Online Monitoring of Stimulus-Induced Gene Expression in Pancreatic β-Cells

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Fluorescent proteins have been extensively used as protein "tags" to study the subcellular localization of proteins and/or their translocation upon stimulation or as markers for transfection in transient and stable expression systems. However, they have not been frequently used as reporter genes to monitor stimulus-induced gene expression in mammalian cells. Here we demonstrate the use of fluorescent proteins to study stimulus-induced gene transcription. The general applicability of the approach is exemplified by doxycyclin-(Tet-On) and phorbol 12-myristate 13-acetate–induced (c-*fos*) promoter activation, with green fluorescent protein (GFP) and red fluorescent protein (DsRed) as semiquantitative and immediate reporters, of transcription activation. Under the control of β-cell–specific promoters, such as the rat insulin 1 promoter or the rat upstream glucokinase promoter, this approach allowed us to monitor online glucose-induced gene transcription in primary β-cells at the single-cell level as well as in the context of the islet of Langerhans. Applying discretely detectable fluorescent proteins, for example GFP and DsRed, enabled us to simultaneously monitor stimulus-induced transcription by two different promoters in the same cell. *Diabetes* 50 (Suppl. 1):S15–S19, 2001

The use of fluorescent proteins as a "protein-tag" in cell biology sheds new light on the mechanisms that determine key processes in cell function, such as subcellular localization of proteins, colocalization of proteins, physical interaction of proteins, and protein translocation. Used in a rational experimental set-up and in combination with techniques that allow high resolution and/or high dynamics of the obtained images, fluorescent proteins provide a tool that makes the phrase "seeing is believing" a reality in cell physiology. The generation of different colored members of the green fluorescent protein (GFP) family (1), i.e., blue (BFP, EBFP), cyan (ECFP), green (GFP, EGFP), and yellow (EYFP), as well as the cloning of new fluorescent proteins, such as the red fluorescent protein DsRed from *Discosoma sp.* (2), permitted fluorescent probes exhibiting distinct spectra of light/energy excitation and emission. This allowed on the one hand the use of a combination of different colors to monitor distinct proteins at the same time; on the other hand, it made it possible to develop tailored molecular probes to monitor protein interactions, substrate concentrations, enzyme activities, etc., at the subcellular level, partially based on fluorescence resonance energy transfer. A timely review on the potential application of fluorescent proteins based on the GFP family was published by Tsien (1).

The use of fluorescent proteins as reporter genes, although proposed as genetic markers from the beginning by Chalfie et al. (3), has been scarce, except as a marker in transfection experiments, despite their potential for use as dynamic "reporters" compared with classical reporter genes.

Here we show that fluorescent proteins, i.e., GFP_{S65T} and DsRed, can serve as semiquantitative and dynamic reporters for stimulus-induced gene expression in general and in the context of stimulus-induced cell type–specific gene expression, exemplified in the insulin-producing pancreatic β-cell. Moreover, we show that fluorescent proteins do not only allow monitoring of stimulus-induced gene expression at the single-cell level but also at the level of a micro-organ—here, the islet of Langerhans. Finally, use of fluorescent proteins with discrete excitation/emission properties, i.e., GFP_{S65T} and DsRed, allows the simultaneous monitoring of two genes in the same cell, here exemplified by the stimulus-induced rat insulin 1 (rIns1) promoter–driven DsRed expression and the rat β-cell–active glucokinase (rβGK) gene promoter–driven GFP expression.

RESEARCH DESIGN AND METHODS

Expression vectors. The construction of prIns1.GFP and pCMV.GFP was described elsewhere (4). Adenovirus (Ad)-rIns1.GFP (5) was provided by Dr. Lina Moitoso de Vargas (New England Medical School, Boston, MA). Plasmid prIns1.DsRed was generated by exchanging the GFP_{S65T}-bGHpA cassette with the DsRed-SV40pA cassette from pDsRed1-1 (Clontech, Palo Alto, CA). Plasmid prβGK.GFP was constructed by exchanging the CAT-SV40pA cassette in prβGK-278.CAT (6) with the GFP_{S65T}-bGHpA cassette. All vector constructions were verified by DNA sequence analysis.

