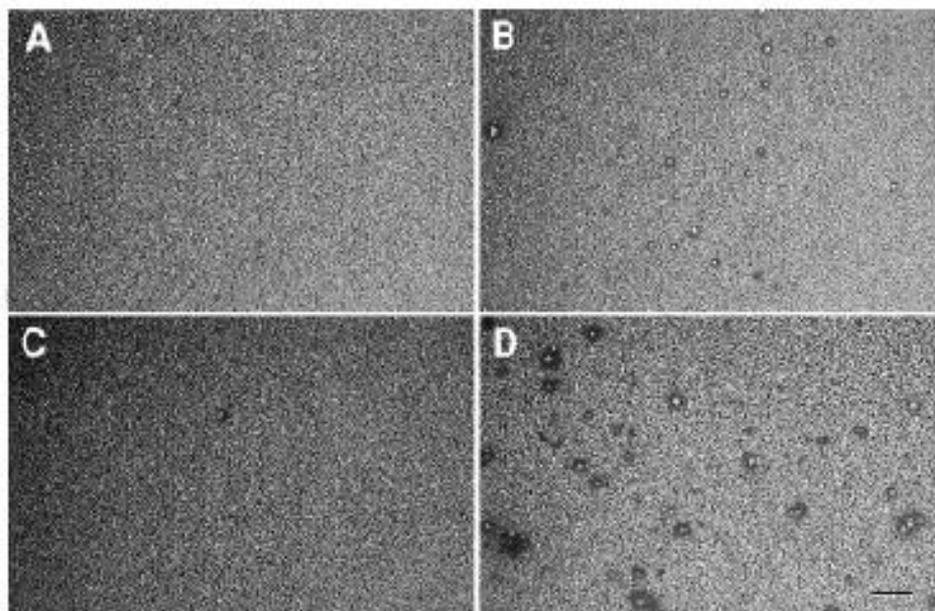
FIG. 2. Insulin secretion of isolated **β**-cells is stimulated on 804G matrix. Dark field views of plaque assay chambers after 1 h of incubation in the presence of either low (2.8 mmol/l) (A and C) or high (22.2 mmol/l) glucose (B and D). Cells were attached on either control glass (A and B) or glass coated with 804G matrix (C and D). At this low magnification, hemolytic plaques are seen as black areas. At low glucose, virtually no plaques are visible on either glass (A) or 804G matrix (C). At high glucose, some hemolytic plaques are seen on glass (B). The number and size of plaques were increased on 804G matrix (D). Bar = 300 μm.
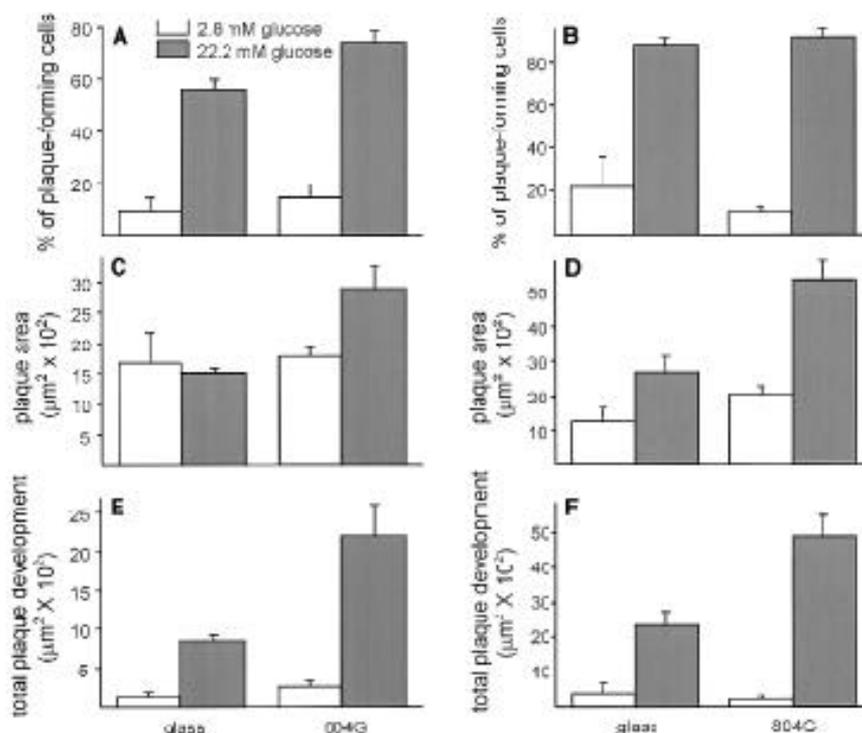

