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Protein Kinase B Is Expressed in Pancreatic β Cells and Activated upon Stimulation with Insulin-like Growth Factor I

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Protein kinase B (PKB) is involved in signaling to a multitude of important cellular events and is activated by insulin and growth factors, including insulin-like growth factor I (IGF-I). We show here expression of PKB in pancreatic islets and in the β cell lines HIT-T15, INS-1, and RINm5F. Expression of PKB mRNA and the presence of PKB isoforms (α, β, and γ) were assessed by Northern blot analysis and RT-PCR, respectively. Antibodies recognizing different parts of PKB isoforms were employed to demonstrate PKB protein expression by immunoblot analysis. By use of immunohistochemistry in rat and mouse pancreatic tissue sections, PKB was localized to predominantly β cells. Regulation of PKB was examined in INS-1 and RINm5F cells; upon stimulation with IGF-I (5–10 min), PKB was phosphorylated and activated (≈3-fold) by a wortmannin-sensitive mechanism, indicating involvement of phosphatidylinositol-3 kinase. The possible participation of PKB in signal transduction pathways modulating cAMP-dependent insulin secretion and in proliferation of β cells is discussed.

Serine/threonine protein kinases are involved in the initiation and modulation of insulin secretion from pancreatic β cells. While insulin release is controled by nutrients, primarily glucose, this responsiveness is modulated by circulating hormones and by paracrine neuronal factors within the islet (1). The pathways transducing these signals are incompletely characterized. Studies employing inhibitors of protein kinase A isoforms, Ca2+/calmodulin activated protein kinases and the various isoforms of protein kinase C have demonstrated that members of these kinase families are important potentiators of glucose-induced insulin secretion (2). In most of these cases, however, substrates for the kinases remain to be identified.

Recently, a signal transduction pathway involving mitogen-activated protein kinases (MAPKs) was identified in islets (3-5). Although no requirement of MAPKs in β cell stimulus-secretion coupling was demonstrated, a possible involvement of MAPKs in proliferative actions of β cells was discussed. Considering the known capacity of islets to increase their cell mass in situations of unusual insulin demands, e.g., in insulin resistant states, β cell proliferation may, in fact, be another means by which insulin secretion is increased.

Insulin-like growth factor I (IGF-I) has been proposed to elicit signals influencing proliferation of β cells (6) as well as insulin secretion (7). The components involved in this signaling have yet to be thoroughly described. Previously, an IGF-I-stimulated, wortmannin-sensitive pathway leading to attenuation of glucose-induced insulin release in the MIN6 β cell line has been reported (8), indicating involvement of the lipid kinase phosphatidylinositol 3-kinase (PI3K). Although the role of PI3K in insulin secretion has not been established, there is firm evidence for the expression of the kinase protein (9) and formation of its lipid product, phosphatidylinositol 3,4,5-trisphosphate (10), in β cells. Targets downstream of PI3K have as yet not been identified in these cells. However, in other systems a suggested target for PI3K is protein kinase B (PKB), which is known to be activated by growth factors and to have a role in prevention of apoptosis (11-13) and stimulation of differentiation (14). PKB has also been shown to be involved in insulin-controled metabolic ac-

Abbreviations used: PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; IGF-I, insulin-like growth factor; MAPK, mitogen-activated protein kinase; PH, pleckstrin homology; RT-PCR, reverse-transcription polymerase chain reaction; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid; IRS, insulin receptor substrate.

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tions, such as glucose uptake and glycogen synthesis in 3T3-L1 adipocytes and hepatocytes (15-17). Three distinct, although structurally similar, mammalian isoforms of PKB have been cloned and characterized (18), (19), the functional differences of which remain unclear. While PKBα and PKBβ seem to be ubiquitously expressed, PKBγ expression is more restricted, being most abundant in brain and testis (19). However, comparison of activation and kinetic properties demonstrated substantial similarities between all forms (20, 21). The activation mechanism proposed for PKB involves translocation to the plasma membrane and subsequent phosphorylation of Thr308 (α) (22)/Thr309 (β) (20)/Thr305 (γ) (21) and for full activity also of the C-terminally located Ser473 (α) (22)/Ser474 (β) (20). PKBγ, which due to a C-terminal truncation lacks the Ser473/474 equivalent, is thought to be fully activated by phosphorylation at Thr305 (21).