Cell culture and transfection. HIT-T15 cells were obtained from ATCC. HIT cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l glutamine, and 10% fetal calf serum at 5% CO_2 and 37°C. Pancreatic islets were isolated from fed male Wistar rats (200–250 g body wt) by collagenase digestion. Isolated islets and cells of disaggregated islets were cultured overnight at 5% CO_2 and 37°C in RPMI 1640 medium containing 5.6 mmol/l glucose and supplemented as above. Transfection of HIT-T15 cells and islet cells was performed overnight by the lipofectamine technique in RPMI 1640 medium containing 11.1 or 5.6 mmol/l glucose, respectively, without serum and antibiotics. Pancreatic islets were infected with Ad-rIns1-GFP by incubating the islets with the virus for 90 min. HIT cells after transfection were incubated overnight in RPMI 1640 medium, supplemented as above but containing 0.1 mmol/l glucose. Islets, islet cells, and HIT cells were stimulated with 16.7 mmol/l for 15 min in supplemented RPMI 1640 medium. When studying c-*fos* promoter–driven GFP expression, transfected cells were stimulated for 15 min with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) in supplemented RPMI 1640 medium.

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CMV, cytomegalovirus; GFP, green fluorescent protein; PMA, phorbol 12-myristate 13-acetate; rβGK, rat β-cell–active glucokinase; rIns1, rat insulin 1.

Detection of fluorescence by digital imaging fluorescence microscopy. Transfected cells were grown on 24-mm glass coverslips. After stimulation, the coverslips were placed into a perifusion chamber that was mounted on an inverted microscope (Zeiss Axiovert 135TV; Carl Zeiss GmbH, Göttingen, Germany). Temperature was kept at 37°C, and the cells were perifused with RPMI 1640 medium. The objective lens used was a Zeiss-plan-NEOFLUAR ×25/0.8 Imm Korr (Carl Zeiss). Fluorescence imaging was performed with a cooled charged-coupled device camera (CH250 with KAF 1400; Photometrics, Tucson, AZ) connected to an imaging system (Inovision, Durham, NJ) with fluorescence excitation from a SPEX fluorolog-2 CM1T11I spectrofluorometer (Spex Industries, Edison, NJ). The following filter settings were used: for GFP_{S65T}, excitation at 485 nm, a 505-nm dicroic mirror, and an emission band-pass filter of 500–530 nm; for DsRed, excitation at 558 nm, a 565-nm dicroic mirror, and a 580-nm long-pass filter for emission. Fluorescence intensity was calculated using the ISEE software for UNIX (Inovision).

Detection of fluorescence by laser scanning confocal microscopy. Transfected cells were grown on 24-mm glass coverslips. After stimulation, the coverslips were placed into a perifusion chamber that was mounted on the inverted microscope of a Leica CLSM (Leica Lasertechnik, Leica, Germany). Temperature was kept at 37°C, and cells were perifused with RPMI 1640 medium. Laser scanning confocal microscopy was then performed using the following settings: ×40/1.30 oil Leitz Fluotar objective lens, excitation wavelength 488 nm (Krypton/Argon Laser), and a band-pass emission filter from 500 to 540 nm.

For the detection of fluorescence in islets, the islet was placed after stimulation into a perifusion chamber and fixed with a metallic mesh to stabilize the position of the islet during the experiment. The settings used for confocal microscopy were the same as above, except that a ×25/0.75 oil Leitz NPL Fluotar objective lens was used.

RESULTS

Fluorescent proteins can be used as semiquantitative and dynamic reporters. To test fluorescent proteins as suitable reporters for semiquantitative as well as for short-term–induced gene transcription, we first studied whether GFP can be used as a quantitative reporter gene using the Tet-On system. Here we placed the GFP gene under the control of the Tet-On promoter and monitored GFP expression in transfected cells, using increasing amounts of doxycycline (0–2,000 ng/ml) as the stimulator. Indeed, GFP expression, measured by digital imaging fluorescence microscopy, was elevated in a dose-dependent manner. When estimated as fluorescence/pixel of monitored cell cluster, where the cell cluster reflects the sum of both GFP-expressing and nonexpressing cells, GFP fluorescence at maximum was increased >100-fold (see Fig. 4*A* in the study by Leibiger et al. [4]). When measured as fluorescence/pixel of GFP-expressing cell, GFP expression was elevated more than fourfold (see Fig. 4*B* in the study by Leibiger et al. [4]).