FIG. 3. Quantitative measurement of the increased insulin secretion of **β**-cells plated on 804G matrix. Purified **β**-cells were incubated for 1 h in the presence of 2.8 or 22.2 mmol/l glucose. Single (A, C, and E) and paired (B, D, and F) **β**-cells were analyzed separately. Secretion is expressed as follows: A and B: percentage of cells forming plaques (proportion of cells secreting insulin); C and D: the area of the plaque (amount of insulin secreted per cell); and E and F: total plaque development (integration of both parameters). At 2.8 mmol/l glucose, no significant difference was observed between cells attached on glass or on 804G matrix for all parameters analyzed, whether cells were single or in pairs. At 22.2 mmol/l glucose, the percentage of **β**-cells surrounded by a hemolytic plaque was higher for single cells attached on 804G matrix than for cells attached on control glass (74.3 ± 4.4 vs. 55.9 ± 3.9%, P < 0.02). The same observation was made for the mean plaque area (2,902 ± 364 vs. 1,514 ± 73 μm^2) and, consequently, for the total plaque development (2,202 ± 408 vs. 848 ± 75 μm^2, P < 0.02). In the case of **β**-cell pairs, almost all cells respond to glucose, whether seeded on plain glass or on 804G matrix. Plaque area, however, and consequently total plaque development, were significantly higher on 804G matrix compared with glass (P < 0.01); the difference was more pronounced than for single cells. Values are means ± SE of three (2.8 mmol/l glucose) or four (22.2 mmol/l glucose) independent experiments.
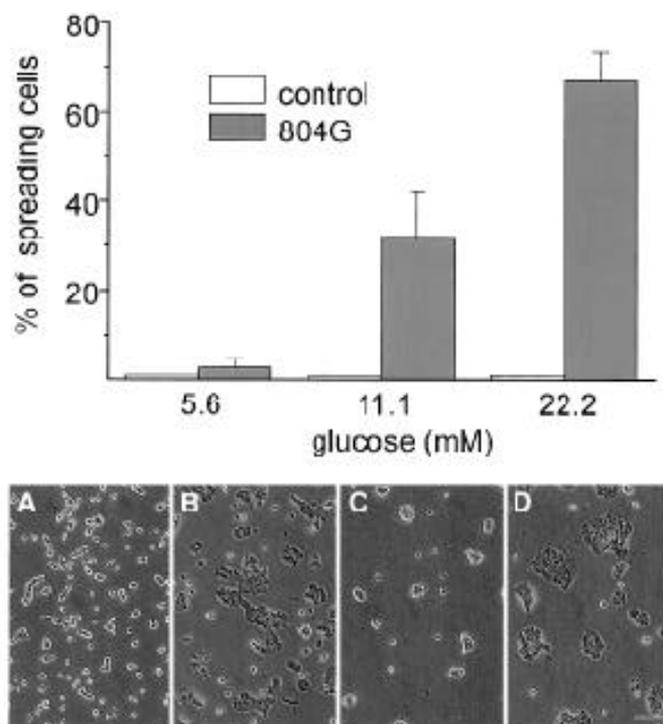
FIG. 4. Glucose increases β-cell spreading on 804G matrix in a reversible fashion. Top: FACS-sorted β-cells were incubated for 24 h in either untreated petri dishes or petri dishes coated with 804G matrix. In control petri dishes, no spreading was observed, whatever the concentration of glucose. On 804G matrix, the percentage of spread cells increased (P < 0.02) with concentration of glucose, reaching 32 ± 10% and 67 ± 6.5% at 11.2 and 22.4 mmol/l, respectively. Values are means ± SE of three independent experiments. Bottom: Phase contrast representation of the effect of glucose on β-cell spreading. At the end of a 24-h incubation at 5.6 mmol/l glucose, β-cells remained round (A). At 22.2 mmol/l glucose, most β-cells spread (B). After lowering the glucose concentration to 5.6 mmol/l glucose for another 24 h, cells or aggregates regained a round morphology (C). Finally, 22.2 mmol/l glucose concentration was restored, resulting again in the spreading of cells over the next 24 h (D). Bar = 100 μm.
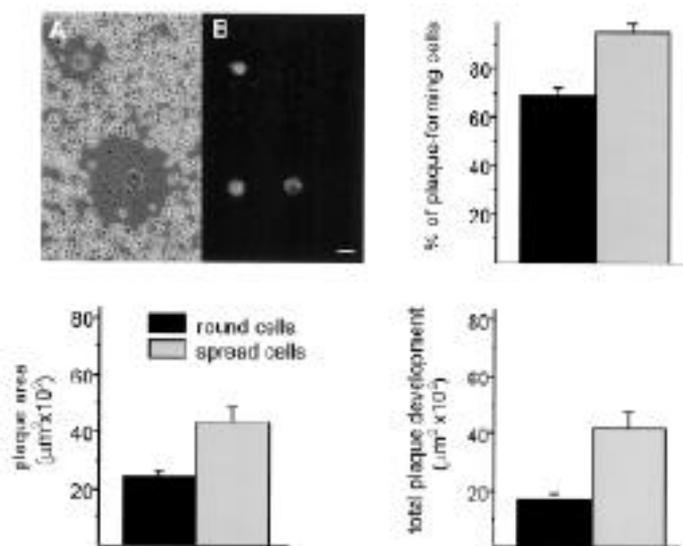

FIG. 5. Insulin secretion from spread cells is higher than that from cells that remain rounded. Phase-contrast (A) and corresponding anti-insulin immunofluorescence (B) views of β-cells allowed to attach to 804G matrix for 3 h in the presence of 2.8 mmol/l glucose and then stimulated for 1 h with 22.2 mmol/l glucose are shown. The round β-cell at the top is surrounded by a smaller hemolytic plaque than the lower spreading β-cell (bar = 20 μm). The proportion of plaque-forming cells, the mean plaque area, and the total plaque development were higher (P < 0.05; n = 4) for spread β-cells than for round cells.

centration of glucose in the medium. On plastic coated with 804G matrix, only a few β-cells were able to spread at 5.6 mmol/l glucose. However, a glucose-dependent increase in β-cell spreading was observed (Fig. 4, top). The effect of glucose on cell spreading was reversible (Fig. 4, bottom).