To address whether PKB is involved also in β cell signal transduction, rat islets of Langerhans and three insulinoma cell lines were investigated for expression of PKB mRNA and protein. PKB activity was studied in INS-1 cells and RINm5F cells under basal and IGF-I-stimulated conditions.

MATERIALS AND METHODS

Chemicals and antibodies. IGF-I was from Upstate Biotechnology, Lake Placid, NY, USA; Protein G-Sepharose from Pharmacia Amersham, Uppsala, Sweden; cAMP protein kinase inhibitor and wortmannin from Sigma, St Louis, MO, USA. [γ-^{32}P]ATP was synthesized as described (23). The peptide Crosstide (24) was synthesized at the Biomolecular Resource Facilities, Lund University. Cell culture media were from Life Technology.

Three antibodies directed towards different peptides of PKBα were used: anti-CT (carboxyterminal) PKB antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-PH (pleckstrin homology domain) PKB antibody from Kinetek Biotechnology (Vancouver, Canada) and anti-phospho(Ser473) antibody, which recognizes PKBα phosphorylated on serine 473, from New England Biolabs (Beverly, MA, USA). Antibodies specific for PKBβ and PKBγ, respectively, were purchased from Upstate Biotechnology.

Isolation of pancreatic islets and cell culture. Langerhans islets were isolated from Sprague-Dawley rats (B&K Universal, Stockholm) using the collagenase digestion method (25). The β cell lines HIT-T15, INS-1 and RINm5F cells were cultured in RPMI 1640 (including L-Alanyl-L-Glutamine and 11 mM glucose unless otherwise stated) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin. Medium for INS-1 cells was, in addition, supplemented with 50 μM β-mercaptoethanol.

RNA isolation, Northern blot analysis, and RT-PCR. Total RNA was isolated from isolated pancreatic islets and from cell monolayers using the RNAzol B reagent (Tel-Test Inc., Friendswood, TX). To obtain adipocyte RNA, rat epididymal adipose tissue was digested with collagenase and adipocytes prepared as described (23, 26), followed by RNA isolation according to the method of Chomczynski and Sacchi (27). For Northern blot analysis, total RNA (different amounts as stated in legend to Fig. 1) was electrophoresed under denaturing conditions and blotted to nylon membrane. Blots were probed with full length PKBα cDNA, obtained as described (28) and an oligonucleotide complementary with 18S rRNA. ^{32}P-labeled RNA was detected and quantified using digital imaging (Fujix Bas 2000).

For reverse-transcription polymerase chain reaction (RT-PCR), oligo-$(dT)_{18}$ was used to prime one strand cDNA synthesis from 1 μg of total RNA using 200 U of Moloney Murine Leukemia Virus transcriptase (Clontech). PCR amplification was performed using isoform-specific primers for PKBα (rat PKBβ, amino acids 138-145 and 459-466) and for PKBβ (rat PKBβ, amino acids 140-146 and 459-466), using the numbering of sequences published by Konishi *et al* (18). PKBγ-specific primers were from amino acids 136-142 and 449-454 of rat PKBγ sequence (19). After initial heating to 94°C for 9 min, cycling was performed for 45 sec at 94°C, 45 sec at 60°C and 1 min at 72°C (40 cycles) using AmpliTaq Gold Polymerase (Perkin-Elmer, Warrington, UK).

Immunoblot analysis. Lysates from 400 freshly isolated islets (400 μg of total protein) and from the clonal β-cells (100 μg of total protein) were mixed with SDS sample buffer and subjected to SDS-PAGE in 8% polyacrylamide. Resolved proteins were electrotransferred to polyvinylidene membranes, which were then probed either with antibodies to the three PKB isoforms (the anti-CT PKBα, the anti-PKBβ and the anti-PKBγ antibody, respectively) or, when lysate from islets were examined, a mixture of these antibodies, for detection of PKB protein. The anti-phospho(Ser473) antibody was used to detect phosphorylated (activated) PKBα. Immunoblot analysis was performed using the Super Signal (Pierce, Rockford, IL, USA) chemiluminiscent substrate.

