Glucose induces early growth response gene (Egr-1) expression in pancreatic beta cells

K. Josefsen[1], **L. R. Sørensen**[1], **K. Buschard**[1], **M. Birkenbach**[2]

[1] Bartholin Institutet, Kommunehospitalet, Copenhagen, Denmark
[2] Majorie B. Kovler Viral Oncology Laboratory, University of Chicago, Chicago, Illinois, USA

Summary A copy deoxyribonucleic acid (cDNA) clone of the immediate early growth response gene, *egr-1* (Krox-24, Zif268, NGFI-1), was isolated through subtractive hybridization screening to identify glucose-induced genes in pancreatic beta cells. Glucose rapidly and transiently induced *egr-1* mRNA in the SV40-transformed murine beta-cell line, MIN6. Glucose also increased *egr-1* mRNA expression in INS-1, βTC3 and RINm5F beta-cell lines, although with different kinetics. Expression of the 82 kDa Egr-1 protein was induced both in MIN6 cells stimulated with glucose in vitro and in primary rat islet cells stimulated in vivo or in vitro. This response is unique to beta cells since glucose did not affect *egr-1* expression in NIH-3T3 fibroblasts or glucose-sensitive hepatocytes. In beta cells *egr-1* induction is specifically associated with insulin secretion, as it was not observed after stimulation with serum or insulin but was elicited by insulin secretagogues, including membrane depolarizing agents and cAMP agonists. Moreover, induction of *egr-1* by glucose was inhibited by EDTA, indicating dependence on influx of extracellular Ca^{2+}. Other immediate early response genes, *c-fos* and *junB,* were also induced following glucose stimulation with kinetics similar to *egr-1*, whereas *c-jun* and *junD* expression were not affected. Since the zinc-finger protein encoded by *egr-1* is highly homologous to transcription factors that control expression of glucose-regulated genes in yeast, Egr-1 could mediate delayed adaptive responses of beta cells to sustained glucose stimulation through transcriptional regulation. [Diabetologia (1999) 42: 195–203]

Keywords Glucose stimulation, beta-cell line, MIN6, transcription factor, Egr-1.

Glucose homeostasis is maintained by beta cells of pancreatic islets through precisely regulated release of insulin from preformed granules. To maintain constant intracellular insulin stores while meeting wide physiological variations in demand, insulin synthesis is modulated in parallel with secretion rates [1]. Immediately following glucose stimulation, synthesis is primarily augmented through enhanced translation [2] and decreased degradation of existing insulin mRNAs [3]. Glucose also induces delayed responses that are manifested several hours after stimulation [4] by increasing insulin gene transcription [5] and expression of other genes involved in beta-cell function. Increased gene expression of glucose transporter 2 (GLUT-2) [6], pyruvate kinase [7], acetyl-coenzyme A-carboxylase [8], and 64 kDa glutamic acid decarboxylase (GAD65) [9] have been observed in primary rat islets in vitro and in rodent insulin-producing tumour cell lines following exposure to glucose. Together these changes comprise a delayed adaptive response that could be necessary to meet increased metabolic and secretory demands during extended or repeated periods of hyperglycaemia.

Mechanisms regulating glucose-activated transcription are only partly understood. Immediate glucose effects on insulin transcription in rodents have been attributed to glucose-dependent activation of the constitutively expressed islet-cell-specific transcription factor, insulin promoter factor 1 (IPF-1) and factors that bind E1 and C1 control elements [10–12]. Activity of the insulin upstream factor 1 (IUF1) complex, which may comprise the human equivalent of rodent IPF-1, is also glucose-dependent [13]. Increased expression of acetyl-coenzyme A-carboxylase results in part from glucose-induced binding of the ubiquitous stimulating protein 1 (Sp1) factor to glucose-response promoter elements [14]. Factors regulating glucose-induced transcription of most other genes, however, are still largely not known.

The relevance of glucose-regulated transcription has not been directly shown. Considerable evidence implicates, however, altered gene expression and transcription factor activity in beta-cell dysfunction. Loss of IPF-1 in hyperglycaemic rats correlates closely with diminished beta-cell insulin secretion in response to a glucose challenge [15]. Similarly, prolonged in vitro propagation of HIT-T15 cells in a high glucose medium results in reduced responsiveness which has been attributed to loss of IPF-1 expression [16]. Development of diabetes in Zucker diabetic fatty (ZDF) rats is associated with reduced expression of the transcription factor ISLET-1 [17], as well as several other genes, including glucokinase, mitochondrial glycerol-3-phosphate dehydrogenase, voltage-dependent Ca^{2+}- and K^+-channels, Ca^{2+}-ATPase and GLUT-2. Together these results suggest that the ability to modulate transcription in response to glucose could be necessary for normal beta-cell function.

To investigate further the effects of glucose on beta-cell gene transcription we have used subtraction cloning to identify genes induced by glucose stimulation. Using this approach, we have isolated a clone encoding the zinc-finger transcription factor Egr-1. Experiments reported here indicate that early growth response gene-1 (*egr-1*) expression is specifically and transiently upregulated in beta-cell lines and primary islet cells in response to glucose and other secretagogues. This is the first time specific induction of transcription factor expression in response to glucose has been shown in beta-cells. In view of its close homology with transcription factors that regulate carbohydrate metabolism in yeast, Egr-1 could mediate other delayed transcriptional changes and play a pivotal role in adaptive responses to sustained glucose stimulation. Moreover, these results suggest that target genes transcriptionally regulated by Egr-1 could be important in normal beta-cell function.

