Glucose Stimulates the Tyrosine Phosphorylation of Crk-associated Substrate in Pancreatic β-Cells*

Robert J. Konrad‡, Gerald Gold, Thomas N. Lee, Robert Workman, Carol L. Broderick, and Michael D. Knierman

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

Several years ago, we demonstrated that glucose induced tyrosine phosphorylation of a 125-kDa protein (p125) in pancreatic β-cells (Konrad, R. J., Dean, R. M., Young, R. A., Bilings, P. C., and Wolf, B. A. (1996) J. Biol. Chem. 271, 24179–24186). Glucose induced p125 tyrosine phosphorylation in β-TC3 insulinoma cells, B-TC6,F7 cells, and in freshly isolated rat islets, whereas increased tyrosine phosphorylation was not observed with other fuel secretagogues. Initial efforts to identify p125 were unsuccessful, so a new approach was taken. The protein was purified from βTC6,F7 cells via an immunodepletion method. After electrophoresis and colloidal Coomassie Blue staining, the area of the gel corresponding to p125 was excised and subjected to tryptic digestion. Afterward, mass spectrometry was performed and the presence of Crk-associated substrate (Cas) was detected. Commercially available antibodies against Cas were obtained and tested directly in β-cells, confirming glucose-induced tyrosine phosphorylation of Cas. Further experiments demonstrated that in β-cells the glucose-induced increase in Cas tyrosine phosphorylation occurs immediately and is not accompanied by increased focal adhesion kinase tyrosine phosphorylation. Finally, it is also demonstrated via Western blotting that Cas is present in normal isolated rat islets. Together, these results show that the identity of the previously described p125 β-cell protein is Cas and that Cas undergoes rapid glucose-induced tyrosine phosphorylation in β-cells.

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‡ To whom correspondence should be addressed: Eli Lilly Corporate Center, Building 88–358C, Indianapolis, IN 46285. Tel.: 317-655-9290; E-mail: konrad_robert@lilly.com.

§ The abbreviations used are: p125, 125-kDa protein; LC, liquid chromatography; MS, mass spectrometry; SH, Src homology; KRB, Krebs-HEPES buffer; CCH, carbachol; HBSS, Hanks’ balanced salt solution; Cas, Crk-associated substrate; FAK, focal adhesion kinase.

MS/MS was used to analyze p125 prepared from βTC6,F7 cells using a method of selective immunodepletion followed by electrophoretic separation.

This approach identified p125 as Crk-associated substrate (Cas), an adapter molecule that has been reported to be tyrosine-phosphorylated in numerous other cell types but whose existence has not previously been described in β-cells (2). To confirm the MS/MS-assigned identity of p125 as Cas, monoclonal and polyclonal commercially available anti-Cas antibodies were obtained and tested directly in β-cells, confirming the glucose-induced tyrosine phosphorylation of Cas. In addition, the presence of Cas in normal rat islets was confirmed via Western blotting. Together, these results show for the first time that the previously described p125 β-cell protein is in fact Crk-associated substrate.

In other cell types, Cas functions as an adapter protein and is involved in such diverse processes as cytoskeletal organization, localization of focal adhesions, and association with focal adhesion kinase (FAK) and growth regulation (3–12). The midportion of the molecule contains at least 15 YXXP repeats that can become tyrosine-phosphorylated and function to bind Src-homology 2 (SH2) containing proteins while the N terminus contains a SH3 domain. In addition, the C terminus of Cas binds a variety of proteins including phosphatidylinositol 3-kinase (13–16). In light of the importance of Cas in other cell types, our results suggest that glucose-induced Cas tyrosine phosphorylation may be important in β-cell function.

EXPERIMENTAL PROCEDURES

βTC6,F7 Cell Culture—Cells were cultured in 10-cm or 15-cm dishes in the presence of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine. Cells were trypsinized and subcloned weekly. Media were changed three times weekly and on the day prior to an experiment. Cells were maintained at 37 °C under an atmosphere of 95% air, 5% CO2. In each experiment, preincubation and all subsequent incubations were performed at 37 °C and under an atmosphere of 95% air, 5% CO2.