Stimulation of INS-1 cells and determination of PKB activity. INS-1 and RINm5F cells, grown to ≈70% confluency on 10 cm plates, were incubated in medium depleted of serum and containing 3 mM glucose for 4 h prior to treatment. The cells were stimulated with IGF-I at 100 ng/ml for 5 or 10 min and then harvested in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1% $C_{13}E_{12}$ (a non-ionic detergent from the alkyl polyoxyethylene group), leupeptin (10 μg/ml), antipain (10 μg/ml and pepstatin (1 μg/ml). The cells were in some cases pretreated with 100 nM wortmannin prior to IGF-I stimulation. After preclearing the lysates with protein G-Sepharose for 30 min, PKB was immunoprecipitated using the polyclonal anti-CT antibody (see above), raised against PKBα but possibly cross-reactive with PKB β (4-8 μg of antibody was used to bring down PKB in lysates containing 400 μg of total protein). Immunoprecipitates were captured after two hours of incubation at 4°C by addition of protein G-Sepharose and incubation was continued for an additional 60 min. Precipitates were washed three times in phosphate-buffered saline, pH 7.4, supplemented with 1% $C_{13}E_{12}$ and 500 mM NaCl, and once in a kinase buffer consisting of 50 mM TES, pH 7.4, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 1 mM dithioerythritol, 40 mM phenyl phosphate, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride and antipain, leupeptin and pepstatin at the concentrations given above. To assay kinase activity, protein G-Sepharose-bound PKB (10 μl) was incubated with 5 μl of a mixture containing 150 mM TES, pH 7.5, 150 μM [γ-^{32}P]ATP (6 μCi), 40 mM $MgCl_2$, 250 mM sucrose, 4 mM dithioerythritol, 5 μM cAMP protein kinase inhibitor. Crosstide (1 μg) was used as substrate and reactions were terminated after 25 min at 30°C by addition of 10 μl of 1% bovine serum albumin, 1 mM ATP, pH 3.0 and 10 μl of 30% trichloroacetic acid. After centrifugation, 15 μl of the supernatants were applied to Whatman p81 filter paper, washed three times with 75 mM phosphoric acid and once with acetone and the ^{32}P incorporated was measured by scintillation. Phosphorylated/activated PKB was also detected, without prior immunoprecipitation, in lysates (100 μg of total protein) using the anti-phospho(Ser473) PKB antibody for immunoblot analysis (see above).

Immunocytochemistry. Pancreata were excised from sacrificed rats and mice and immersed overnight in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 7.2), followed by repeated rinsing in Tyrode's solution (10% sucrose in phosphate buffer, pH 7.2) for cryoprotection prior to freezing on dry ice. INS-1 cells and RINm5F cells were cultured on poly D lysin coated microscopic slides (Culture Slides Biocoat, Falcon, Becton

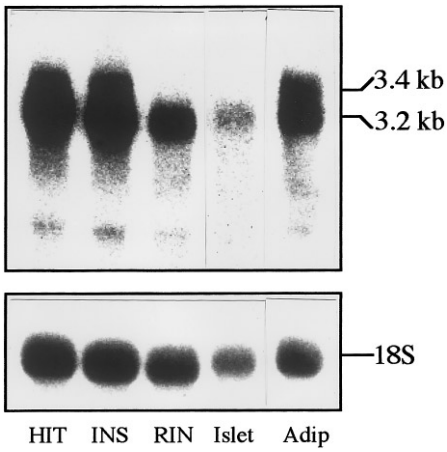

FIG. 1. Northern blot of total RNA from the β cell lines HIT-T15 (15 μg), INS-1 (15 μg), RINm5F (10 μg), from rat islets (7 μg) and rat adipocytes (10 μg). Hybridization was performed with a full-length PKBα cDNA probe (*top*) and an oligonucleotide complementary with 18S rRNA (*bottom*).

Dickinson, New Jersey, USA), fixed in ice-cold methanol for 8 min and rinsed in phosphate-buffered saline. Single and double indirect immunofluorescence were performed on cultured cells or tissue sections as described (29). For detection of PKB, the anti-CT PKBα (125 ng) and anti-PH PKB (625 ng) antibodies, respectively, were used; previously characterized antibodies (29) to insulin (9003, Euro-Diagnostica, Malmö, Sweden) and glucagon (7811, Euro-Diagnostica) were used for double immunofluorescence experiments. For specificity controls, the PKB antibodies were preabsorbed by partially purified recombinant PKB (10 μg/ml antibody at working dilution) prior to incubation of slides.