Materials and methods

Reagents. Thyrotropin, thyroid stimulating hormone (TSH) was from Armour Pharmaceuticals (Rhône Poulenc, Courbevoie, France), insulin a gift from Novo Nordisk (Bagsvœrd, Denmark) and Rp-cAMPS from BioLog Life Science Institute (Bremen, Germany). Phorbol 12-myristate 13-acetate (TPA), EDTA, ionomycin, isobutylmethylxanthine (IBMX), forskolin, arginine, rA · rU, dextran sulphate and okadaic acid were from Sigma (St. Louis, MO., USA).

Cell line cultures. The SV40-transformed murine beta-cell lines, MIN6 [18] and βTC3 [19] were cultured in 90% DMEM (22.5 mmol/l glucose) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Gaithersburg, MD., USA). Hamster HIT-T15 [20], rat RINm5F [4] and INS-1 [19] insulinoma cell lines were maintained in 90% RPMI 1640 (11 mmol/l glucose), 10% FBS with necessary additives [19]. 3T3 fibroblasts (ATCC) were grown in 90% DMEM (22.5 mmol/l glucose), 10% donor calf serum (Life Technologies). Human HepG2 hepatoma cells were grown in 90% RPMI 1640, 10% FBS. All cells were routinely tested for mycoplasma and were consistently negative.

Primary cell cultures. Primary islets of Langerhans were isolated from adult male Lewis rats by standard collagenase digestion. For in vitro stimulation experiments, islets were initially incubated for 24 h in 90% RPMI 1640 (11 mmol/l glucose), 10% FBS, followed by 24 h in 90% glucose-free-RPMI, 10% dialysed FBS, supplemented with 2.8 mmol/l glucose. Islets were either stimulated for 90 min by addition of glucose to 20 mmol/l, or were left for an additional 90 min in a low glucose medium (unstimulated control). For in vivo stimulation studies, male Lewis rats were injected intravenously with 2 ml of either 1 mol/l glucose or saline after overnight starvation. After 90 min, both stimulated and unstimulated animals were injected with cycloheximide (5 mg/kg, i. p.). Islets were isolated 30 min later. Primary rat liver cells, obtained by in situ collagenase digestion, were grown in defined medium with or without insulin [21]. Human thyroid cells were obtained as surgical specimens of healthy gland adjacent to non-toxic thyroid adenomas and seeded as monolayers in RPMI 1640 containing growth factors (insulin, somatostatin, transferrin, hydrocortisone and glycyl-histidyl-lysine acetate) [22]. After 48 h in vitro culture, cells were stimulated for 30 min with 1 U/l TSH.

RNA isolation and characterization. The MIN6, HIT, RIN or INS-1 cells were glucose deprived (1, 0.1, 0.01 and 1 mmol/l, respectively) for 48 h and stimulated in a high glucose medium (20 mmol/l) for 30 min. Alternatively, they were starved for 24 h and stimulated for 24 h. Stimulation was assessed by insulin RIA of culture supernatants. After washing with PBS, cells were lysed in situ in acid guanidinium phenol [23], and RNA was isolated after chloroform extraction. Total cellular RNA (7 μg/lane) was fractionated on 1% agarose gel, and transferred to activated nylon membranes (GeneScreen Plus, New England Nuclear Research Products, Boston, MA., USA). The RNA blots were hybridized overnight with ^{32}P-labelled probes. Autoradiographs were quantified by transmission densitometry with a HP4 scanner (Hewlett Packard, Palo Alto, Calif., USA) and SigmaGel software (Jandel Scientific, San Rafarel, CA, USA). The latter method was linear over a range of at least tenfold differences in autoradiographic band intensity.

Subtractive cDNA cloning. Subtracted ^{32}P-labelled cDNA probes were synthesized from 2 μg polyadenylated RNA [24] from glucose-stimulated or unstimulated MIN6 cells using Su-

perscript reverse transcriptase (BRL) and 18.5 MBq ^{32}P-dCTP in each reaction. After alkaline hydrolysis of RNA templates, copy deoxyribonucleic acid (cDNA) probes were hybridized at 65 °C with 20 μg biotinylated mRNA from unstimulated cells (Photobiotin, Vector Laboratories, Burlingame, CA, USA) for 36 h. Biotin-RNA: ^{32}P-cDNA duplexes were removed with streptavidin. Residual single-stranded ^{32}P-cDNA was used to screen a lambda gt10 bacteriophage cDNA library constructed from stimulated (20 mmol/l glucose) MIN6 cell RNA. Duplicate filters were hybridized with probes from stimulated or unstimulated cells, respectively, at 42 °C for 48 h in hybridization buffer [50 % formamide, 6X SSPE (0.9 mol/l NaCl, 60 mmol/l NaH$_2$PO$_4$, 6 mmol/l EDTA, pH 7.4), 10 % dextran sulphate, 1 % SDS, 2 × Denhardt, 100 μg/ml herring testes DNA and 16 μg/ml rA · rU). Filters were washed with three high stringency washes for 20 min each at 65 °C in 0.2 × SSC (30 mmol/l NaCl, 3 mmol/l C$_6$H$_5$O$_7$Na$_3$, pH 7.0), 1 % SDS, 0.05 % sodium pyrophosphate, and autoradiographed for 3 days. Plaques detected more strongly with the stimulated cell probe than the control probe were selected for further characterization. cDNA inserts were subcloned into pBluescript (Stratagene, La Jolla, CA, USA) and sequenced by the dideoxy-nucleotide chain termination method.

