Upstream Stimulatory Factor-2 (USF2) Activity Is Required for Glucose Stimulation of L-Pyruvate Kinase Promoter Activity in Single Living Islet \(\beta\)-Cells

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Elevated glucose concentrations stimulate L-pyruvate kinase (L-PK) gene transcription in liver and islet \(\beta\)-cells. A glucose response element termed the L4 box (two noncanonical E-boxes located −165 and −154 base pairs upstream of the transcriptional start point) has previously been defined within the proximal promoter region of the gene. However, the identity of the trans-acting factor(s) which binds to this site remains unclear. We have used photon counting digital imaging of firefly luciferase activity to monitor promoter activity continuously in single living islet \(\beta\) and derived INS-1 cells, and to analyze the molecular basis of the regulation by glucose. L-PK promoter activity, normalized to cytomegalovirus promoter activity using the distinct Renilla reniformis luciferase, was 6-fold higher in cells cultured at 16 mM glucose or above compared with cells cultured at 3 mM glucose. Microinjection of antibodies against the ubiquitous transcription factor USF2 inhibited L-PK promoter activity in \(\beta\)- and INS-1 cells incubated at 30 mM glucose by 71–87%. Anti-USF2 antibodies had a much smaller effect on promoter activity in INS-1 cells cultured at 3 mM glucose, and on the activity of a modified promoter construct lacking an L4 box. These data support the view that glucose enhances L-PK gene transcription in \(\beta\)-cells by modifying the transactivational capacity of USF2 bound to the upstream L4 box.

Increases in extracellular glucose concentration stimulate transcription of the L-pyruvate kinase (L-PK) gene in liver and islet \(\beta\)-cells (1, 2). This effect is mediated by a cis-acting DNA sequence termed the L4 box centered 160 base pairs upstream of the transcriptional start site (3–5) and consisting of a tandem repeat of non-canonical E-boxes (underlined) as follows: 5′-CACGCGGCTCCCGTG-3′. This site appears also to confer glucose responsiveness on the L-PK promoter in islet \(\beta\)-cells (6). Oligonucleotides derived from this sequence bind to major-late transcription factor or USF (upstream stimulatory factor), a ubiquitous member of the basic helix-loop-helix and leucine zipper (LZ) family (7). Members of this family form dimers through the interaction of helix-loop-helix and leucine zipper motifs, and then bind to cognate sites on DNA via their basic regions. Two forms of USF, USF1 (43 kDa) and USF2 (44 kDa), were first identified in HeLa cells (8, 9) and are encoded by separate genes (9–12). Furthermore, alternative splice variants of USF2, USF2a, and USF2b have recently been identified, while heterodimers of USF1 and USF2a appear to represent the predominant form in vivo (13). Consistent with a role for USF in the response of the L-PK promoter to glucose in hepatocytes, the expression of truncated USF proteins lacking the DNA-binding domain inhibits the stimulation of promoter activity by glucose, probably by dimerizing non-productively with endogenous USF (14).

To provide a more specific assay of the role of USF in the transcriptional activation of the L-PK gene in the \(\beta\)-cell type, we have developed a technique whereby the activity of the L-PK promoter can be constantly imaged at the single cell level using firefly luciferase. Simultaneous measurement of the activity of a second reporter protein, luciferase from the sea flower Renilla reniformis, then allows L-PK promoter activity to be normalized to the activity of a constitutive viral promoter. We report here that inhibition of USF2 function with specific antibodies microinjected directly into the nucleus of living clonal \(\beta\)-cells (INS-1) substantially prevents the induction of L-PK gene transcription by glucose. This combination of techniques should provide a general means with which to investigate the molecular mechanisms underlying the control of transcription in living cells.

Experimental Procedures

Materials—Beetle luciferin (K′ salt) was obtained from Promega Ltd., United Kingdom, and coelenterazine from Molecular Probes, or was a gift from Dr. B. Sherf (Promega). Bovine serum albumin solutions of predefined density were obtained from Dr. R. F. L. James (Leicester, UK). Collagenase (Pan Plus) was from Serva (Wokingham, UK). Rabbit polyclonal antisera to USF2 was raised as described earlier (13), or was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies were lyophylized and microdialyzed versus 10 mM Tris, pH 8.0, 0.2 mM EDTA, and quantitated by Bradford assay (15) with bovine serum albumin as standard. Plasmid pRL.CMV, containing cDNA encoding Renilla luciferase, was purchased from Promega.