RESULTS AND DISCUSSION

To demonstrate the expression of PKB mRNA in β cells we first probed total RNA from rat pancreatic islets and the insulinoma cell lines HIT-T15, INS-1 and RINm5F with full-length PKBα cDNA in a Northern blot experiment (Fig. 1). In accordance with the size of PKBα mRNA in rat adipocytes (Fig. 1) and sizes reported from other tissues (18) a PKBα transcript of 3.2 kb was visible in the islets as well as in the cell lines. Quantification of the expression (after normalization for the different amounts of RNA applied) revealed a considerably lower ($\approx$8 times) amount of PKBα mRNA in islets than in rat adipocytes. When compared to the expression in the β cell lines, islet mRNA expression was $\approx$6 times lower than in RINm5F cells and $\approx$20 times lower than in INS-1 cells and HIT-T15 cells. The apparent transcript of $\approx$3.4 kb in adipose, HIT-T15 and INS-1 cells (Fig. 1) indicates expression of PKBβ, which previously has been identified as an mRNA species of this size in rat tissues (18). To further substantiate the expression of PKB isoforms, RT-PCR using primers specific for PKBα, PKBβ and PKBγ, respectively, was performed (data not shown). In agreement with the Northern blot results, cDNA representing

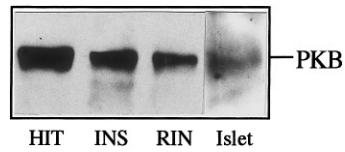

FIG. 2. Immunoblot of PKB from HIT-T15, INS-1 and RINm5F cells and from rat islets. Total lysates of the HIT-T15, INS-1 and RINm5F cells (100 μg of total protein) and 400 rat islets ($\approx$400 μg of total protein) were subjected to SDS-PAGE and immunoblot analysis with an anti-CT PKBα antibody (cell lines) and a mixture of the anti-CT PKBα, the anti-PH PKB and anti-PKBβ antibodies (islets).

PKBα was detected in rat islets and in the clonal β cells as well as in rat adipocytes. In addition, both PKBβ and PKBγ cDNAs were detected in all cell types.

We next employed immunoblot analysis to identify PKB protein in extracts from pancreatic islets and the three β cell lines. As shown in Fig. 2, in lysates from the cell lines the anti-CT-PKBα antibody recognized a protein with an apparent molecular weight of $\approx$60 kDa. The identity of this protein was confirmed by reprobing the immunoblot with the anti-PH-PKB antibody (not shown). Since lysates containing equal amounts of total protein were analysed, these results suggested that whereas similar amounts of PKB was present in HIT-T15 and INS-1 cell lysates, the RINm5F cells expressed less PKB protein. This difference in expression was independent of the antibody used and is consistent with the lower level of mRNA expression in the RIN cells (Fig. 1). To examine whether not only PKB mRNA but also protein representing PKBβ and γ was expressed, an immunoblot of lysate from INS-1 cells was probed with the antibodies recognizing the different isoforms. As shown in Fig. 3, all isoforms indeed appeared to be expressed in INS-1 cells; however, due to differences in sensitivity between the antibodies we can not estimate the relative abundance. By using a cocktail of PKB antibodies we were able to demonstrate expression of PKB, albeit at low abundance, in lysates from rat islets (Fig. 2). The reasons for the seemingly lower degree of expression in islets may be technical; although the amount of total protein in the lysate from 400 islets was higher ($\approx$400 μg) than in the lysates from the cell lines (100 μg), staining of the immunoblot

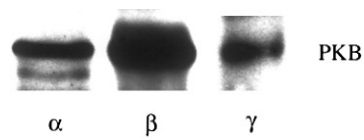

FIG. 3. Immunoblot of PKB isoforms in INS-1 cells. A total lysate from INS-1 cells was subjected to SDS-PAGE (100 μg of total protein in each lane) and immunoblot analysis using the anti-CT PKBα, anti-PKB β and anti-PKBγ antibodies, respectively.