Immunoblotting. Cells were rinsed in PBS, lysed in 2 × SDS loading buffer (125 mmol/l TRIS pH 6.8, 20 % glycerol, 4 % SDS, 10 % 2-mercaptoethanol) and electrophoresed on 8 % SDS-polyacrylamide gel. Proteins were electroblotted to nitrocellulose, incubated with affinity-purified Egr-1 antibody (588 or C-19, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 dilution for 1 h, and made visible by autoradiography using ^{125}I-protein A, or by enhanced chemiluminescence (Supersignal Substrate, Pierce, Rockford, Ill., USA) using horseradish peroxidase-conjugated swine-anti-rabbit secondary antibody (1:1000, Dako, Glostrup, Denmark).

Results

We used subtractive hybridization cloning to identify genes induced by glucose stimulation of the transformed murine beta-cell line, MIN6. These cells closely resemble primary beta cells in several respects [25]. Most importantly, MIN6 cells: (1) synthesize and secrete fully processed insulin in response to physiologic concentrations of glucose (2–25 mmol/l) [18, 25, 26]; (2) regulate proinsulin synthesis primarily at the translational level [27]; (3) express the GLUT-2 glucose transporter characteristic of islet beta cells and fail to express inappropriately the low Km 'brain-type' transporter [28]; (4) predominantly use glucokinase to catalyse glucose phosphorylation [28].

Subtracted, ^{32}P-labelled cDNA probes enriched for sequences complementary to mRNAs unique to stimulated cells, were used to screen a glucose-stimulated MIN6 cell cDNA library. Out of 66 000 recombinant clones screened, 53 representing six different genes were isolated. Four genes encoded enzymes involved in glucose metabolism: triose phosphate isomerase; phosphoglycerate kinase; lactate dehydrogenase; and enolase. Several clones encoding preprodynorphin were isolated [28a]. A single clone encoded
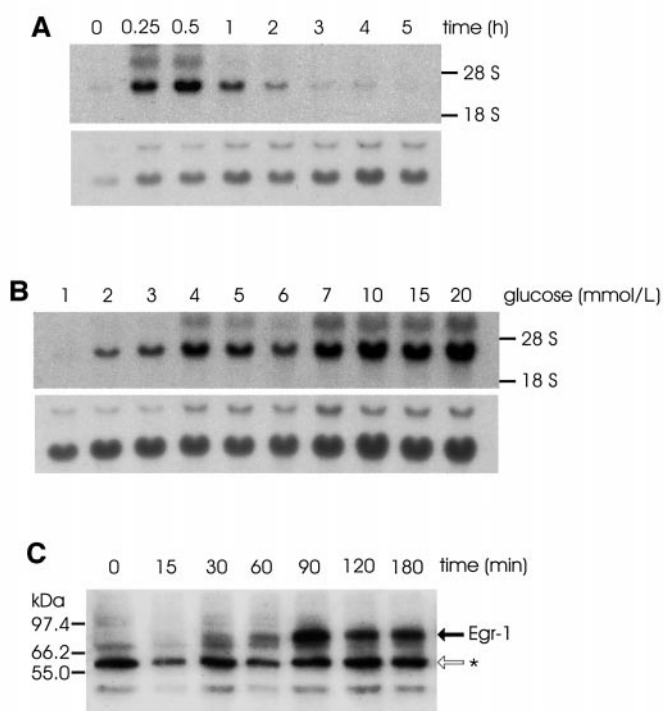
Fig. 1 A–C. Induction of early growth response gene-1 by glucose in MIN6 cells. **A** Time-course of *egr-1* mRNA response. After 48 h glucose-deprivation, cells were stimulated by incubation in medium containing 20 mmol/l glucose and harvested after the indicated times. RNA was extracted, fractionated on agarose gel and hybridized with ^{32}P-labelled murine *egr-1* cDNA (upper panel). Positions of rRNA bands are indicated (18 S, 28 S). **B** Magnitude of *egr-1* induction is dependent upon stimulatory glucose concentration. After 24-h glucose deprivation, MIN6 cells were incubated in medium containing the indicated glucose concentrations for the next 24 h (upper panel). Lower panels in **A** and **B** show respective blots rehybridized to human β-actin cDNA probe. **C** Egr-1 protein induction. MIN6 cells were glucose-deprived for 24 h, stimulated with 20 mmol/l glucose at t = 0 and harvested after the indicated times. Proteins were analysed by immunoblot using anti-Egr-1 antibody. Positions of molecular mass standards are indicated on the left. Egr-1 is detected as an 82 kDa protein which first appears at 30 min post-stimulation. A 59 kDa protein uniformly expressed at all time points is a cross-reactive molecule (*). For results used for quantification in this and the following figures the number of experiments are indicated in Table 1. All other results were confirmed in at least one independent experiment

the zinc-finger transcription factor *egr-1* (also called Krox-24, Zif268, NGFI-1; Genbank M28 845).