To test whether fluorescent proteins can be used as reporters to study short-term regulation of gene transcription, we analyzed the ability of the immediate early gene c-*fos* promoter to drive GFP expression upon stimulation with PMA. The formation of the fluorophore of GFP_{S65T} has been reported to take ~30–60 min after translation of the GFP mRNA (7). Considering this and the time necessary for transcription initiation, transcription elongation, transcript processing, nuclear-cytoplasmic mRNA transport, and translation, we expected a significant increase in GFP fluorescence 80–120 min after the start of stimulation. In transient expression studies using a variety of cell lines, we observed that the expression of GFP by individual cells within a transfected cell cluster is heterogeneous probably because of differing numbers of plasmids expressed in each cell. To quantify GFP expression, we set the intensity of GFP fluorescence of each individual cell at minute 20 as the basal value 1.0. The intensity of GFP fluorescence of each individual cell monitored was related to its basal value. By using digital imaging fluorescence microscopy, we were able to show that PMA stimulation results in increased GFP fluorescence between 80 and 100 min after start of stimulation and reaches a maximum at 240–260 min (see Fig. 5 in the study by Leibiger et al. [4]). Specificity was demonstrated by blocking the PMA effect with the pharmacological inhibitor of protein kinase C bisindolylmaleimide-I.

Fluorescent proteins as reporters in β-cell–specific gene expression. To investigate the usability of fluorescent proteins as reporter genes in β-cell–specific stimulus-induced gene expression, we studied their expression under the control of the rIns1 promoter (–410/+1 bp) and the rβGK upstream promoter (–278/+123 bp) in insulin-producing cell lines as well as in primary β-cells.

We have recently shown that short-term glucose stimulation upregulates insulin gene expression at the level of transcription initiation (4). When we compared glucose-stimulated (16.7 mmol/l) rIns1 promoter–driven GFP expression with PMA-stimulated c-*fos* promoter–driven GFP expression, we obtained similar kinetics. Monitoring glucose-induced rIns1 promoter–driven GFP expression online showed that GFP fluorescence remained constant between 20 and 60 min after the start of stimulation, was elevated from minute 80, and reached a maximum at 240 min. The glucose effect was specific for the rIns1 promoter because high glucose did not elevate GFP expression when driven by the human cytomegalovirus (CMV) promoter (see Fig. 5 in the study by Leibiger et al. [4]).

In our studies, GFP fluorescence was monitored by either digital imaging fluorescence microscopy or confocal microscopy. Both imaging techniques led to similar results (see Fig. 6 in the study by Leibiger et al. [4]). Whereas digital imaging fluorescence microscopy enabled us to monitor more cells in parallel, laser scanning confocal microscopy allowed us to monitor rIns1 promoter–driven GFP expression in β-cells in the context of the islet of Langerhans (Fig. 1). Upon stimulation with either 16.7 mmol/l glucose (15 min) or 5 mU insulin/ml (5 min), we were able to monitor upregulated rIns1 promoter activity in insulin-producing HIT-T15 cells (Fig. 2*A*), in β-cells of disaggregated islets (Fig. 2*B*), and in β-cells within the intact islet (Fig. 2*C*).

Fluorescent proteins as reporters to simultaneously monitor transcription by multiple promoters in the same cell. We questioned whether the use of different fluorescent proteins would allow us to monitor distinct promoters simultaneously in the same cell. Because of their different excitation and emission profiles, it should be possible to discretely measure the signals generated by GFP_{S65T} and DsRed in the same cell. To test this, we fused the transcription factor pancreatic duodenum homeobox-1 (PDX1) with DsRed and the insulin receptor with GFP, thus targeting DsRed fluorescence to the nucleus and GFP fluorescence mainly to the plasma membrane. When monitored by digital imaging fluorescence microscopy using the appropriate filter sets (DsRed, excitation at 558 nm, emission filter 580LP; GFP_{S65T}, excitation at 488 nm, emission filter BP525/50), PDX1-DsRed was detected only in the nucleus (Fig. 3*B*), whereas HIR-GFP was detected only outside the nucleus (Fig. 3*A*). An overlay of the obtained fluorescence images revealed no overlap of the two signals (Fig. 3*C*).

A second requirement to simultaneously monitor DsRed and GFP_{S65T} is that both fluorophores exert similar kinetics.