Plasmid Construction—A fragment of the L-PK gene corresponding to the region −183 to +10 nucleotides with respect to the transcriptional start site was subcloned from plasmid 183PKCAT (16) via flanking KpnI and XhoI sites into plasmid pGL3.basic (Promega). The resulting plasmid (p.LPK.Luc\(_{\text{p}}\)) contained a modified version of the firefly luciferase cDNA gene, lacking the three amino acid C-terminal peroxisomal targeting sequence, and engineered for optimal codon usage in mammalian cells (17). Use of the modified construct provides 10–50-fold greater firefly luciferase synthesis compared with the unmodified cDNA.2 A plasmid which lacked the L4 box containing the putative USF-binding sites (pL4.LPK.Luc\(_{\text{p}}\)) was generated by polymerase chain reaction amplification of base pairs −148 to +10 from

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1 The abbreviations used are: L-PK, L-pyruvate kinase; USF, upstream stimulatory factor; LZ, leucine zipper; CMV, cytomegalovirus.

2 G. A. Rutter and J. M. Tavare, unpublished data.
plasmid 183PKCAT, using primers: 5'-TTTGTAGTCGTTCTG-GACTCTGGCC-3' (IgH site underlined) and 5'-TTTGTAGTCGTTCTG-GACTCTGGCC-3' (HindIII site underlined). Standard molecular biology cloning procedures were followed (18) and all plasmids were purified on CsCl gradients.

Isolation of Primary Islet β-Cells—Rat islets were isolated from adult males (220–250 g) by in situ collagenase digestion, followed by purification on a discontinuous bovine serum albumin gradient (19). Islets were dissociated into single cells by incubation for 2 min, at 20–22 °C in modified, nominally Ca2+-free Krebs-Ringer-bicarbonate medium (20) containing trypsin (Type XI, Sigma; 0.5 mg·ml−1) and poly-L-lysine (0.012 mg·ml−1) or Cell-Tak 228 (1.5 dilution; Becton-Dickinson, Bedford, MA). After allowing 5–30 min for cell adherence, culture was continued for 16 h at 37 °C in 2 ml of modified Eagle’s medium plus 10% (v/v) fetal calf serum, 100 IU penicillin and 100 μg of streptomycin·ml−1, in the presence of 5% CO2.

Culture of INS-1 Cells—Prior to microinjection, cells were cultured for 48 h on 24-mm diameter poly-L-lysine-coated coverslips as described previously (21).

Microinjection and Cell Imaging—Intracellular pressure microinjection was performed using an Eppendorf 5172 transjector as described before (20, 22). IgG and anti-USF2 antibodies were always microinjected into cells (22) and (ii) permits the introduction of other molecules such as antibodies at the same time.

Whereas firefly luciferase uses luciferin as cofactor, Renilla luciferase oxidizes only coelenterazine (23). These specificities were retained in living cells, with neither enzyme, when expressed alone, showing any activity except in the presence of its cognate substrate (data not shown). When β-cells were micro-injected with pLPK.LucFp plus pRL.CMV and incubated 16 h at either 3 or 16 mM glucose, cells maintained at the higher glucose concentration displayed a 6-fold higher ratio of firefly to Renilla luciferase activity compared with those at 3 mM glucose (Fig. 1). This change can be ascribed entirely to enhanced transcription of the L-PK promoter, since any nonspecific alterations in basal transcriptional or translational efficiency are normalized (with Renilla luciferase). Furthermore, this effect of glucose was mediated by the L4 box of the L-PK promoter, since glucose was ineffective in altering the transcriptional activity of a truncated promoter lacking this element (pΔL4-LPK.LucFp; firefly:Renilla luciferase activity ratio 0.009 ± 0.013, n = 10 cells, and 0.020 ± 0.01, n = 11, after incubation for 24 h at 3 and 16 mM glucose, respectively).

Effects of USF1/2 Inhibition on L-PK Promoter Activity in Primary β-Cells—To determine whether isoforms of USF may be involved in the response to glucose we co microinjected either an antibody (USF-G) raised to the 49-amino acid N-terminal domain of USF2/αb or control IgG. This domain may be involved in transactivation by USF (24). In three separate experiments, the ratio of firefly:Renilla luciferase activity was lowered 3 h after injection from 0.63 ± 0.009 × 10−2 (n = 12 cells) to 0.08 ± 0.0001 × 10−2 (n = 16) in the presence of the microinjected antibody (86.7% inhibition).