Glucose stimulation of beta cells induces expression of egr-1. Early passage MIN6 cells maintained 48 h in low glucose (1.0 mmol/l) medium expressed low or undetectable levels of *egr-1* mRNA (Fig. 1A). Within 15 min after stimulation with 20 mmol/l glucose, however, *egr-1* mRNA expression became detectable and achieved maximum by 30 min. Expression gradually declined thereafter, returning to baseline by 3 h post-stimulation (Fig. 1A). Despite continous glucose stimulation, the *egr-1* mRNA signal did not return

during the time interval studied (24 h). The kinetics of *egr-1* mRNA accumulation in MIN6 cells after glucose stimulation are identical to those reported previously after anti-μ activation of murine B lymphocytes and serum stimulation of Balb/c 3T3 fibroblasts [29]. Maximum *egr-1* RNA induction in several experiments averaged over 50-fold as determined by scanning densitometry of autoradiographs.

The magnitude of *egr-1* mRNA induction increased in proportion to the stimulatory glucose concentration over a physiologic range from 2 to 20 mmol/l (Fig. 1B). Previous studies with MIN6 cells [18] and our own observations (data not shown) have indicated graded insulin release over a similar range of glucose concentrations. At high passage numbers (> 40), MIN6 cells exhibited reduced insulin release in response to glucose and failed to increase *egr-1* mRNA expression (data not shown). Together these results suggest that *egr-1* induction could be coupled to physiologic mechanisms that regulate specific beta-cell responses to glucose. Importantly, insulin released from stimulated cells was not responsible for this effect, as induction of *egr-1* was not detected after addition of exogenous human insulin (see below).

Since Egr-1 expression in many settings is regulated at the translational level [30, 31] and expression of several genes in beta cells, including insulin [5, 27] and glucokinase [32], is largely regulated translationally, we examined Egr-1 protein expression in glucose-activated cells by immunoblotting using a rabbit polyclonal anti-Egr-1 antibody. The 82 kDa Egr-1 protein accumulated in glucose-activated MIN6 cells with similar but slightly delayed kinetics relative to changes in *egr-1* RNA (Fig. 1C). We detected Egr-1 30 min after the shift to high glucose medium, but it did not reach maximum until 90 min post-stimulation. It persisted beyond 3 h, by which time *egr-1* RNA levels had already returned to baseline. A similar time course of Egr-1 protein induction has been observed following serum stimulation of Balb/c-3T3 fibroblasts [33].

Glucose also induced *egr-1* in other insulin-producing cell lines and in primary pancreatic islets. In glucose-responsive rat INS-1 beta cells, *egr-1* mRNA increased within 30 min after stimulation (Fig. 2A). In murine βTC-3 beta cells, 30-min stimulation with glucose failed to increase *egr-1* expression above basal level. Cells cultivated continuously in high glucose medium, however, expressed *egr-1* mRNA at high levels compared with 48-h glucose deprivation (Fig. 2A, and data not shown). The RINm5F rat and late-passage HIT-T15 hamster beta-cell lines are generally regarded as non-responsive or responsive in a non-physiological manner [25, 26]. We found RINm5F cells showed weak induction after 30 min stimulation or prolonged cultivation in a high glucose medium and HIT-T15 cells were totally unresponsive (Fig. 2A). Insulin RIA measurements of 24-h culture
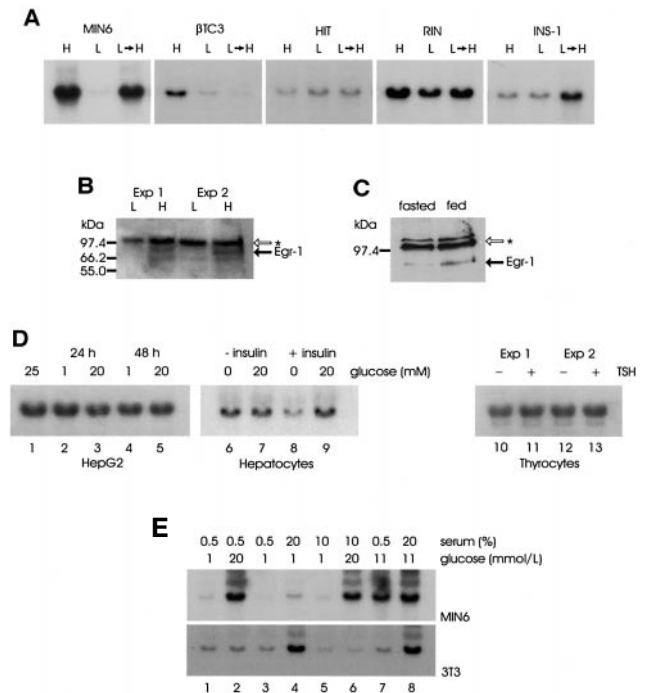
Fig. 2 A–E. *egr-1* mRNA expression in beta- and non-beta-cells. **A** Expression of *egr-1* mRNA in beta-cell lines MIN6, βTC3, HIT-T15, RINm5F and INS-1 after continuous cultivation in high glucose (20 mmol/l) medium (H), 48-h glucose-deprivation (L), and 30-min glucose stimulation after glucose-deprivation (L- > H). Glucose induced *egr-1* mRNA expression in glucose-responsive MIN6, INS-1, βTC3 and RINm5F cells but minimally affected expression in non-responsive late passage HIT-T15 cells. Loading of equal amounts of RNA was verified by β-actin hybridization (not shown). **B** Egr-1 protein expression in primary rat islets of Langerhans ex vivo. After 24 h in vitro glucose-deprivation, two separate islet cell preparations (Exp 1 and Exp 2, respectively) were stimulated with glucose (20 mmol/l) for 90 min (H), or were left unstimulated in low glucose (2.8 mmol/l) medium (L). Expression of the 82 kDa Egr-1 protein is detected only in stimulated cells. The position of a previously described 105 kDa cross-reactive protein, present in all lanes, is indicated (*). **C** Egr-1 protein induction in vivo. Islets of Langerhans were isolated after cycloheximide infusion from rats fasted over night and injected with either glucose or saline. The 105 kDa cross-reactive protein is indicated (*). **D** Expression of *egr-1* mRNA was not affected by glucose stimulation after glucose deprivation of the human liver cell line HepG2 or of primary rat hepatocytes, or after TSH (1 U/l) stimulation of primary human thyroid cells (actin control hybridization showed a reduced amount of RNA in lane 8). **E** Egr-1 induction in MIN6 cells is glucose-dependent and serum-independent. *Egr-1* expression in glucose-deprived MIN6 cells was induced by stimulation with glucose (lanes 2, 6) relative to unstimulated controls (lanes 1, 5) under both low serum (0.5 %, lanes 1, 2) and high serum (10 %, lanes 5, 6) conditions. *Egr-1* expression was not significantly induced in serum deprived MIN6 cells stimulated with 20 % serum (lanes 4, 8) relative to unstimulated controls (0.5 % serum, lanes 3, 7) under either low (1 mmol/l, lanes 3, 4) or high (11 mmol/l, lanes 7, 8) glucose conditions. In the lower panel the same conditions were used to stimulate NIH-3T3 fibroblasts. Serum induced *egr-1* expression (lanes 4,8) in the presence of either low (lanes 3, 4) or high (lanes 7, 8) glucose. Glucose stimulation of NIH-3T3 cells (lanes 2, 6) failed to change *egr-1* expression relative to low glucose-treated cells (lanes 1, 5), regardless of the serum concentration

