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Mechanisms of transcriptional regulation underlying temporal integration of signals

Dominique A. Glauser and Werner Schlegel*

Fondation pour Recherches Médicales, University of Geneva, Switzerland

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ABSTRACT

How cells convert the duration of signals into differential adaptation of gene expression is a poorly understood issue. Signal-induced immediate-early gene (IEG) expression couples early signals to late expression of downstream <*target*> genes. Here we study how kinetic features of the IEG-<*target*> system allow temporal integration of stimuli in a pancreatic beta cell model of metabolic stimulation. Gene expression profiling revealed that beta cells produce drastically different transcriptional outputs in response to different stimuli durations. Noteworthy, most genes (87%) regulated by a sustained stimulation (4 h) were not regulated by a transient stimulation (1 h followed by 3 h without stimulus). We analyzed the induction kinetics of several previously identified IEGs and <*targets*>. IEG expression persisted as long as stimulation was maintained, but was rapidly lost upon stimuli removal, abolishing the delayed <*target*> induction. The molecular mechanisms coupling the duration of stimuli to quantitative <*target*> transcription were demonstrated for the AP-1 transcription factor. In conclusion, we propose that the network composed of IEGs and their <*targets*> dynamically functions to convert signal inputs of different durations into quantitative differences in global transcriptional adaptation. These findings provide a novel and more comprehensive view of dynamic gene regulation.

INTRODUCTION

Gene expression in mammalian cells is controlled by a wide variety of extracellular and intracellular signals. Most signals, which regulate gene expression, are initially transduced in the cytoplasm through the production of second messengers and the activation of cascades of protein modifications (1). These cascades end in the nucleus with the activation of transcription factors, which control the transcription of specific genes. Genes that are regulated directly downstream of these signal transduction pathways are called immediate-early responsive genes (IEGs) (2,3). The regulation of IEG transcription occurs very rapidly since it is independent of *de novo* protein synthesis. Many IEGs code for transcription factors that in turn will regulate the transcription of downstream target genes; we will refer to these genes as <*targets*> in the rest of the manuscript. A 'transcriptional network' links IEG transcription factors to the expression of cell specific sets of <*target*> genes. Thus transmission of information involved in gene transcription control occurs initially in signaling pathway networks and is then pursued in transcriptional networks.

Signaling networks have been extensively studied. Substantial efforts have been devoted to their qualitative description and to the quantification of their dynamic properties. Based upon such knowledge, modeling and computational analysis provides insights on emergent properties of these complex systems that underlie the processing of signal information (4). The kinetic behavior of specific components within signaling networks is a key element in the functioning of these networks, since these latter include complex feedback loops and generate distinct outputs in the various cellular compartments depending on signal duration. Thus, it is now well accepted that progression of information through signaling pathway networks constitutes a mode of signal integration.

The question that we address here is whether a similar integration of signals occurs in transcriptional networks. In particular, we ask whether a system composed of IEGs and their downstream <*target*> genes would differentiate between long and short signals to produce distinct transcriptional outputs. This question is particularly relevant for metabolic signaling in the pancreatic beta cell. Beta cells function as sensor of the blood nutrient availability. They respond e.g. to an elevation in blood glucose concentration by increased insulin secretion, which in turn favors glucose uptake into muscle and fat, thereby lowering blood glucose. This secretory response is potentiated by hormones released from the gut, such as glucagon-like peptide-1 (GLP-1), which activates cAMP production in beta cells (5).

In addition to activating insulin secretion, nutrients, in conjunction with cAMP, also regulate gene expression (6,7)

*To whom correspondence should be addressed at Fondation pour Recherches Médicales, Avenue de la Roseraie 64, 1211 Geneva, Switzerland. Tel: +41 22 382 38 11; Fax: +41 22 347 59 79; Email: werner.schlegel@medecine.unige.ch

mediating thereby long term adaptation of beta cells according to changes in diet and insulin demand (8–10). Circulating nutrients are fluctuating widely upon food intake or due to varying energy expenditure or the mobilization of reserves during stress responses. Since metabolic activation of the beta cell is fluctuating accordingly, the initial signaling input into gene expression control is widely variable. To adapt the beta cell performance, gene expression should take into account the longer term behavior of the system. To this end, metabolic signals have to be integrated over time.