Many paracrine and endocrine modulators of insulin secretion involve changes in the levels of cAMP. Spreading of β-cells was therefore studied in response to known stimuli of cAMP. Cells were attached to glass coated with 804G matrix in the presence of 5.6 mmol/l glucose. After 3 h, the fraction of spread β-cells was almost doubled (29.7 ± 1.8% vs. 15.8 ± 3.7%, P < 0.05, n = 3) by the addition of 0.5 mmol/l IBMX. By 20 h on plastic coated with 804G matrix, virtually all cells were spread in the presence of either 0.5 mmol/l IBMX or 1 μmol/l glucagon.

Correlation between cell spreading and insulin secretion. When attached to 804G matrix-coated glass for only 3 h in the presence of 2.8 mmol/l glucose, most β-cells still display a round morphology (top cell in Fig. 5A and B), as do cells attached to glass. A few β-cells, however, appear to spread out and acquire a flatter morphology (bottom cell), possibly suggesting β-cell heterogeneity in the expression of matrix

receptors. We took advantage of the RHPA to analyze separately round and spread cells for insulin secretion on the same coverslip. Quantitative analysis of the results (Fig. 5) indicated that, in the same culture conditions, both the percentage of plaque-forming cells and the mean plaque area and, consequently, total plaque development were higher for spread than for round cells.

To further correlate cell spreading and secretion, β-cells were attached to either plain glass or 804G matrix and incubated at 2.8 mmol/l glucose for 3 h in the absence or presence of IBMX before the secretion assay. When β-cells were attached to glass, preincubation with IBMX had no effect on the subsequent insulin response to glucose (Fig. 6); under these conditions, virtually no spreading was observed. However, when β-cells were attached on 804G matrix, preincubation with IBMX resulted in increased spreading, together with a 75% increased insulin response to glucose. These results support the hypothesis that promoting cell spreading results in an increased insulin response to glucose.

Expression of α6β1 integrin on isolated β-cells and pancreas. Immunofluorescence for the α3 integrin subunit resulted in labeling of all β-cells, as expected from our previous studies (15). Because of the absence of heterogeneity in expression between β-cells, and lack of stimulation by secretagogues (data not shown), it was estimated that α3β1 was not responsible, at least by itself, for the observed effects of β-cell–matrix interactions. Based on coimmunoprecipitation studies, we previously made the hypothesis that α6 might represent another integrin subunit associating with β1 in rat islet β-cells (15). Indirect immunofluorescence, using a specific monoclonal antibody, was used to detect the expression of α6β1 integrins on purified islet β-cells (Fig. 7A–H). Labeling was present on most cells and was confined to the plasma
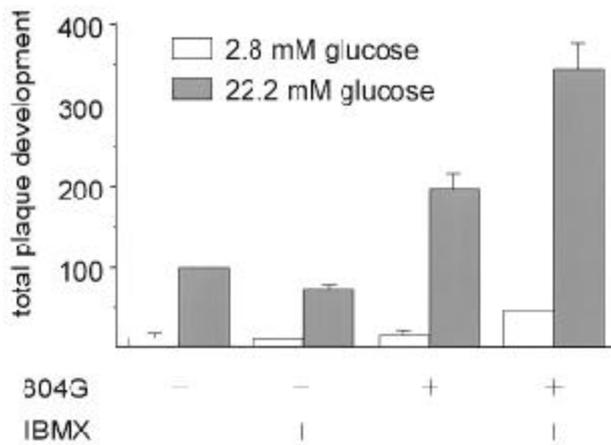
FIG. 6. IBMX potentiates the effect of glucose on insulin secretion of β-cells attached to 804G matrix. β-Cells were attached for 3 h in Cunningham's chambers coated (+) or not (–) with 804G matrix in the presence (+) or absence (–) of IBMX. Insulin secretion from single cells was evaluated by RHPA after a 1-h incubation with 2.8 or 22.2 mmol/l glucose and was expressed as total plaque development. Results (means ± SE; n = 3) are expressed relative to the secretion of β-cells stimulated by 22.2 mmol/l glucose in the absence of IBMX and 804G matrix. IBMX (0.1 mmol/l) had no effect on insulin secretion of cells attached to control glass. By contrast, on 804G matrix, IBMX increased insulin secretion stimulated with 22.2 mmol/l glucose (P < 0.02).

membrane, as expected for an adhesion receptor. Interestingly, under all conditions tested, marked heterogeneity in the intensity of labeling was evident between β-cells.

To exclude the possibility that α6β1 integrin is only expressed under in vitro conditions, frozen sections of rat pancreas were analyzed by immunofluorescence (Fig. 7J). Strong staining was apparent on both acinar and islet cells. As was observed in vitro, marked differences in labeling occurred between cells within an islet, independently of whether cells were located in the core (mostly β-cells) or at the periphery (non–β-cells) of the islet. On both acinar and islet cells, labeling was localized at the plasma membrane.

To further confirm the expression of α6β1 integrins at the surface of β-cells, sorted β-cells were surface-biotinylated, and total cell extracts were used for immunoprecipitation with the anti-α6β1 antibody. The immunoprecipitated material was analyzed by SDS-PAGE under reducing conditions: two specific bands were identified at 130 and 120 kDa apparent molecular weight, corresponding to α6 and β1 integrin subunits, respectively (Fig. 7, immunoblot lane 1).