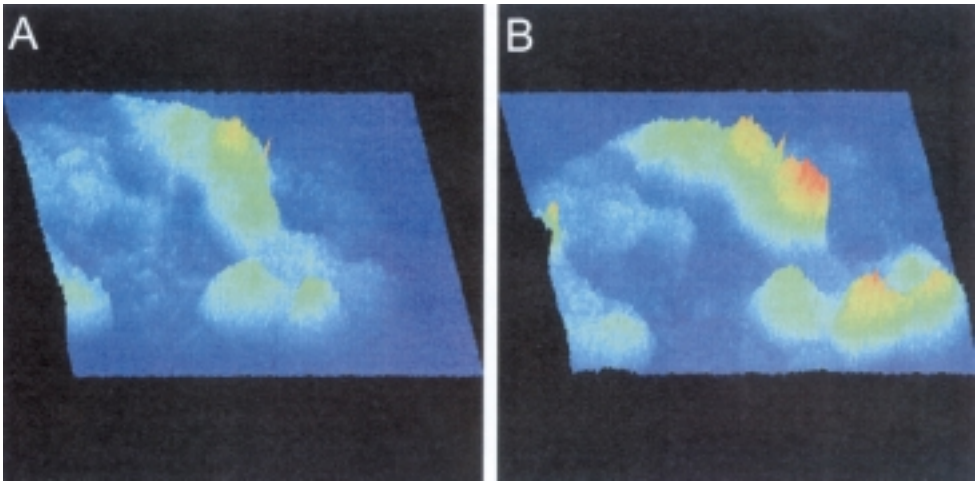

FIG. 1. Online monitoring of rIns1 promoter–driven GFP expression in infected rat islets by laser-scanning confocal microscopy. Isolated islets were infected with Ad-rIns1.GFP (5) and stimulated for 15 min with 16.7 mmol/l glucose. To visualize the increase in fluorescence, the original confocal images of a partial section through the islet obtained at 60 min (*A*) and 240 min (*B*) after the start of stimulation were converted into "intensity-surface-plots" (ISEE software for UNIX). This experiment was repeated three times.

To test this, we placed both DsRed and GFP_{S65T} under the control of the rIns1 promoter and separately monitored DsRed and GFP_{S65T} fluorescence in response to glucose stimulation in transfected HIT cells. As shown in Fig. 4*A*, indeed, both DsRed and GFP_{S65T} show a similar fluorescence profile when their expression is driven by the same promoter.

Next we wanted to know whether DsRed and GFP_{S65T} can be used as reporters to monitor stimulus-induced transcription in the same cell. Therefore, we cotransfected HIT cells with rIns1.DsRed and rIns1.GFP and monitored DsRed and GFP_{S65T} fluorescence in response to glucose stimulation. As shown in Fig. 4*B*, again, both reporters exhibited a similar fluorescence profile. Finally, to demonstrate that this approach can be used to monitor stimulus-induced activation of two different promoters in the same cell, we placed GFP under the control of the rat glucokinase upstream promoter (rbGK.GFP) (–278/+123 bp) and DsRed under the control of the rIns1 promoter (rIns1.DsRed). We have previously shown that the rat glucokinase upstream promoter (–278/+123 bp) can be activated by glucose stimulation (8). After cotransfection of the HIT cells with the two constructs, glucose-stimulated rIns1 (16.7 mmol/l, 15 min) and rβGK promoter activities were monitored as DsRed and GFP fluorescence, respectively. As shown in Fig. 4*C*, this approach allowed us to monitor two different promoter activities in the same cell.

DISCUSSION

In the present study, we demonstrate the applicability of fluorescent proteins as reporter genes to monitor stimulus-induced gene expression online in highly differentiated mammalian cells (i.e., pancreatic β-cells).

Although the potential application of GFP as a reporter gene has been suggested (1,9–12), so far, fluorescent proteins have mainly been used as markers for transfected cells in both tran-