Islet Isolation—Islets were isolated aseptically from Sprague-Dawley rats as described previously (1). Islets were isolated using Hanks’ balanced salt solution (HBSS) and Ficol containing 5.5 mM glucose, penicillin (25 units/ml), and streptomycin (25 units/ml). During surgery, the pancreas was inflated with 10–20 ml of HBSS. The distended pancreas was excised, and lymph nodes, fat, blood vessels, and bile ducts were removed. Tissue was chopped extensively, rinsed 5–6 times with HBSS, and digested with collagenase (2 mg/ml tissue) at 37 °C for 3 min. Digested tissue was rinsed four times with HBSS. Islets were harvested, washed once with HBSS, and washed six times in 3 ml glucose KRB (Krebs-HEPES buffer containing 25 mM HEPES, pH 7.40, 115 mM NaCl, 1 mM NaH2PO4, 24 mM NaHCO3, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2) via hand picking under a stereomicroscope prior to use.

Antibodies—PY20 anti-phosphotyrosine antibody, mouse monoclonal anti-Cas antibody, and mouse monoclonal anti-FAK antibody were obtained from Transduction Laboratories (Lexington, KY), whereas rabbit...
cells, and boiling for 5 min. Afterward, 20 μl of protein A-Trisacryl beads (Pierce) were added to the tubes and the incubation was continued overnight. At the end of the incubation, beads were washed three times with wash buffer (150 mM NaCl, 10 mM HEPES, pH 7.40, 1% Triton X-100, 0.1% SDS) and once with wash buffer 2 (10 mM HEPES, pH 7.40, 1% Triton X-100, 0.1% SDS). After the final washing step, 0.4 μl of 2% sample buffer (100 mM Tris, pH 6.80, 4% SDS, 20% glycerol, 20 μl/liter bromphenol blue, 15 mg/ml dithiothreitol) was added to each tube. Samples were vortexed, boiled for 5 min, vortexed again, and stored at −20 °C prior to subsequent analysis.

Western Blotting—Samples were loaded onto 7.5% SDS-polyacrylamide gels. Colored molecular weight markers (Amersham Biosciences) were run on each gel. Proteins were separated for 1 h at 175 V at room temperature using a Bio-Rad Mini-PROTEAN II dual slab cell. Proteins were transferred to ECL nitrocellulose paper (Amersham Biosciences) for 1.5 h (100 V, 4 °C). Nitrocellulose blots were blocked for 1 h at room temperature in blocking buffer (5% bovine serum albumin in 10 mM Tris, pH 7.40, 150 mM NaCl, 0.1% sodium azide, 0.05% Tween 20). After blocking, blots were probed with PY20 or other appropriate antibodies (in blocking buffer) for 1 h at room temperature. Blots were washed six times (5 min each) with TBST (10 mM Tris, pH 7.40, 150 mM NaCl, 0.05% Tween 20). After washing, blots were probed with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Biosciences) at a 1:1500 dilution in TBST for 1 h at room temperature. Blots were washed again as above and developed with ECL reagent (Amersham Biosciences). After air-drying, blots were exposed to BioMax x-ray film (Eastman Kodak Co.).