Expression of α6β1 is influenced by insulin secretagogues. The expression of α6β1 integrin was studied by immunofluorescence of sorted β-cells maintained for 18–20 h either on 804G matrix or in suspension under various stimulating conditions. When attached on 804G at 2.8 mmol/l

FIG. 7. α6β1 integrin is expressed on rat islet β-cells. FACS-sorted β-cells: phase-contrast (A–D) and corresponding immunofluorescence (E–H) views of β-cells incubated in the presence of 11.1 mmol/l glucose and labeled by indirect immunofluorescence using a specific monoclonal antibody against α6β1 integrin. Labeling was restricted to the surface of purified β-cells and was heterogeneous: some β-cells, whether single or within aggregates, were strongly stained (E, G, and H), while others (F, G, and H) showed little if any labeling for α6β1. Bar = 12 μm. Rat islet cells in situ: phase-contrast (I) and immunofluorescence (J) views of a section of rat pancreas. α6β1 integrin is detected at the surface of pancreatic islet and acinar cells. Bar = 30 μm. Immunoblots: Triton X-100 extracts of biotin-labeled β-cells purified by FACS were immunoprecipitated with the anti-α6β1 antibody. The precipitated material was subjected to PAGE and transferred to nitrocellulose. Peroxidase-streptavidin detection of biotinylated proteins revealed two major bands, of 130 and 120 kDa, corresponding to α6 and β1 integrin subunits, respectively (lane 1). Lane 2 is a control experiment with an irrelevant mouse ascites used for immunoprecipitation.
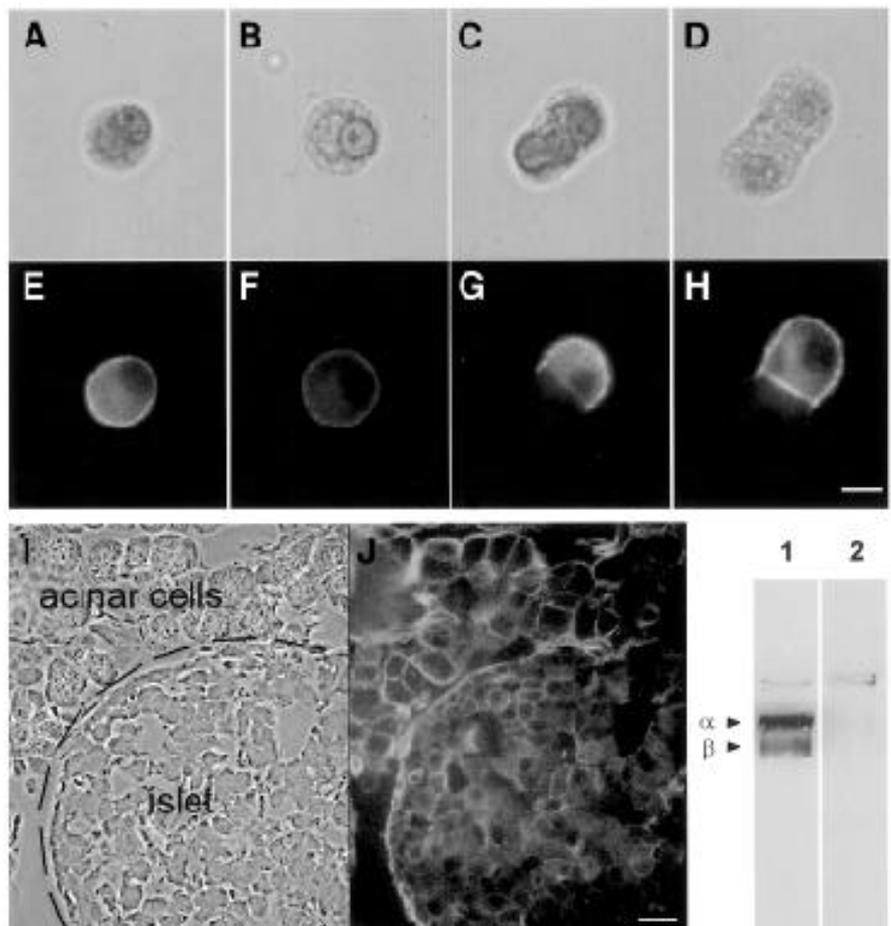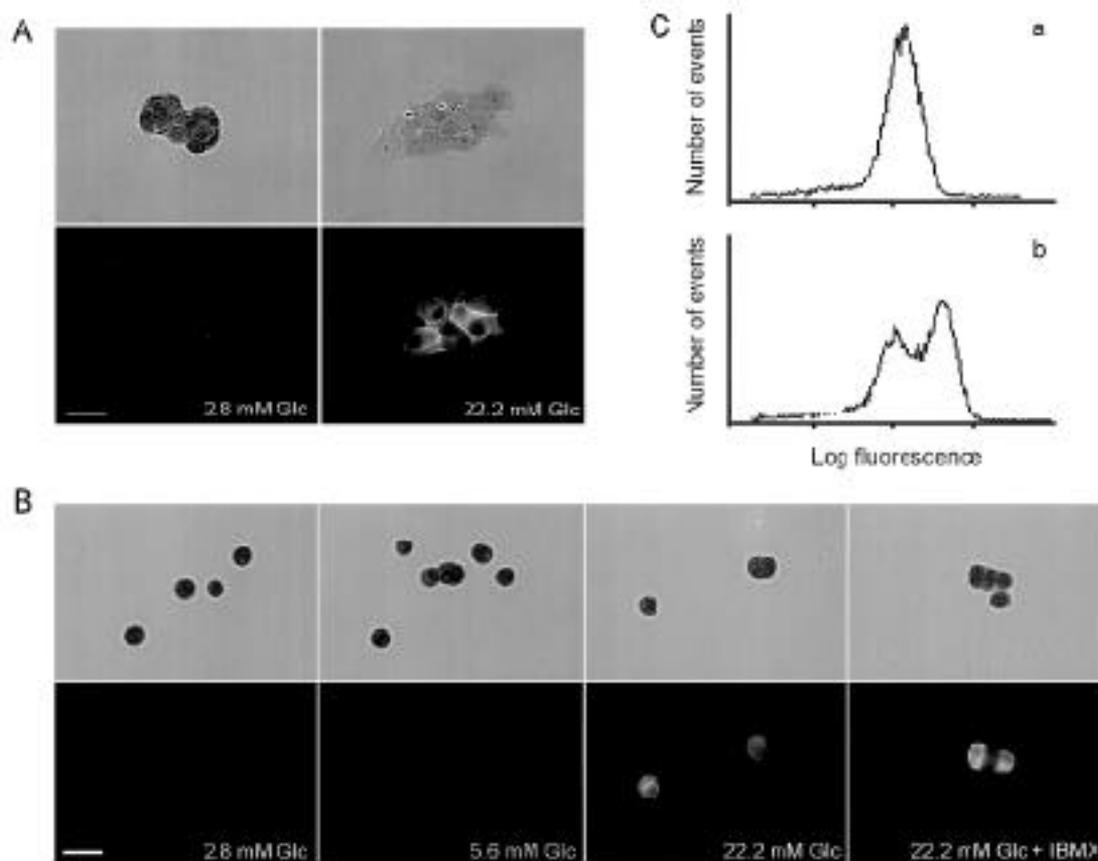
FIG. 8. Insulin secretagogues increase β-cell expression of α6β1. A: Cells attached to 804G matrix: phase-contrast (top) and immunofluorescence (bottom) views of β-cells incubated with the anti-α6β1 antibody. When incubated for 24 h in the presence of 2.8 mmol/l glucose, β-cells did not spread and show weak labeling for α6β1. At 22.2 mmol/l glucose, β-cells spread out and were strongly labeled for α6β1. B: Cells in suspension: phase-contrast (top) and α6β1 immunofluorescence (bottom) of cells incubated for 24 h in the presence of increasing concentrations of glucose ± IBMX. At 5.6 mmol/l glucose or below, very weak labeling for α6β1 is detected. At 22 mmol/l glucose, strong surface labeling is observed; surface staining was increased further with the addition of IBMX. Marked heterogeneity was nevertheless always observed in the stimulated states. This was confirmed by immunoflow cytometry of cells labeled with the anti-α6β1 antibody (C). In the presence of 5.6 mmol/l glucose, one single population of cells is detected (a). With IBMX added (b), a second population of increased α6β1 immunofluorescence is seen, representing ~50% of the cells.