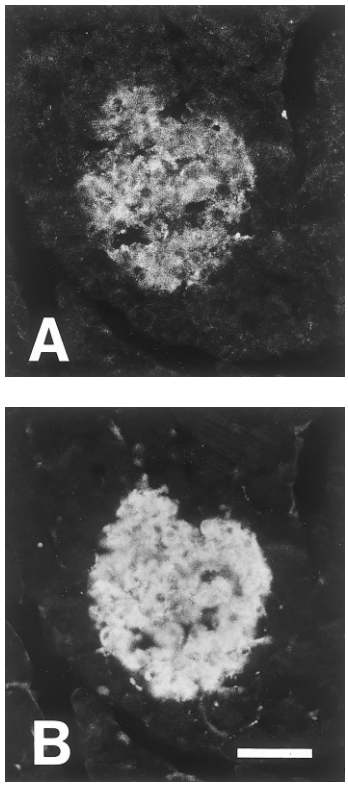

FIG. 4. Double immunofluorescence for PKB (A) and proinsulin (B) in a section of rat pancreas. Bar = 50 μm.

for protein demonstrated ≈10 times less protein in the lane containing islet lysate. Given the multitude of proteolytic activities in pancreas, degradation of PKB is thus a likely explanation for the poor signal. On the other hand, considering the known expression of PKB in mammalian cancers, [see for instance (30)]. and its suggested anti-apoptotic role [reviewed in (31)], it is also possible that PKB is upregulated in the insulinoma cells.

To localize PKB in islets, immunochemical detection using the anti-CT-PKBα antibody was performed on tissue sections from rat and mouse pancreas. Immunoreactive PKB was typically demonstrated in all islets in the sections of both rat (Fig. 4) and mouse pancreas (data not shown), but not in the surrounding exocrine tissue. INS-1 and RINm5F cells also harboured PKB immunoreactivity (data not shown); HIT-T15 cells were not examined. Furthermore, in rat islets, double immunofluorescence demonstrated that the PKB immunoreactivity predominantly localized to the β cells; additionally, also a number of glucagon cells harboured PKB immunoreactivity. Immunofluorescent detection with the anti-PH-PKB antibody demonstrated a similar localization of PKB. Further, preabsorption with partly purified recombinant PKB abolished the PKB immunoreactivity (not shown).

PKB is known to be activated by insulin and several growth factors, including IGF-I [see for instance (22)]. In this study, as a first step to study the regulation of PKB, we examined the effect of IGF-I stimulation on phosphorylation and activation of PKB in INS-1 cells and RINm5F cells. Upon stimulation (5 or 10 min) with IGF-I at a physiologically relevant concentration, PKB was activated ≈3 fold [fold activation 3.2 ± 0.5 (mean ± SEM; n=3)] (Fig. 5a). These results correlated with immunoblot analysis of total extracts using an antibody specifically recognizing PKB phosphorylated sites in PKBα (32) (Fig. 5b). Similar results were obtained upon stimulation of RINm5F cells with IGF-I (data not shown). Pretreatment of INS-1 cells (Fig.5) or RINm5F cells (not shown) with wortmannin, a selective PI3K-inhibitor, completely blocked basal activity and IGF-I-induced activation of PKB, indicating that in these cells PKB is a down-stream target for PI3K.

The significance of the IGF-I-mediated activation of PKB in β cells is not known. Because the major task