These experiments demonstrated that monitoring glucose-regulated gene expression with single microinjected β-cells was feasible and implicated USF1/2 in the effects of glucose. To achieve a detailed analysis of the regulation of the L-PK promoter in a large and homogeneous cell population we turned to highly differentiated INS-1 β-cells (21, 25) whose flattened morphology (Figs. 2 and 3) facilitated microinjection.

L-PK Promoter Activity Can Be followed in Real Time in Single INS-1 Cells—Using photon counting digital imaging in the presence of luciferin alone it was possible to follow the
In line with the repression of USF2 in the context of L-PK promoter activity, we used two antibodies raised against the leucine zipper domain of USF2 proteins. The antibody termed LZ2 (see Ref. 13, Fig. 2A) was raised against amino acids 298–346 of human USF2a corresponding to the entire leucine zipper motif and the antibody termed USF2-SC, developed by Santa Cruz, was directed against amino acids 327–346 including 2 of the 4 leucine residues of the leucine repeat of mouse USF2. This latter antibody also recognizes the analogous epitope in USF1. We analyzed the effects of comicroinjecting these antibodies at the earliest possible time point after microinjection, i.e. 3 h. As shown in Table I, antibodies LZ2 and USF2-SC caused a decrease in the ratio of firefly:Renilla luciferase activity of 85.1 and 70.9%, respectively. Microinjection of these antibodies had no significant effect on L-PK promoter activity in cells maintained at 3 mM glucose (Table I). In addition, antibody USF-G substantially inhibited (74.8%) glucose-dependent L-PK promoter activity. By contrast, an antibody (USF-F) raised to a central epitope of USF2 (resides 143–193 of USF2a, corresponding to residues 76–126 of USF2b) was without effect on apparent activity. However, this antibody inhibited by 77.6% the expression of a luciferase construct (pCol.Luc), bearing the fragment −517 to + 63 of the human collagenase promoter (22), which possesses a non-canonical E-box (−408CGGTTG). No significant change in firefly:Renilla luciferase activity was observed in INS-1 cells microinjected with LPK.LucFF using an irrelevant antibody, anti-c-Fos (Table I), nor after microinjection of anti-USF2 antibodies into the cell cytosol (data not shown).

To confirm that the effect of USF2a/b inhibition was the result of disrupting an interaction between this transcription factor and the L4 box, we examined the effect of the antibody on the expression of luciferase in cells microinjected with the truncated plasmid pLPK.LucFF (see above). As observed after transfection of the analogous CAT construct into liver cells (5), the L4-LPK promoter in this plasmid was unable to mediate a response to glucose (Table I). Indeed, in cells microinjected with the construct and incubated at 30 mM glucose, the ratio of firefly:Renilla luciferase activity was lower in identically injected cells maintained at 3 mM glucose, than in cells maintained at 30 mM glucose, or a small effect in cells maintained at 3 mM glucose (antibody G).

**DISCUSSION**

**Single Cell Analysis of Transcription**—The microinjection/imaging technique we have developed here provides a rapid, non-invasive and highly quantitative assay of gene expression in single living cells (26). The technology we describe extends our earlier studies (22), by allowing the easy assay of the activity of two reporters in the same living cell, simply by switching between two cofactors (luciferin and coelenterazine). In this way, the activity of a regulated promoter (L-PK) can be normalized to a promoter which is constitutively active (CMV). Extremely high sensitivity is achieved due to the absence of significant background signals and the high sensitivity of the intensified imaging camera. Typically, firefly luciferase activities corresponded to 50–500 photon cell−1 15 min−1 (intact...
TABLE I

Effect of microinjection of anti-USF2 antibodies on L-PK promoter activity in individual INS-1 cells