Purification of β-Cell p125—TC6,F7 cells grown in 15-cm dishes were stimulated for 30 min with 15 mM glucose + 0.5 mM carbachol (CCH). Afterward, cells were washed twice with ice-cold phosphate-buffered saline supplemented with 1 mM NaVO₄, 1 ml of ice-cold lysis buffer (50 mM HEPES, pH 7.40, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 30 mM Na₃P₂O₇, 1 mg/ml bacitracin, 1 mM phenylmethylsulfonylfluoride), 1 μg/ml leupeptin, 1 μg/ml aprotinin) was added to each tube. Cells were scraped into 50-ml conical tubes, vortexed, placed on ice for 15 min, and vortexed again before centrifugation at 14,000 × g for 15 min. Afterward, the supernatant was transferred to new 50-ml conical tubes, which were precleared for 2 h with 200 μl of protein A-Trisacryl beads. After preclearing, p125 was immunoprecipitated on a rocker with 5 μl of PY20 antibody. After 2 h, 10 μl of protein A-Trisacryl beads was added to the tubes and the incubation was continued overnight. At the end of the incubation, beads were pooled and transferred to a 1.5-ml Eppendorf tube, washed once with wash buffer and once with wash buffer 2. After the final washing step, 25 μl of 2% sample buffer (50 mM Tris, pH 6.80, 4% SDS, 20% glycerol, 20 μl/liter bromphenol blue, 15 mg/ml dithiothreitol) was added to the tube. The sample was vortexed, boiled for 5 min, vortexed again, and loaded onto one lane of a 7.5% SDS-polyacrylamide gel. Following one-dimensional electrophoresis as described above, the gel was stained with colloidal Coomassie Blue (Novex) and the portion of the gel corresponding to p125 was excised for LC/MS/MS analysis.

In-gel Digestion of p125—The region of interest from the gel was cut into 2-mm pieces and subjected to reduction, alkylation, and tryptic digestion on a Tecan Genesis 150 robotics platform. One hundred microliters of 50 mM dithiothreitol in 50 mM NH₄HCO₃, 50% acetonitrile was added to each of the stained gel pieces and incubated at room temperature in a 37°C water bath. The solution was aspirated, and 100 mM iodoacetamide (in 50 mM NH₄HCO₃, 50% acetonitrile) was added to the gel pieces and incubated for 15 min at 37°C. The solution was decanted, and gel pieces were washed twice with 50 mM NH₄HCO₃ in 50% acetonitrile. One hundred microliters of acetonitrile was added to the gel pieces and incubated for 5 min at room temperature. After the 5-min incubation, gel pieces were placed in an incubator for 10 min at 37°C. Ten microliters of 20 μg/ml Promega modified trypsin was added to the dry gel pieces and incubated overnight at 37°C. The following day, gel pieces were extracted with 70 μl of 0.1% trifluoroacetic acid in water. The sample was desalted on a Magic ZipTip (Millipore) by passing excess over the ZipTip twice and washing with 0.1% trifluoroacetic acid. Bound material was eluted into a 96-well 200-μl polypyrene PCR plate with 1 μl of 0.1% trifluoroacetic acid in 50% acetonitrile and diluted with 9 μl of 0.1% trifluoroacetic acid in water. The sample plate was placed in a FAMOS autosampler (LC Packings).
Glucose-induced β-Cell Cas Tyrosine Phosphorylation

**Table I**

LC/MS/MS identification of p125 as Crk-associated substrate (Cas)

Left, a gel slice containing p125 was processed as described under “Experimental Procedures.” Afterward, LC/MS/MS analysis was performed, which demonstrated the presence of peptides matching those of Cas. Shown is the MS/MS ion list for peptide TQQGQLYPAPGNQPGQSPPAK. **Bold numbers** indicate masses seen in the MS/MS spectrum. Right, shown is the MS/MS ion list for peptide GLLPNQYQEVYDTPPM*AVK. **Bold numbers** indicate masses seen in the MS/MS spectrum.