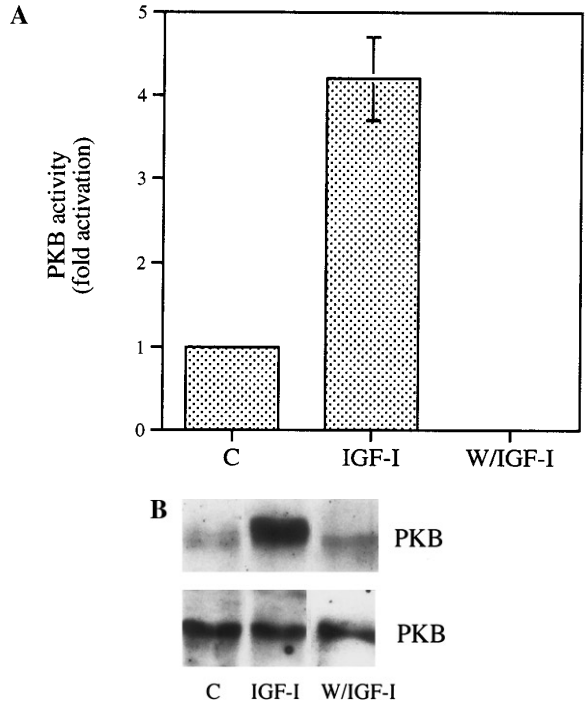

FIG. 5. Activation of PKB with IGF-I. Kinase activity (**a**) of PKB immunoisolated from lysates (200 μg of total protein) of INS-1 cells, either non-stimulated (C; control), IGF-I-stimulated (100 ng/ml for 10 min) or treated with 100 nM wortmannin for 10 min prior to stimulation with IGF-I (100 ng/ml for 10 min) (W/IGF-I). Activation is calculated relative basal activity (taken as 1) and the fold activation of PKB is shown as mean ± SEM of three independent experiments. Wortmannin treatment inhibited both IGF-I-mediated activation and basal activity of PKB. Immunoblot analysis (**b**) of PKB in aliquots of the same lysates as used in 5a. Total lysates (100 μg of total protein) were subjected to SDS-PAGE and immunoblot analysis using an anti-phospho(Ser473) PKBα antibody (*top*) and an anti-CT PKBα antibody (*bottom*).

of these cells is to produce and subsequently release insulin, the possible involvement of PKB in insulin secretion is of great interest. Data to support such a hypothesis exist inasmuch as signal transduction pathways induced by both IGF-I and insulin and involving PI3K have been reported to attenuate glucose-stimulated insulin secretion (7, 8, 33). A model for IGF-I signaling in β cells was proposed by Zhao *et al.* (33), in which increased insulin release yields, in addition to its attenuating effect on its own secretion by a negative feed-back mechanism, increased IGF-I synthesis in and release from the liver. Circulating IGF-I would then act back on the β cell to inhibit insulin secretion via activation of phosphodiesterase (PDE) 3B, which causes reduction of cellular cAMP. Accordingly, in their study (33) selective inhibitors of PDE3 blocked IGF-I-mediated attenuation of insulin secretion and thus acted as insulin secretagogues, presumably through an increase in cAMP and activation of protein kinase A. In adipocytes, the insulin-mediated phosphorylation/activation of PDE3B involves activation of PI3K (34). As PKB was recently proposed to be the kinase phosphorylating PDE3B in adipocytes (28, 35) it is conceivably that PKB plays a similar role in the β cell.

Another possible role of PKB in β cells became evident through the recently published report describing the knock-out of IRS-2 in the mouse (36). Such mice develop insulin resistance but lack the normal capacity of pancreatic β cells to compensate for hyperglycemia through proliferation, highlighting of a possible new role for IRS-2-dependent signaling. While both PKB and PI3K have been implicated in signal transduction mediating mitogenesis and prevention of apoptosis, it remains to be addressed whether they could be part of such an IRS-2-mediated pathway in β cells.

In summary, we have demonstrated the expression of PKB isoforms in pancreatic β cells and, in addition, a wortmannin-dependent activation of PKB upon stimulation with IGF-I. Although we do not as yet have data regarding the function of PKB in β cells, given the versatility of PKB we speculate both on a role for the kinase in signal transduction pathways influencing insulin secretion and in neogenesis of β cells.