supernatants showed that these findings correlated with the ability of the various cell lines to secrete insulin in response to glucose. Glucose stimulation (20 mmol/l) of 24 h glucose-deprived MIN6 cells increased the insulin concentrations more than 15-fold (1070 ± 104 vs 17085 ± 2831 ng/ml, $n = 20$, $p < 0.001$, subsequent values mentioned in this paragraph are measured in the same units). Similar results were obtained for βTC3 cells (2640 ± 771 vs 9007 ± 2213, $n = 8$, $p = 0.006$). Substantially weaker responses were obtained with INS-1 (63 ± 6 vs 119 ± 13, $n = 5$, $p = 0.005$) and RIN cells 53 ± 4 vs 95 ± 13, $n = 11$, $p = 0.002$), whereas stimulation could not be shown for HIT (125 ± 39 vs 65 ± 14, $n = 12$, $p = 0.16$). Together these data indicate that *egr-1* induction after glucose stimulation correlates with other variables of glucose-responsiveness.

In primary islets, Egr-1 expression was assessed at the protein level by immunoblot. In isolated rat islets cultured ex vivo in low glucose medium Egr-1 protein was undetectable but it was induced after 90 min incubation in a high glucose medium (Fig. 2B). To minimize possible artifacts of the isolation procedure or the in vitro cultivation, islets were also isolated from rats injected with the protein synthesis inhibitor, cycloheximide, after overnight starvation and subsequent glucose or saline injection. A protein of 80 kDa that most likely represents Egr-1 protein was present at higher concentrations in lysates of islets from animals injected with glucose than from control animals (Fig. 2C). Although non-beta cells present in the unfractionated islets used in these studies could have contributed to the Egr-1 protein induction, taken together these results strongly suggest that increased Egr-1 expression is a component of the beta-cell response to glucose in vivo.

Induction of egr-1 by glucose is unique to beta-cells. Expression of *egr-1* was not affected by stimulation in any of the non-insulin-producing primary cell explants or cell lines examined. Glucose failed to induce *egr-1* expression in glucose-depleted HepG2 human hepatoma cells or primary rat hepatocytes cultured ex vivo either with or without insulin (Fig. 2D). Though HepG2 cells do not synthesize insulin, they are glucose-responsive and increase expression of genes encoding metabolic enzymes, such as fatty acid synthase, after glucose stimulation [34]. Stimulation of primary human thyrocyte cultures [22] with TSH also did not affect *egr-1* mRNA expression (Fig. 2D), demonstrating that *egr-1* induction is not a general feature of activation, increased intracellular cAMP or exocytosis in other endocrine cell types. Similarly, whereas mitogenic serum stimulation induced *egr-1* mRNA in serum-depleted NIH-3T3 fibroblast cultures (sevenfold induction), glucose stimulation of glucose-deprived 3T3 cells failed to affect *egr-1* expression (1.5-fold induction, Fig. 2E, lower panel). These results

show that the effects of glucose on *egr-1* expression are highly specific to beta-cells. Interestingly, glucose induction of *egr-1* mRNA in primary explanted renal mesangial cells has also been reported [35].