| Seq | No. | b | y | (+1) | Seq | No. | b | y | (+1) |
|-----|-----|---|---|------|-----|-----|---|---|------|
| T   | 1   | 102.1 | 2254.1 | 21 | G   | 1   | 58.0 | 2236.1 | 20 |
| Q   | 2   | 230.1 | 2153.1 | 20 | L   | 2   | 171.1 | 2179.1 | 19 |
| Q   | 3   | 358.2 | 2620.5 | 19 | L   | 3   | 284.2 | 2066.0 | 18 |
| G   | 4   | 415.2 | 1897.0 | 18 | P   | 4   | 381.3 | 1952.9 | 17 |
| L   | 5   | 528.3 | 1839.9 | 17 | N   | 5   | 495.3 | 1859.9 | 16 |
| Y   | 6   | 691.3 | 1726.9 | 16 | Q   | 6   | 623.4 | 1741.8 | 15 |
| Q   | 7   | 819.4 | 1563.8 | 15 | Y   | 7   | 786.4 | 1613.8 | 14 |
| A   | 8   | 980.4 | 1435.7 | 14 | G   | 8   | 843.4 | 1450.7 | 13 |
| P   | 9   | 987.5 | 1564.7 | 13 | Q   | 9   | 971.5 | 1383.7 | 12 |
| G   | 10  | 1044.5 | 1287.6 | 12 | E   | 10  | 1100.5 | 1285.6 | 11 |
| P   | 11  | 1141.6 | 2120.6 | 11 | V   | 11  | 1199.6 | 1136.6 | 10 |
| N   | 12  | 1255.6 | 1113.6 | 10 | Y   | 12  | 1362.7 | 1037.5 | 9  |
| P   | 13  | 1352.7 | 999.9  | 9  | D   | 13  | 1477.7 | 874.4  | 8  |
| Q   | 14  | 1400.7 | 902.5  | 8  | E   | 14  | 1578.7 | 759.4  | 7  |
| F   | 15  | 1627.8 | 774.4  | 7  | P   | 15  | 1675.8 | 658.4  | 6  |
| Q   | 16  | 1755.8 | 627.3  | 6  | P   | 16  | 1772.8 | 561.3  | 5  |
| S   | 17  | 1842.9 | 499.3  | 5  | M*  | 17  | 1919.9 | 464.3  | 4  |
| P   | 18  | 1939.9 | 412.3  | 4  | A   | 18  | 1990.9 | 317.3  | 3  |
| P   | 19  | 2037.0 | 315.2  | 3  | V   | 19  | 2090.2 | 246.2  | 2  |
| A   | 20  | 2108.0 | 218.2  | 2  | K   | 20  | 2218.1 | 147.1  | 1  |
| K   | 21  | 2236.1 | 147.1  | 1  |

LC/MS/MS Analysis of p125—LC/MS/MS analysis was performed on a LCQ deca ion trap mass spectrometer (ThermoFinnigan) fitted with a custom capillary high pressure liquid chromatography column containing Aquasil C18 packing (New Objective). Five microliters of the sample was loaded onto the column at 1.5 μl/min, and after 9 min, the flow rate was reduced to 200 nanoliters/min by opening a tee in the flow-path just above the column and running a linear gradient from 5 to 45% acetonitrile over 60 min. The LCQ deca was run in triple play mode with a custom nano electron spray ionization interface. Data from the mass spectrometer were processed for signal quality, and protein identification was performed by Sequest software (ThermoFinnigan).

Data Analysis—Films were photographed using a digital camera. Intensities of bands were quantitated with results expressed as the mean ± S.E. using the Windows-compatible version 2.98 of the program FigP (Biosoft, St. Louis, MO). Statistical analysis was performed using the same program. Data were analyzed by one-way analysis of variance followed by comparisons between the means using the least significant difference test. A probability of p < 0.05 was considered to indicate statistical significance.

RESULTS

Fig. 1A shows that βTC6,F7 cells contained p125 that undergoes increased tyrosine phosphorylation in response to stimulation of the β-cells with glucose and carbachol. The increased tyrosine phosphorylation was apparent either when crude cell lysates were used for Western blotting or when tyrosine-phosphorylated proteins were first immunoprecipitated prior to Western blotting. Fig. 1B demonstrates that glucose and the combination of glucose plus carbachol both stimulate p125 tyrosine phosphorylation.

These preliminary data demonstrated that βTC6,F7 cells are a suitable cell source for purification of p125. To purify the protein, cells were grown in 15-cm dishes and stimulated for 30 min with 15 mM glucose + 0.5 mM CCH. After preclearing, p125 was immunoprecipitated with PY20 antibody and separated electrophoretically, allowing the portion of the subsequently Coomassie Blue-stained gel to be excised and analyzed via LC/MS/MS.