glucose, 94 ± 5% of cells remained rounded, while at 22.2 mmol/l glucose 70 ± 7% spread out. At 2.8 mmol/l glucose, α6β1 labeling was weak and barely detectable (Fig. 8A). At the higher glucose concentration (22.2 mmol/l), labeling was stronger and became easily visible on many cells. Similar heterogeneity and effect of glucose were obtained in β-cells maintained in suspension (Fig. 8B).

The addition of either IBMX (Fig. 8B) or glucagon (not shown) to high glucose further increased the number of β-cells strongly positive for α6β1 integrin. Analysis by flow cytometry of purified β-cells immunostaining for α6β1 (Fig. 8C) revealed a striking rightward shift (increase in fluorescence intensity) of ~50% of purified β-cells in response to IBMX, suggesting the presence of a subpopulation of β-cells with a different threshold of response to insulin secretagogues.

Correlation between β-cell spreading and α6β1 integrin expression. To assess a putative role of α6β1 integrin on islet cell spreading and regulation of function, β-cells were attached on 804G matrix, and labeling was compared between spread and round cells. The results (Fig. 9) show that although some labeling was present on all cells, the proportion of cells with strong labeling was significantly higher for flattened (65 ± 10%) than for round cells (25 ± 8%; P < 0.01,

n = 3). A role of α6β1 integrins in β-cell attachment and/or spreading was confirmed by incubating purified β-cells on petri dishes coated with 804G matrix in the presence or absence of anti-α6β1 integrin–blocking antibodies. Under these conditions, cells remained attached to the matrix, but spreading was decreased (Fig. 10B). As expected, spreading was also inhibited by an antibody raised against the β1 integrin subunit (Fig. 10C).

The extracellular ligand is probably laminin-5, since β-cells spread with about the same kinetics on purified laminin-5 (Fig. 10F) as they do on 804G matrix (Fig. 10A and D). Furthermore, spreading on both substrates was prevented by an antibody against laminin-5 (Fig. 10E and G).