Although *egr-1* can be induced by mitogenic stimuli in many cell types, serum stimulation of serum-deprived MIN6 cells elicited minimal induction (threefold, Fig. 2E). In contrast, glucose stimulation resulted in characteristically large increases in *egr-1* RNA under both high (20%) and low (0.5%) serum conditions (greater than 20-fold). Since MIN6 cells failed to proliferate in 0.5% serum under either high or low glucose conditions, activation of *egr-1* expression did not require induction of mitosis.

Glucose induces other immediate early response genes. Expression of other immediate early response genes in MIN6 cells after glucose treatment was examined by RNA blot hybridization. Glucose induced expression of *c-fos* mRNA with kinetics similar to *egr-1* message accumulation (Fig. 3A). Maximum *c-fos* mRNA expression was reached 15 to 30 min after stimulation and declined rapidly thereafter to baseline by 1 to 2 h. Similar kinetics of *c-fos* mRNA accumulation have been shown after stimulation with mitogens [36]. Glucose also induced *junB* mRNA, but did not affect expression of *junD* or *c-jun*, which were expressed constitutively at readily detectable levels (Fig. 3A). The latter result is consistent with previous studies showing high constitutive *c-jun* expression in beta cells in the absence of glucose and reduced expression after glucose treatment [37]. As was observed for *egr-1*, prolonged cultivation in a high glucose medium also resulted in constitutive, high expression of both *junB* (Fig. 3A, lane H) and *c-fos* (data not shown).

Regulation of egr-1 induction. Intracellular signals involved in early growth response gene induction were analysed using pharmacologic agents. Results of these studies are summarized in Table 1. Though insulin induces *egr-1* expression in several cell types [38], it did not alter the expression of *egr-1* mRNA in MIN6 cells (Fig. 3B), even when added in concentrations up to 10 times higher than those achieved in post-stimulatory culture supernatants. These results rule out autocrine insulin stimulation as a mechanism for *egr-1* induction. Treating MIN6 cells with membrane depolarizing agents, arginine or potassium chloride, induced *egr-1* mRNA to levels comparable to induction by glucose (Fig. 3B, Table 1). As with glucose-stimulated insulin release, *egr-1* induction was largely inhibited by EDTA and thus appears to be dependent upon influx of extracellular Ca^{2+} (Fig. 3C). Treating MIN6 cells with the Ca^{2+}-ionophore, ionomycin, did not, however, increase *egr-1* mRNA expression (Fig. 3B), indicating that Ca^{2+} influx alone is insufficient to induce *egr-1*.
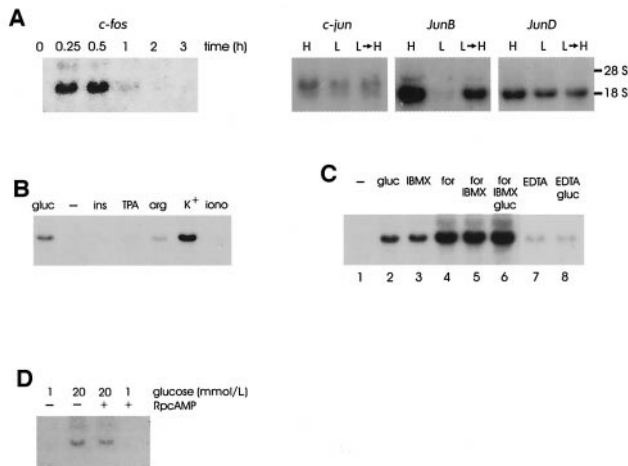
Fig. 3 A–D. Stimulation of early growth response genes by glucose and secretagogues. **A** Glucose stimulation of early growth response genes. Expression of *c-fos* mRNA increased to maximum within 15 to 30 min after stimulation of glucose-deprived MIN6 cells and declined rapidly thereafter. Expression of *junB* was also induced by 30 min glucose stimulation of glucose-deprived MIN6 cells or by continuous cultivation in high glucose medium (lane H). Expression of *c-jun* and *junD* were unaffected by transient stimulation with glucose (lane L- > H) or continuous high glucose cultivation (lane H). H, L and L- > H refer to stimulation conditions as in Fig. 2A. **B–D** Effects of pharmacologic agents and insulin secretagogues on *egr-1* mRNA expression. After 48 h culture in low glucose medium, MIN6 cells were treated for 30 min with the indicated agents. Concentrations for each agent are indicated in Table 1. EDTA (**C**, lane 8) and Rp-cAMPS (**D**) were added 30 min prior to addition of glucose

Table 1. Induction of *egr-1* mRNA expression after 30-min stimulation of glucose-deprived MIN6 cells with various secretagogues, quantification of *egr-1* expression was by transmission densitometry on autoradiographs of RNA blots. Induction ratios were calculated by dividing *egr-1* signal intensity for each secretagogue by the signal intensity for the 20 mmol/l glucose stimulation standard included with each experiment. The average number of (*n*) separate stimulation/hybridization experiments is indicated