LC/MS/MS analysis of the gel slice revealed the presence of peptides matching those described for a protein known as Cas, an adapter protein that has been described to be highly tyrosine-phosphorylated in many different cell types, but had not been so far described to exist in β-cells (2). The sequences of two such peptides are shown in Table I, whereas Fig. 2 shows the LC/MS/MS spectra of the actual peptides.

In light of the high quality of the MS/MS spectra obtained and the indication that Cas had been previously described as being highly tyrosine-phosphorylated in other cell types (2), commercially available monoclonal and polyclonal anti-Cas antibodies were obtained to confirm the identity of the β-cell protein as Crk-associated substrate. Fig. 3 shows that when these antibodies were used to immunoprecipitate Cas from βTC6,F7 cells, glucose dramatically increased the tyrosine phosphorylation of Cas. This increased tyrosine phosphorylation was observed using independent monoclonal and polyclonal anti-Cas antibodies.

Likewise, when PY20 antiphosphotyrosine antibody was used to immunoprecipitate tyrosine-phosphorylated proteins from β-cells, increased Cas was detected by Western blotting following glucose stimulation of the cells (Fig. 3). The increase in tyrosine-phosphorylated Cas content in PY20 immunoprecipitates was also observed with Western blotting using independent monoclonal and polyclonal anti-Cas antibodies. As expected, glucose stimulation did not affect the total amount of Cas immunoprecipitates from the β-cells as determined by immunoprecipitation of Cas followed by Western blotting with anti-Cas antibody.

As Fig. 4 (A and B) demonstrates, glucose alone induced a 180.8 ± 11.3% increase in Cas tyrosine phosphorylation (p < 0.05 compared with control) while the muscarinic agonist carbachol alone caused a 123.7 ± 11.6% increase in Cas tyrosine phosphorylation (p = 0.08 compared with control). The combination of glucose and carbachol induced a 224.2 ± 43.5% increase in Cas tyrosine phosphorylation (p < 0.05 compared with control). This increase, while significant compared with control, was not significantly greater than that caused by glucose alone. Fig. 4C shows the insulin secretion data corresponding to Fig. 4 (A and B). Glucose alone induced a 151.8 ± 17.5% increase in insulin secretion (p < 0.05 compared with control), whereas carbachol alone caused a 138.4 ± 3.5% increase in insulin secretion (p < 0.05 compared with control). The combination of glucose and carbachol induced a 365.9 ± 83.2% increase in insulin secretion (p < 0.05 compared with control).
immediate, occurring within 2 min of stimulation. Together, the data shown in Figs. 3–5 confirmed that the protein that we had previously named p125, which undergoes rapid increased tyrosine phosphorylation in \(/H9252\)-cells in response to glucose, is in fact Cas.

In light of reports in other cell types that increased Cas tyrosine phosphorylation is also accompanied by increased FAK tyrosine phosphorylation and that Cas and FAK can co-localize at focal adhesions (3–7), we examined FAK tyrosine phosphorylation in \(/H9252\)-cells. This analysis was also warranted in light of the fact that the molecular mass of FAK is 125 kDa (3–7). To determine whether FAK underwent glucose-induced tyrosine phosphorylation, \(/H9252\)TC6,F7 cells were stimulated with glucose prior to immunoprecipitation with either anti-FAK antibody or PY20 anti-phosphotyrosine antibody. Subsequent Western blotting demonstrated that FAK immunoprecipitates had no glucose-induced increase in tyrosine phosphorylation (Fig. 6). Similarly, PY20 immunoprecipitates did not demonstrate any increase in tyrosine-phosphorylated FAK content (Fig. 6), indicating that \(\beta\)-cell glucose-induced tyrosine phosphorylation of Cas is not accompanied by increased FAK tyrosine phosphorylation.