Correlation between α6β1 integrin expression and insulin secretion. To further test the correlation between spreading, α6β1 expression, and insulin secretion, secretion was studied in response to α6β1 upregulation. To this end, cells were maintained in suspension in the presence of 22.2 mmol/l glucose, supplemented with IBMX. Under these conditions, two populations of β-cells were easily distinguished under the microscope: one with slight immunostaining and the other, representing 60% of the cells, with strong staining for α6β1. Cells were then allowed 3 h to

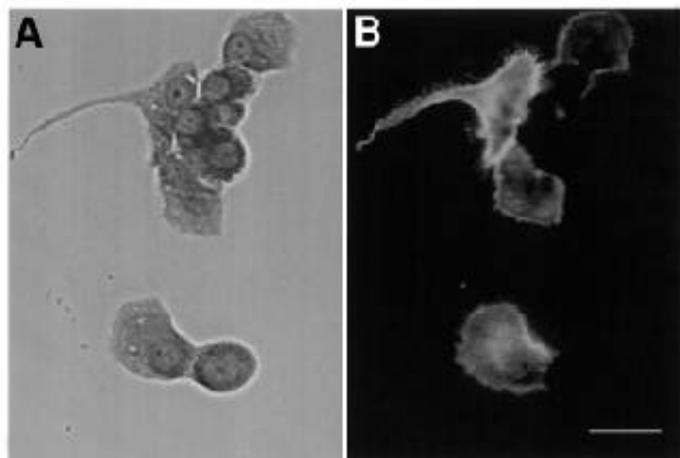FIG. 9. α6β1 labeling is increased on spread β-cells. Phase-contrast (A) and immunofluorescence (B) views of β-cells attached on 804G matrix for 24 h in the presence of 22.2 mmol/l glucose and labeled by immunofluorescence for α6β1 integrin. Direct visualization confirms that strong cell-surface labeling for α6β1 was predominantly seen on spreading β-cells compared with round cells. See text for statistical analysis of the results.

attach to 804G matrix at 2.8 mmol/l glucose, followed by a 1-h stimulation with 16.7 mmol/l glucose. At the end of the test, cells were analyzed by immunofluorescence for expression of α6β1 integrin and by RHPA for insulin secretion (Fig. 11). Total plaque development was 200 ± 100 μm^2 (n = 3) for

β-cells expressing weak labeling for α6β1 (as in the left), but reached 630 ± 80 μm^2 (n = 3) for strongly labeled cells (as in the right) (P < 0.05 compared with β-cells expressing weak labeling), thus supporting the hypothesis of a role of α6β1 signaling in the regulation of insulin secretion.

DISCUSSION

Various ECM components have been shown to improve the maintenance and function of islet cells in vitro. In the present study, we show that both the number of secreting cells and the amount of insulin secreted by individual cells in response to secretagogues are increased when cells are cultured on 804G matrix. The positive influence of the matrix appears to be related to its ability to promote islet cell spreading. Interestingly, the magnitude of spreading is under the influence of insulin secretagogues. The identification of α6β1 integrin on islet cells, the finding that anti-α6β1 antibodies reduced cell spreading, and the fact that there is a correlation between expression of α6β1, cell spreading, and insulin secretion suggest that this integrin plays an important role in the maintenance of an optimally regulated β-cell function.

Adhesion to 804G matrix. Previous studies have shown that the 804G matrix produced by a rat bladder carcinoma cell line is able to induce attachment and spreading of many epithelial cell types, including pancreatic β-cells (27,28). It is thus regarded as a good in vitro model to study the molecular basis of β-cell attachment, spreading, and signaling from the ECM. In the present study, we confirm that purified and isolated adult rat islet β-cells spread out on plastic or glass