Compound	Induction ratio Means ± SEM	*n*
Glucose-deprived: 24 h, 1 mmol/l glucose	0.02 ± 0.04	10
Glucose 20 mmol/l	1.0	15
Insulin 0.3 U/ml	0.0 ± 0.0	6
IBMX 100 µmol/l	6.9 ± 5.4	3
Forskolin 10 µmol/l	7.0 ± 4.0	3
IBMX 100 µmol + forskolin 10 µmol	10.0 ± 4.4	7
IBMX 100 µmol + forskolin 10 µmol/l + Glucose 20 mmol/l	40.3 ± 17.0	3
Rp-cAMPS 100 µmol/l + glucose 20 mmol/l	2.2 ± 0.5	4
Arginine 20 mmol/l	0.4 ± 0.1	4
K+ 25 mmol/l	1.8 ± 1.0	4
Ionomycin 1 µmol/l	0.2 ± 0.1	4
EDTA 10 mmol/l	0.1 ± 0.1	2
EDTA 10 mmol/l + glucose 20 mmol/l	0.3 ± 0.2	3
TPA 10 nmol/l	0.2 ± 0.1	9
TPA 10 nmol/l, 30 min + glucose 20 mmol/l	28.6 ± 21.7	2
TPA 100 nmol/l, 24 h + glucose 20 mmol/l	2.8 ± 1.7	2
Okadaic acid 1 µmol/l	0.2 ± 0.2	2
Okadaic acid 1 µmol/l + glucose 20 mmol/l	3.7 ± 1.2	4

Activation of protein kinase C by TPA did not increase *egr-1* mRNA. Treating cells with TPA for 30 min prior to glucose stimulation, however, potentiated the effects of glucose (Table 1). Down-regulating protein kinase C by 24-h incubation with TPA did not appreciably reduce the response to glucose (Table 1). Previous studies have shown that protein kinase C activation augments, but is not necessary for, glucose-induced insulin secretion [39]. Similarly, treating MIN6 cells with the phosphatase inhibitor, okadaic acid, did not alter *egr-1* expression, but potentiated *egr-1* induction by glucose (Table 1). Together these results suggest an important role for protein phosphorylation in modifying transcriptional responses to glucose stimulation.

Finally, IBMX and forskolin, stimulators of the cAMP system, both induced *egr-1* mRNA in the absence of glucose (Fig. 3C). The magnitude of induction with each drug individually was on average sevenfold higher than standard induction with glucose (Table 1). In combination, IBMX and forskolin induced *egr-1* synergistically (Fig 3 C, Table 1). These agents in combination also strongly potentiated the effects of glucose, inducing a 40-fold greater increase in *egr-1* mRNA than glucose alone (Fig. 3C, Table 1). A competitive cAMP inhibitor, Rp-cAMPS, did not,

however abolish the stimulatory effect of glucose (Fig. 3D), showing that cAMP signalling pathways can potentiate but are not essential for glucose-induced *egr-1* mRNA accumulation.

Discussion

These experiments show that glucose stimulation of beta cells induces expression of the immediate early growth response genes *egr-1*, *c-fos* and *junB*. Expression of *c-jun* was not induced in MIN6 cells, in agreement with previous studies showing down-regulation of *c-jun* in HIT beta cells by glucose [37]. Induction of *egr-1* correlated with insulin secretory capacity and was not observed in thyrocytes stimulated with thyrotropin or in glucose-treated hepatocytes. Among beta-cell lines, *egr-1* was strongly induced in highly glucose-responsive MIN6 and *β*TC3 lines and in INS-1 cells, least induced in weakly responsive RINm5F cells [25, 26], and was not induced detectably in late passage MIN6 and HIT-T15 cells, which fail to secrete insulin in response to glucose [16]. Importantly, Egr-1 protein was also induced in primary islets stimulated either in vivo or in vitro. These results argue that immediate early gene activation is a unique and fundamental aspect of the beta-cell response to glucose.

Although immediate early growth response genes are typically induced by stimuli that promote mitosis [29, 36, 40], their induction by glucose is independent of the beta-cell proliferative state. Glucose increased *egr-1* expression under low serum conditions which do not support proliferation of MIN6 cells, whereas serum stimulation of serum-deprived MIN6 cells induced *egr-1* expression only weakly. Moreover, mitogenic stimuli characteristically induce *c-jun* coordinately with *c-fos* and *junB* [36, 40, 41], whereas glucose did not alter *c-jun* expression. Failure to activate *c-jun* is probably an important aspect of the unique beta-cell response to glucose since c-Jun represses insulin gene transcription [37].

The correlation between *egr-1* induction and insulin secretion suggests that shared or overlapping intracellular signalling pathways regulate these two responses. During beta-cell stimulation, products of glucose metabolism initiate secondary signals that trigger closure of ATP-sensitive K^+-channels, depolarization of beta-cell membranes and opening of voltage-sensitive, L-type Ca^{2+}-channels [26]. The resulting increase in intracellular Ca^{2+}-ion concentration causes insulin release. In this light it is of relevance that *egr-1* expression was both dependent on Ca^{2+}-influx and inducible by membrane depolarizing agents.