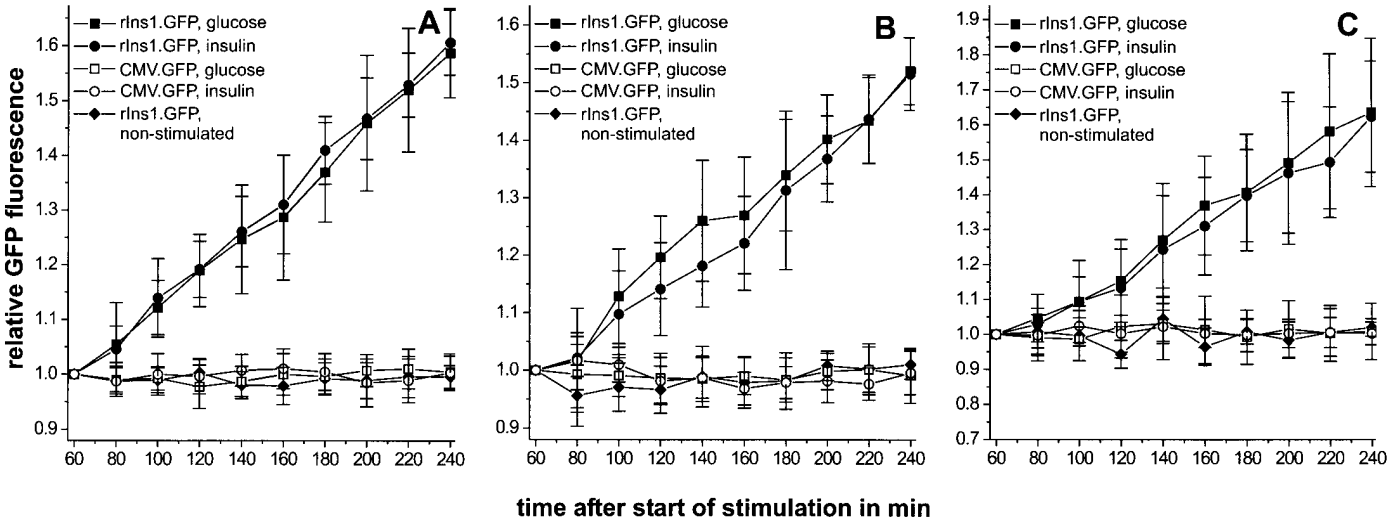

FIG. 2. Online monitoring of GFP expression in transfected HIT cells (*A*), primary β-cells of disaggregated islets (*B*), and infected islets (*C*). Cells transfected with prIns1.GFP (■, ●) and with pCMV.GFP (□, ○) were stimulated for 15 min with 16.7 mmol/l glucose (■, □) or for 5 min with 5 mU/ml insulin (●, ○). Nonstimulated cells transfected with prIns1.GFP are shown (◆). Data are means ± SD (*n* = 8).

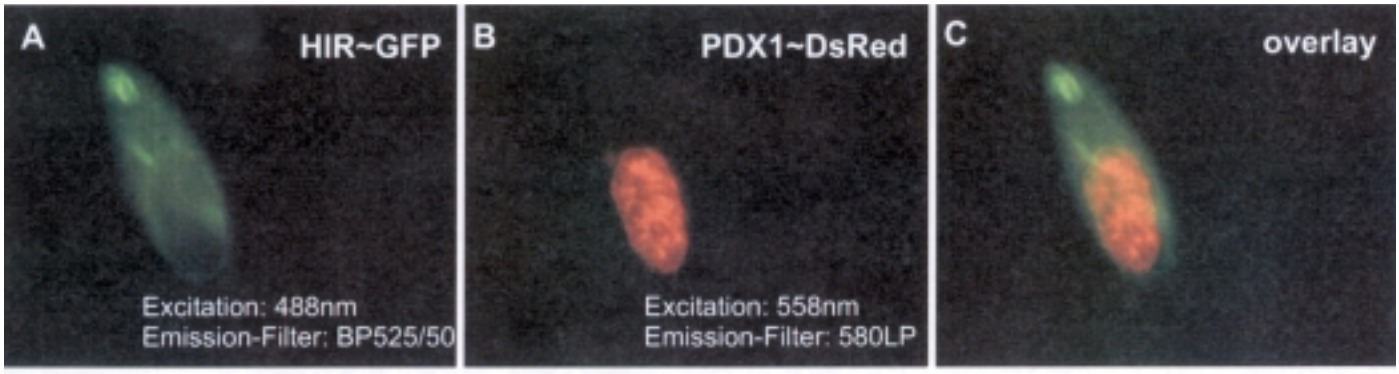

FIG. 3. Discrete imaging of GFP and DsRed fluorescence in cells coexpressing HIR-GFP (*A*) and PDX1-DsRed (*B*) by digital imaging fluorescence microscopy. The overlay (*C*) demonstrates the distinct separation of the two colors. Representative images of a total of nine are shown.