Cells were microinjected with plasmids pLPK.LucFF, or pΔL4-LPK.LucFF, plus pRL.CMV (0.3 mg/ml each) and antibody (0.9–1.2 mg/ml) and incubated 3 h in culture medium containing the glucose concentration shown. Firefly and Renilla luciferase activities were then determined by sequential incubation with luciferin and coelenterazine (Fig. 1). Photon release from individual cells was determined by defining a circular “region of interest” approximately 20 pixel in diameter, around the luminescent area (see Figs. 1 and 3). The ratio (R) of firefly: Renilla luciferase activity expressed in each cell was calculated from center of gravity images according to,

\[ R = \frac{FF}{bg1(T - FF)}, \]

where FF is the number of photons detected in the presence of luciferin alone, and T is the number of photons detected in the simultaneous presence of luciferin and coelenterazine. The background value, bg1, was determined by measuring the mean photon number in three randomly selected areas of the image where no cell was located. Data are given as the mean value for R ± S.E. (×100) for the number of cells given in parentheses (number of cells, number of separate cultures). Where the number of separate cultures is not shown, all cells were from the same culture. Statistical significance was determined by Student’s t test assuming equal variance: *, p < 0.05; **, p < 0.01; ***, p < 0.001. The extent of inhibition achieved with each antibody is presented as a percentage of the IgG control.

| Promoter [glucose] | L-PK | ΔL4-LPK | Collagenase, 30 |
|-------------------|------|---------|-----------------|
| mm                |      |         |                 |
| IgG               | 1.47 ± 0.29 (54, 2) | 9.17 ± 1.98 (62, 2) | 2.43 ± 0.56 (132, 3) |
| USF-2 (SC) %      | NS*  | 1.37 ± 0.21 (85, 2)**  | 1.40 ± 0.30 (137, 3) |
| IgG %             |      | 85.1    | 0.43 ± 0.05 (118, 2) |
| LZ2   %           | 7.47 ± 3.56 (23) | 9.86 ± 1.35 (61, 4) | 8.89 ± 1.14 (57, 2) |
| USF-G %          | NS*  | 2.87 ± 0.35 (66, 4)** | 6.28 ± 0.63 (54, 2)* |
| Collagenase, 30   |      | 70.9    | 29.4            |
| IgG %            | 9.96 ± 2.30 (26) | 1.63 ± 0.16 (55, 3) |
| USF-F %          | 2.51 ± 0.37 (53)** | 0.91 ± 0.13 (48, 3) |
| USF-G %          |      | 74.8    | 1.71 ± 0.16 (55, 3) |
| USF-F %          | 4.23 ± 0.68 (29, 2) | 6.56 ± 1.67 (26) |
| USF-F %          | 4.63 ± 0.73 (53, 2) | 1.47 ± 0.28 (28)** |
| USF-F %          |      | 77.6    | 77.6         |
| c-Fos %          | 6.48 ± 1.4 (23) | 2.44 ± 0.4 (68) |
| c-Fos %          | 4.66 ± 0.81 (49) | 1.94 ± 0.32 (68) |
| USF-F %          |      | NS      | 85.1            |

**NS, not significant.

L-PK promoter, 30 mM glucose) versus a background of about 20 photons (i.e. signal:noise, S:N, ratio between 2.5:1 and 25:1). Renilla luciferase activity produced 1000–10,000 photoncell−15 min, with a background of 50–100 photonscell−15 min−1 (S: N = 20–200:1). The S:N ratios are at least 1 order of magnitude higher than we have been able to achieve using enhanced mutants of green fluorescent protein (22, 26, 27).

Molecular Basis of the Regulation of the L-PK Gene by Glucose in β-Cells—Previous studies have established that an intact L4 box is crucial for the induction of the L-PK gene in the β-cell type (6) as well as in liver cells (5, 28). Whereas a role for USF proteins, interacting with this site, is implicated in liver cells (29), no data has been available up to now on the role of this family in regulating expression in islet β-cells. Here we provide evidence that USF2, probably as a heterodimer with USF1, is responsible for the activation by glucose of L-PK gene transcription in β-cells. Thus, in preliminary experiments, an antibody to the N terminus of USF2a/b inhibited L-PK promoter activity in primary β-cells. In INS-1 cells, antibody USF-G and two further antibodies against the leucine zipper motif (LZ2, USF2-SC) each inhibited L-PK promoter activity. However, an antibody (USF-F) to the central portion of the
protein was without effect. Although the reason for this difference is unclear, it might be speculated that the three inhibitory antibodies, but not USF-F, are able to: (i) disrupt the interaction of USF2 with the L4 box; (ii) to prevent the transactivation function of the factor; or (iii) to block an interaction with an ancillary activating protein. Of these, it might be noted that option (i) would seem the least likely since the antibodies are able to supershift complexes between USF2 and L4 box-derived oligonucleotides (13).

How might glucose control the transactivational capacity of USF proteins? Since in the liver, glucose has no effect on the occupancy of the L4 site as determined by USF2 to interact with L4-derived oligonucleotides (13).

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