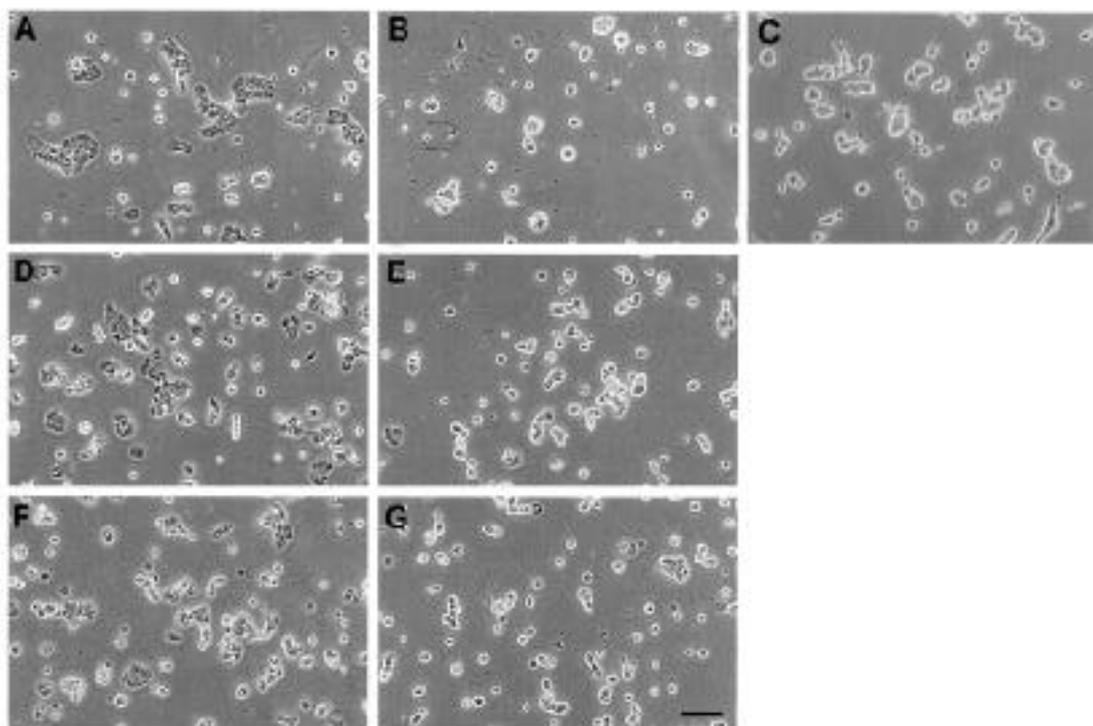FIG. 10. Laminin-5 is involved in spreading of β-cells incubated on 804G matrix. β-Cells were cultured for 3 h on either 804G matrix (A–E) or laminin-5 (F and G). Medium was either control (A, D, and F) or supplemented with anti-integrin α6β1 (1:30) (B), anti-integrin β1 (1:100) (C), or anti–laminin-5 (100 μg/ml) (E and G) antibodies. Under phase-contrast microscopy, spreading β-cells were identified by the loss of the white refringent borders that characterized round cells. A total of 150 cells were counted in two independent experiments. The 804G matrix (A and D) and laminin-5 (F) induced spreading of 75 and 68% β-cells, respectively. On 804G matrix, spreading was prevented (spreading <15%) by either anti-integrin α6β1 (B), anti-integrin β1 (C), or anti–laminin-5 (E) antibodies. On laminin, spreading was prevented by anti–laminin-5 antibodies (G). A is the control for B and C, D is the control for E, and F is the control for G. Bar = 80 μm.
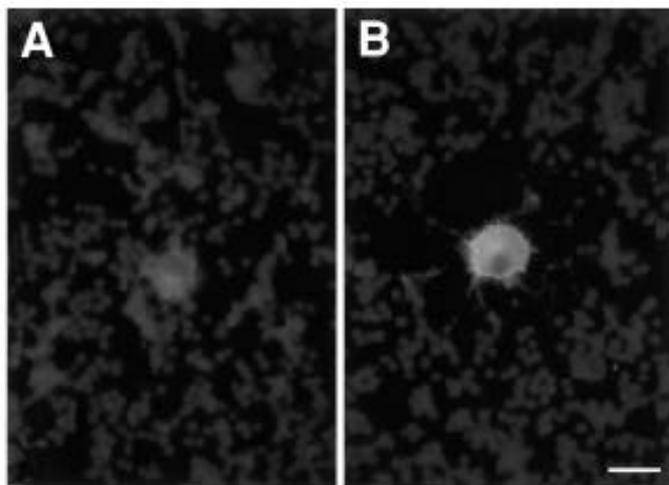
FIG. 11. Cells expressing strong labeling for $\alpha6\beta1$ secrete more insulin. β-Cells were cultured in suspension for 20 h in the presence of 0.5 mmol/l IBMX, allowed to attached to 804G matrix for 3 h, and insulin secretion was measured after 1 h of stimulation with 16.7 mmol/l glucose using the RHPA. Cells were then labeled by immunofluorescence using the anti-$\alpha6\beta1$ integrin antibody. A: Fluorescence view of a β-cell that is not surrounded by a hemolytic plaque: very weak, if any labeling for $\alpha6\beta1$ integrin is seen. B: Fluorescence view of one β-cell that is surrounded by an hemolytic plaque: strong cell-surface labeling for $\alpha6\beta1$ integrin is seen. Bar = 25 µm. For quantitative and statistical analysis, see RESEARCH DESIGN AND METHODS.

coated with 804G-conditioned medium. Because laminin-5 is the major protein produced by 804G cells, it is likely that the effects we studied on β-cells were mediated, at least in part, by laminin-5. Two observations support this hypothesis. First, laminin-5 purified from 804G-conditioned medium induced spreading of β-cells comparable to that induced by 804G matrix. Second, a monoclonal antibody against the α-chain of laminin-5 significantly decreased spreading of β-cells attached on plates coated with 804G-conditioned medium or laminin-5. On the other hand, Engelbreth-Holm-Swarm matrix used as source of laminin-1 was unable to induce spreading of β-cells, and a monoclonal antibody against laminin-1 was without effect on β-cells attached on 804G matrix or laminin-5 (not shown). Further studies will be needed to assess whether other unidentified elements of 804G matrix are able to contribute to β-cell attachment, and which ECM elements are expressed in vivo within islets of Langerhans.

Insulin secretion of β-cell attached to 804G matrix. Islets of Langerhans lose glucose responsiveness when maintained for several days in culture. It has been shown that a better preservation of an insulin response to glucose was largely dependent on the substrate used for maintaining the cells in culture (5,27,29). These studies primarily addressed the long-term (days) preservation and growth of primary islet cells in culture. Our interest was to establish the possibility of short-term (1–3 h) signaling events from the ECM to modulate the function of islet β-cells.

A prerequisite for studying the role of cell-matrix interactions on insulin secretion is to be able to measure secretion from individual cells, in order to take into account cellular heterogeneity as well as the impact of cell-cell interactions. The RHPA allows one the unique capability to study insulin secretion from either individual, doublet, or triplet cells and to eas-

ily differentiate between cell-matrix and cell-cell effects (19,23,24). Furthermore, it allows the direct correlation between a morphological and/or molecular change and insulin response to secretagogues. This approach reveals that not only the amount of insulin secreted per cell but also the number of secreting cells are rapidly increased in cells attached to ECM compared with cells seeded on plastic dishes or on poly-L-lysine.