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REFERENCES

1. Holz, G. G., and Habener, J. F. (1992) *Trends Biochem. Sci.* **17,** 388–393.
2. Persaud, S. J., Jones, P. M., and Howell, S. L. (1994) *in* Frontiers of Insulin Secretion and Pancreatic B-Cell Research (Flatt, P. R., and Lenzen, S., Eds.), pp. 251–256, Smith-Gordon, London.
3. Persaud, S. J., Wheeler-Jones, C. P., and Jones, P. M. (1996) *Biochem. J.* **313,** 119–124.
4. Khoo, S., and Cobb, M. H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5599–5604.
5. Benes, C., Roisin, M. P., Van Tan, H., Creuzet, C., Miyazaki, J., and Fagard, R. (1998) *J. Biol. Chem.* **273,** 15507–15513.
6. Zhang, Q., Berggren, P. I., Hansson, A., and Tally, M. (1998) *J. Endocrinol.* **156,** 573–581.
7. Van Schravendijk, C. F., Heylen, L., Van den Brande, J. L., and Pipeleers, D. G. (1990) *Diabetologia* **33,** 649–653.
8. Hagiwara, S., Sakurai, T., Tashiro, F., Hashimoto, Y., Matsuda, Y., Nonomura, Y., and Miyazaki, J. (1995) *Biochem. Biophys. Res. Commun.* **214,** 51–59.
9. Gao, Z., Konrad, R. J., Collins, H., Matschinsky, F. M., Rothenberg, P. L., and Wolf, B. A. (1996) *Diabetes* **45,** 854–862.
10. Alter, C. A., and Wolf, B. A. (1995) *Biochem. Biophys. Res. Commun.* **208,** 190–197.
11. Kulik, G., Klippel, A., and Weber, M. J. (1997) *Mol. Cell Biol.* **17,** 1595–1606.
12. Marte, B. M., and Downward, J. (1997) *Trends Biochem. Sci.* **22,** 355–358.
13. Eves, E. M., Xiong, W., Bellacosa, A., Kennedy, S. G., Tsichlis, P. N., Rosner, M. R., and Hay, N. (1998) *Mol. Cell Biol.* **18,** 2143–2152.
14. Magun, R., Burgering, B. M., Coffer, P. J., Pardasani, D., Lin, Y., Chabot, J., and Sorisky, A. (1996) *Endocrinology* **137,** 3590–3893.
15. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem.* **271,** 31372–31378.
16. Peak, M., Rochford, J. J., Borthwick, A. C., Yeaman, S. J., and Agius, L. (1998) *Diabetologia* **41,** 16–25.
17. Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B. M., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1998) *J. Biol. Chem.* **273,** 5315–5322.
18. Konishi, H., Shinomura, T., Kuroda, S., Ono, Y., and Kikkawa, U. (1994) *Biochem. Biophys. Res. Commun.* **205,** 817–825.
19. Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y., Kameyama, K., Haga, T., and Kikkawa, U. (1995) *Biochem. Biophys. Res. Commun.* **216,** 526–534.
20. Meier, R., Alessi, D. R., Cron, P., Andjelkovic, M., and Hemmings, B. A. (1997) *J. Biol. Chem.* **272,** 30491–30497.
21. Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P., and Alessi, D. R. (1998) *Biochem. J.* **331,** 299–308.
22. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *Embo J.* **15,** 6541–6551.
23. Chang, K. J., Marcus, N. A., and Cuatrecasas, P. (1974) *J. Biol. Chem.* **249,** 6854–6865.
24. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* **378,** 785–789.
25. Lacy, P. E., and Kostianovsky, M. (1967) *Diabetes* **16,** 35–39.
26. Honner, P. C., Dhillon, G. S., and Londos, C. (1985) *J. Biol. Chem.* **260,** 15122–15129.
27. Chomczynski, P., and Sacci, N. (1987) *Anal. Biochem.* **162,** 156–159.

28. Wijkander, J., Holst, L. S., Rahn, T., Resjo, S., Castan, I., Manganiello, V., Belfrage, P., and Degerman, E. (1997) *J. Biol. Chem.* **272,** 21520–21526.
29. Mulder, H., Lindh, A. C., and Sundler, F. (1993) *Cell Tissue Res.* **274,** 467–474.
30. Hausler, P., Papoff, G., Eramo, A., Reif, K., Cantrell, D. A., and Ruberti, G. (1998) *Eur. J. Immunol.* **28,** 57–69.
31. Alessi, D. R., and Cohen P. (1998) *Curr. Opin. Genet Dev.* **8,** 55–62.
32. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) *J. Biol. Chem.* **272,** 31515–31524.
33. Zhao, A. Z., Zhao, H., Teague, J., Fujimoto, W., and Beavo, J. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3223–3228.
34. Rahn, T., Ridderstrale, M., Tornqvist, H., Manganiello, V., Fredrikson, G., Belfrage, P., and Degerman, E. (1994) *FEBS Lett.* **350,** 314–318.
35. Wijkander, J., Landstrom, T. R., Manganiello, V., Belfrage, P., and Degerman, E. (1998) *Endocrinology* **139,** 219–227.
36. Withers, D. J., Sanchez Gutierrez, J., Towery, H., Burks, D. J., Ren, J.-M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., Bonner-Weir, S., and White, M. F. (1998) *Nature* **391,** 900–903.