Although FAK tyrosine phosphorylation did not accompany Cas phosphorylation in \(\beta\)-cells, it was still possible that an additional substrate or substrates of similar molecular weight to Cas might be tyrosine-phosphorylated. To explore this possibility, \(/H9252\)TC6,F7 cells were stimulated with glucose and carbachol. Cas immunodepletion was performed next by incubating the immunoprecipitation lysates with protein A beads pre-adsorbed with anti-Cas antibody. Following this Cas im-

**A: MS/MS spectrum for peptide TQQGLYQAPGPQFPQSQPAK**

**B: MS/MS spectrum for peptide GLLPNQYGQEVYDTPPMAVK**

**Fig. 2.** MS/MS spectra of peptides from the \(\beta\)-cell protein p125 assign an identity of Cas. A, an LC/MS/MS experiment was carried out as detailed under “Experimental Procedures.” Shown is the MS/MS spectrum for p125 peptide-TQQGLYQAPGPQFPQSQPAK. The spectrum is labeled with b and y ions that match peptide-TQQGLYQAPGPQFPQSQPAK corresponding to residues 221–241 of Cas. B, shown is the MS/MS spectrum for p125 peptide-GLLPNQYGQEVYDTPPMAVK. The spectrum is labeled with b and y ions that match peptide-GLLPNQYGQEVYDTPPMAVK corresponding to residues 354–373 of Cas.
munodepletion, immunoprecipitation and Western blotting were performed with PY20 antibody. Fig. 7 shows the results from these experiments. Remarkably, Cas immunodepletion demonstrated that there is an additional protein of slightly higher molecular weight than Cas that also undergoes glucose and carbachol-induced tyrosine phosphorylation. We do not know the identity of this protein and are currently trying to identify it. Possibilities include a variant of Cas, a post-translationally modified form of Cas, or an unrelated protein.

Finally, the expression of Cas in normal isolated rat islets was explored via Western blotting. As Fig. 8 shows, when rabbit anti-Cas antibody was used to immunoprecipitate Cas from isolated rat islets and mouse monoclonal anti-Cas antibody was used for subsequent Western blotting, Cas was clearly observed to be present in isolated islets. It was noted that in Fig. 8, the absolute intensity of the Cas band was less than that compared with experiments with βTC6,F7 cells. This is to be expected since each islet tube contained 1000 islets, which are estimated to correlate to 2 million β-cells (assuming 2,000 β-cells per islet). In contrast, experiments with βTC6,F7 cells were performed in 10-cm dishes, which contain ~20–40 million β-cells (10–20 times as many cells).

**DISCUSSION**

The above results demonstrate that the β-cell protein p125, which undergoes glucose-induced tyrosine phosphorylation, is in fact Cas. Cas has not previously been described in β-cells. Identification of p125 as Crk-associated substrate by LC/MS/MS analysis following purification of the protein from βTC6,F7 cells was confirmed by immunoprecipitation and Western blotting with two different commercially available anti-Cas antibodies. Importantly, the presence of Cas in normal islets is also demonstrated. Interestingly, in the β-cell, glucose-induced Cas tyrosine phosphorylation was not accompanied by increased tyrosine phosphorylation of FAK. This is in contrast to some other cell types in which both molecules have been reported to map to focal adhesions and undergo increased tyrosine phosphorylation in response to the same stimuli (3–7).

These results open up a new area of β-cell investigation because Cas has not been previously described in pancreatic β-cells. In other cell types, Cas appears to function as an adapter protein and is implicated in a number of cellular responses including migration, regulation of growth, focal adhesion localization, and actin organization (8–10; for a detailed review, see Ref. 2). Cas was first described as forming complexes with v-Src and v-Crk in a tyrosine phosphorylation-de-
pendent manner and, when subsequently cloned, appeared to be particularly well suited as an adapter molecule based on its structure (11, 12). The N-terminal portion of the protein contains a SH3 domain, whereas the mid-portion of the molecule contains at least 15 YXXP repeats that can become tyrosine-phosphorylated and function to bind SH2-containing proteins (13, 14). Finally, the C terminus of Cas can also function to bind a variety of proteins, including phosphatidylinositol 3-kinase and 14-3-3 proteins (15, 16).