Recent observations indicate that glucose stimulation could be linked to immediate early gene induction through Ca^{2+}-dependent activation of mitogen-activated protein (MAP) kinases. Transcription of *c-fos* and *egr-1* is initiated by phosphorylation of serum response element (SRE)-binding proteins such as Elk-1 by activated MAP kinases [42, 43]. In INS-1 beta cells, 44-kDa MAP kinase is activated after either glucose stimulation, membrane depolarization or treatment with insulin secretagogues [44]. Depolarization of PC12 pheochromocytoma cell membranes similarly causes Ca^{2+}-dependent activation of MAP kinases and induces expression of immediate early genes *egr-1*, *c-fos*, and *junB* but does not affect *c-jun* expression [45]. These results suggest that MAP kinase activation and immediate early gene induction are consistent features of neuroendocrine-cell activation after membrane depolarization. The role of these kinases in specific beta-cell responses to glucose is, however, not clear since activation of 44-kDa MAP kinase alone is not sufficient to cause insulin release [46, 47].

In addition to triggering beta-cell insulin release, glucose also alters expression of a number of genes, including those encoding insulin, the GLUT-2 glucose transporter [6], and various glucose metabolic enzymes such as acetyl-coenzyme A-carboxylase [8] and pyruvate kinase [7]. In concert, these changes could be necessary for beta-cell adaptation to the increased metabolic and secretory demands of a high glucose environment. Immediate early response proteins could serve an important function in this process

by altering expression of specific target genes in response to metabolic changes. The *egr-1* encodes a zinc-finger protein of the Cys_2-His_2 subfamily that regulates transcription through redox-dependent binding to the consensus DNA sequence 5′-GCGGGGGCG-3′ [48, 49]. The *c-fos* gene product associates with Jun proteins to form stable AP-1 heterodimers. Sequence-specific DNA binding by AP-1 factors is also dependent on reduction at cysteine residues in Fos and Jun components [50]. Since glucose stimulation of beta cells increases cytoplasmic flavin adenine dinucleotide [reduced form] ($FADH_2$) and NADH reducing potential [51], glucose metabolism could be coupled to transcriptional activation by Egr-1 and AP-1 factors.

The possibility that Egr-1 has a central role in regulating beta-cell responses to glucose is supported by close sequence similarities between Egr-1 and transcription factors that control carbohydrate metabolism in yeast. Multicopy inhibitor of *GAL1* promoter (MIG1p) is a Cys_2-His_2 zinc-finger protein of *Saccharomyces cerevisiae* which shares 68% amino acid identity in its zinc-finger region with Egr-1 and binds to GC-rich target sequences that closely resemble the Egr-1 consensus binding site [52]. MIG1p is responsible for glucose-repression of *GAL1*, which encodes galactase and of *GAL4* [52]. The proteins encoded by multicopy suppressor of snf (*MSN)2* and *MSN4* [53] of *S. cerevesiae* share 60% and 52% identity, respectively, with the Egr-1 zinc-finger region and are necessary for metabolic stress responses and for glucose-induced derepression of the alcohol dehydrogenase gene ADH_2.

Though *egr-1* expression is induced in many mammalian cell types after a variety of external stimuli, few genes specifically regulated by Egr-1 have been identified. Moreover, effects on individual target genes are largely cell-type-specific, implying that Egr-1 acts in concert with other transcription factors whose expression or activity is highly restricted [31]. In fibroblasts, Egr-1 activates expression of thymidine kinase after serum stimulation [54], whereas in endothelial cells Egr-1 upregulates platelet-derived growth factor B-chain expression after mechanical injury [55]. In B lymphocytes, Egr-1 activates expression of genes encoding the intercellular adhesion molecules ICAM-1 [56] and CD44 [57]. Genes regulated by Egr-1 in beta cells are currently not known. The Egr-1 consensus sequence has, however, been identified in promoters of several genes that could be involved in beta-cell function. These include genes encoding the beta-cell-specific α_1 subunit of the L-type voltage-dependent Ca^{2+} channel (VDCCα_1), slow sarcoplasmic reticulum Ca^{2+}-ATPase, calcineurin, the cAMP response element binding protein, Na^+/K^+-ATPase $\alpha 1$ subunit (NKAA1) and aldolase. Interestingly, aldolase mRNA is upregulated in MIN6 cells after glucose stimulation (unpublished

observation). Experiments are in progress to determine whether expression of any other of these genes is altered after glucose stimulation.

In endocrine cells Egr-1 induction has not been described previously. Its role in closely related neuronal cells has, however, been studied intensively and could be relevant. Both *c-fos* and *egr-1* are induced in neurons after repetitive depolarization and are essential to adaptive long-term potentiation [58]. Specific transcriptional targets for Egr-1 include genes encoding synaptic vesicle transport proteins synapsins I and II [59]. Beta cells resemble neurons in several respects including activation by membrane depolarization, voltage-dependent Ca^{2+} influx and regulated release of intracytoplasmic vesicle contents via exocytosis. Expression of neuroendocrine-specific transcriptional activators (Islet-1, Pax-6 and Beta2) and neuron-restrictive silencer factor / repressor element silencing transcription (NRSF/REST) in both cell types [60] indicates close similarities in gene regulation. Beta cells also exhibit plasticity and modulate insulin release according to previous glucose exposure. Together, these findings suggest that immediate early response proteins could modulate transcription of genes which are necessary for long-term potentiation or adaptive changes in beta-cell function.

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