sient and stable expression systems. Their use as dynamic and semiquantitative reporters to monitor stimulus-induced promoter activity in mammalian cells has been exploited sparsely (4,13–15). The application of fluorescent proteins to monitor gene expression online has recently been discussed for micro-organisms (11). We have for the first time described the use of GFP_{S65T} as a reporter gene when studying short-term induced insulin gene expression in primary pancreatic β-cells (4,13). This approach not only allowed us to overcome the limitation set by the amount of tissue when working with primary islet cells but also demonstrated its advantage when studying stimulus-induced transcription. Because the set-up allowed us to monitor stimulus-induced transcription in the same cell before, during, and after stimulation, no artificial normalization was required. Because of the sensitivity of the approach, this enabled us to directly evaluate the role of *cis*-elements in glucose/insulin–stimulated insulin promoter activation, studying respective promoter mutants (13). Using adenovirus-based vectors for gene transfer, we were able to monitor stimulus-induced transcription in individual β-cells in an intact micro-organ, i.e., the islet of Langerhans.

Taken together, our results clearly show that fluorescent proteins can be used as reporter genes to monitor online stimulus-induced gene expression. They offer great flexibility, enabling us to measure gene expression in single cells, cell clusters, and intact tissues. The use of fluorescent proteins exhibiting similar expression kinetics and emission profiles that can be distinctly detected, such as GFP_{S65T} and DsRed, as demonstrated in this study, provides the opportunity to simultaneously monitor the activity of more than one promoter within the same cell. The combinatory use of fluorescent proteins and the high sensitivity in signal detection enable studies ranging from stimulus-induced gene expression in poorly transfectable primary cells to the high-throughput screening of pharmacological compounds.

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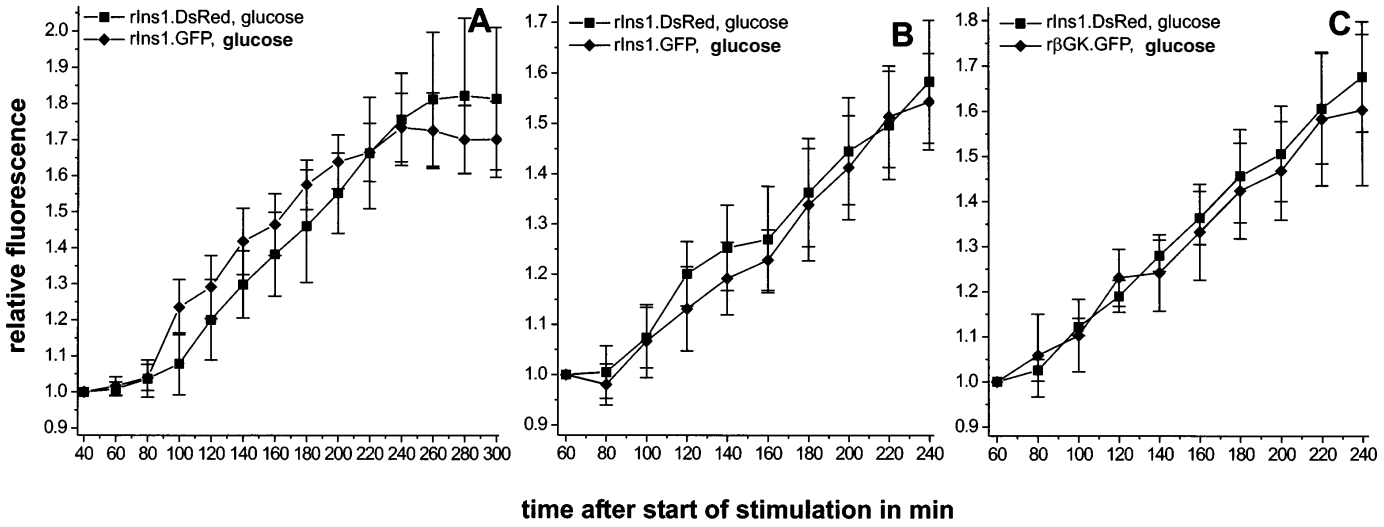

FIG. 4. Online monitoring of glucose-stimulated (16.7 mmol/l, 15 min) expression of GFP and DsRed in transfected HIT cells. *A*: Comparison of the kinetics of rIns1 promoter–driven DsRed (■) with that of rIns1 promoter–driven GFP (◆) when expressed separately. *B*: Simultaneous monitoring of GFP (◆) and DsRed (■) in cells cotransfected with prIns1.GFP and prIns1.DsRed. *C*: Simultaneous monitoring of rIns1 promoter–driven DsRed expression (■) and rβGK promoter–driven GFP expression (◆). Data are means ± SD (n = 11).

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