There appear to be several described inducers of Cas tyrosine phosphorylation in other cell types including G-protein receptor agonists such as cholecystokinin, adhesion receptors such as ICAM (intercellular adhesion molecule) and integrins, and ligands of receptor protein tyrosine kinases such as epidermal growth factor (17–24). In addition, non-receptor protein tyrosine kinases such as Src, Pyk2, and FAK have also been reported to associate with and/or tyrosine-phosphorylate Cas (25–28). Once phosphorylated, it appears that Cas is able to interact with a number of SH2-containing proteins including Src and Crk (29–32). In the latter case, Cas appears to allow Crk to interact subsequently with numerous additional proteins involved in intracellular signaling (29–32).

In the β-cell, the mechanism by which glucose stimulates increased Cas tyrosine phosphorylation is unclear. Glucose does not have a receptor and as such does not fit into any of the

![Fig. 5. Time course of Cas tyrosine phosphorylation. A, βTC6-F7 cells in 10-cm dishes were incubated with 15 mM glucose + 0.5 mM CCH (G15/CCH) for 0–30 min. Afterward, Cas was immunoprecipitated (IP) with rabbit polyclonal anti-Cas antibody. Western blotting was subsequently performed with PY20 anti-phosphotyrosine antibody and mouse monoclonal anti-Cas antibody. Results are representative of two independent experiments. B, quantitation of the time course of increased Cas tyrosine phosphorylation corresponding to A.](http://www.jbc.org/content/281/28/28121)

![Fig. 6. Glucose-induced tyrosine phosphorylation of Cas is not accompanied by increased FAK tyrosine phosphorylation. βTC6-F7 cells in 10-cm dishes were incubated with 0 mM glucose (G0) or 15 mM glucose + 0.5 mM CCH (G15/CCH) for 30 min. Afterward, tyrosine phosphorylated proteins were immunoprecipitated (IP) with PY20 antibody and FAK was immunoprecipitated with anti-FAK antibody. Subsequent Western blotting was performed with PY20 and anti-FAK antibody. Results are representative of two independent experiments.](http://www.jbc.org/content/281/28/28121)

![Fig. 7. Selective immunodepletion of Cas demonstrates an additional protein of slightly greater molecular weight is also tyrosine-phosphorylated in response to glucose. βTC6-F7 cells in 10-cm dishes were incubated with 0 mM glucose (G0) or 15 mM glucose + 0.5 mM CCH (G15/CCH) for 30 min. After the reaction was stopped, Cas was immunodepleted by overnight incubation with protein A-Trisacryl beads preadsorbed with 5 μg of rabbit polyclonal anti-Cas antibody. The following day, remaining tyrosine-phosphorylated proteins were immunoprecipitated (IP) with PY20 antibody and subsequent Western blotting was also performed with PY20 antibody. Results are representative of two independent experiments.](http://www.jbc.org/content/281/28/28121)

![Fig. 8. Cas is present in normal isolated rat islets. Expression of Cas in isolated rat islets was explored via immunoprecipitation and Western blotting. One thousand isolated islets were placed in a 13 × 100 siliconized tube, and Cas was immunoprecipitated with rabbit polyclonal anti-Cas antibody. Western blotting was then performed with mouse monoclonal anti-Cas antibody. Results are representative of two independent experiments.](http://www.jbc.org/content/281/28/28121)
categories of stimulators of Cas tyrosine phosphorylation listed above. We are currently attempting to determine which β-cell kinase or kinases tyrosine-phosphorylate Cas in response to increased extracellular glucose and how these kinases might be linked to β-cell recognition of glucose. Likewise, it is also unclear which molecules may lie downstream of tyrosine-phosphorylated Cas in the β-cell. Similarly, we are attempting to identify which β-cell signaling molecules may bind to tyrosine-phosphorylated Cas in the β-cell. Our identification of p125 as Crk-associated substrate opens up a new and exciting pathway in the β-cell.

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