The proportion of spread cells increased with time of culture on the 804G matrix. By the end of the 3 h necessary to complete the RHPA, cells that had spread showed an increased insulin response to glucose. Other cells displayed a phenotype indistinguishable from cells attached on glass or poly-L-lysine, and a lower sensitivity in terms of insulin response to glucose. It has been hypothesized in the past that functional heterogeneity represents a spectrum of glucose sensitivity among β-cells (19,30). The present findings of a positive effect of glucose on the number of spread cells would be in line with this hypothesis, inasmuch as spread cells secrete more insulin than round cells. Furthermore, they suggest that β-cell sensitivity can be modulated in part by cell-matrix interactions, in addition to the previously described effect of cell-cell interactions (23). Alteration of islet cell function observed in vitro is probably due not only to the loss of direct (via gap junctions) or indirect cell-cell interactions (mediated by hormones, nerve transmitters, and other molecules), but also to an inappropriate substrate unable to activate a valid outside-in signaling pathway.

Expression of $\alpha6\beta1$ integrin. The observation that the insulin secretory response of β-cells attached to the polycationic poly-L-lysine did not differ from control glass, while morphological and functional changes take place on 804G matrix, suggests that specific signaling pathways must arise between matrix and cells.

Our previous studies have shown that $\alpha3\beta1$ is a major $\beta1$ integrin expressed on islet β-cells, but that other unidentified subunits of 130 kDa are also expressed (15). $\alpha6$ is a putative candidate, but previous reports have failed to show its expression within islets (31,32). Evidence from our work for $\alpha6$ expression on islet β-cells is twofold: 1) indirect immunofluorescence shows membrane-associated staining on purified islet β-cells in vitro and on intact islets in situ; 2) immunoblots of purified β-cell extracts separated by PAGE stain a protein with the appropriate apparent molecular weight. To identify candidate β-cell matrix receptors, we searched for integrins that are heterogeneously expressed on β-cells, bind 804G matrix, and are upregulated on spread cells and in response to glucose. $\alpha6\beta1$ expression showed the expected characteristics. Thus, 1) expression was heterogeneous between purified β-cells and was upregulated by glucose; 2) spread cells showed stronger labeling for $\alpha6\beta1$, and spreading is perturbed by anti-$\alpha6\beta1$–specific antibodies; and 3) insulin response to glucose was higher in cells presenting high levels of expression of $\alpha6\beta1$. Although $\alpha3\beta1$ is the specific ligand for laminin-5 (8), a major component of 804G matrix, and is expressed at high levels on islet cells (15), its expression is homogeneous on all β-cells and is not modulated by glucose. It must, however, be kept in mind that integrin activity can be modulated independently of changes in the level of expression (33). How and to what extent $\alpha6\beta1$ is involved in the transduction of a matrix signal to modulate β-cell function is presently unknown.

Correlation between spreading, expression of **α6β1**, and insulin secretion. Several points suggest a role of α6β1 in β-cell spreading and response to glucose. First, glucose added at concentrations that induce insulin secretion increases spreading of cells on 804G matrix. This effect is increased by IBMX, a known synergist of insulin response to glucose. Second, α6β1 expression is increased, as assessed by indirect immunofluorescence, on most spread cells, compared with round cells. The correlation is not absolute, suggesting that there is a redundancy of integrins able to transduce the signal. Third, cells with strong α6β1 staining in response to an overnight incubation with IBMX have the highest acute response to glucose, while cells with little (basal) α6β1 staining do not respond to glucose. This is the first demonstration of a special phenotype (high α6β1 expression) correlating with the known heterogeneity in β-cell response to glucose. Because the correlation is not absolute, heterogeneity in insulin secretion might result from more than two phenotypes.

Taken together, the present work shows that glucose stimulation of insulin secretion in vitro is modulated by cell-matrix interactions, and it suggests that α6β1 integrin signaling pathways are involved. Whether the effect is secondary to the observed increased spreading or more direct on the regulation of secretion is not known and merits further investigations. In that regard, it is worth noting that spreading of cells increases the intracellular influx of calcium (34), which, in the case of β-cells, will stimulate insulin secretion. A positive effect of spreading on secretion is not universal, as exemplified by the secretion of albumin, which is clearly inhibited by spreading of hepatocytes (35). Because of interference with the formation of the plaque, the RHPA cannot be used to study the effect of α6β1 antibodies on secretion. On the other hand, antibodies against integrins, including α6β1 (36), were previously shown to have intrinsic effects mimicking cell attachment to the matrix (37), which would further complicate the interpretation of the results. To circumvent these difficulties, experiments aimed at blocking α6β1 mRNA translation are now under way.

The finding of a role of specific cell-matrix interactions in the regulation of insulin secretion might be of clinical relevance. First, it is tempting to speculate that the known alterations of matrix compositions in type 2 diabetes (38) might contribute, at least in part, to the decreased insulin sensitivity, due to possible interference with integrin signaling (39) and β-cell spreading (40). Second, the characterization of the molecules involved in these interactions might be of help in defining the optimal islet conditioning and appropriate host tissue for improving the success of transplantation to type 1 diabetic patients (41).

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