As the mechanisms of adaptation are crucial to beta cell function and are likely impaired during progression of diabetes, understanding the dynamics of gene regulation in this system is of particular interest. In addition, to our knowledge, this represents the only system in which IEGs and their <*targets*> have been identified at a genomic scale (Glauser,D. A., Brun,T., Gauthier,B. R. and Schlegel,W. Immediate-early genes as 'third messengers': functional genomics bring a new perspective to an old concept, manuscript submitted).

The present study demonstrates that the pancreatic beta cell effectively produces distinct global transcriptional outputs when activated by metabolic stimuli for various times. Emergent properties of the IEG-<*target*> transcriptional system provide a global mechanistic explanation based upon the kinetics of the induction, distinct between IEGs and <*targets*>. IEG expression continuously follows the levels of stimulation, whereas <*targets*> respond with a delay. A proof-of-concept for this mechanism of temporal integration of metabolic signaling was given through the specific analyses of the IEG-encoded AP-1 transcription factor and its downstream <*target*> genes.

MATERIALS AND METHODS

Chemicals

Chlorophenylthio-cAMP (cpt-cAMP) and Glucagon-like peptide-1 (GLP-1)(7–37, human) were purchased from Sigma (Buchs, Switzerland).

Min6 cell culture and incubations

Min6 B1 cells (11) (generously provided by Dr Philippe Halban, Department of Genetic Medicine and Development, University Medical Center, Geneva) (passage 15–28) were cultured in DMEM supplemented with 15% fetal calf serum (FCS), 25 mM glucose, 71 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin. Before stimulation, culture medium was changed to low glucose medium (same as above with 1% FCS and 1 mM glucose) for a pre-stimulatory period of 20 h.

RNA preparations

Total RNA preparations were made using RNeasy Micro Kit (Qiagen) or with Tri Reagent (Molecular Research Center, Inc.).

Microarray experiment

All the stimulations were performed at the same time (in parallel), with the same batch of cells plated at uniform confluence. For each experimental condition (control, sustained stimulation or transient stimulation), transcript profiles were established for three different preparations of total RNA made using RNeasy Micro Kit. Labeled cRNA synthesis, hybridization to the arrays and scanning were essentially performed as described earlier (12). Affymetrix Mouse Genome 430 2.0 oligonucleotide array (containing probe features for 45 101 transcripts) were used. Fluorescence signals from the arrays were analyzed with Affymetrix software GCOS for normalization and calculation of gene-expression values (13,14).

A conjunction of three criteria was used to consider genes as differently expressed. First, a minimal fold-change values of 1.5. Second, a *P*-value by Welch *t*-test below 0.05 when comparing signal values in two experimental conditions. Third, a good concordance between replicates. The strategy to evaluate the concordance of an effect in two different experimental conditions was the following: each replicate of one condition was compared to each replicate of the other, resulting in nine pairwise comparisons. Transcripts were considered as differentially expressed if their levels changed in the same direction in at least 7/9 comparisons.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Each total RNA sample was reverse-transcribed in triplicate with random hexamers as primers and Omniscript reverse transcriptase (Qiagen). Quantitative real-time PCR were performed with the SYBR Green system as described in Brun *et al*. (15). Primers were synthesized by Microsynth (Balgach, Switzerland). Their sequences are described in Supplementary Table S1. For normalization, 18S rRNA was quantified using 0.3× 18S rRNA Predeveloped Assay Reagent and 1× TaqMan® Universal PCR Master Mix (Applied Biosystems). PCR amplicons were quality controlled and all displayed a single homogeneous melting curve as well as the correct size on 2% agarose gels. A cDNA serial dilution standard curve was added to the microtiter plate of each amplification reaction to calibrate each relative quantification in function of PCR amplification efficiency.

Nuclear extract preparation and DNA-binding assay

Nuclear protein extracts were prepared according to the protocol of Schreiber *et al*. (16). The detection of c-FOS and JUND specific binding to AP-1 site was made with the enzyme-linked immunosorbent assay (ELISA)-like TransFactor Kit Inflammation II (BD Biosciences AG, Switzerland) according to the supplier's instructions except that a chemiluminescent detection step replaced the colorimetric one. Briefly, after initial blocking, 12 μg of nuclear extracts were incubated 60 min in AP-1 or STAT consensus oligo coated 96-well plates. Plates were then washed three times, incubated 60 min with primary antibodies (anti-c-FOS or anti-JUND), washed three times and incubated 30 min with HRPO-anti-rabbit-IgG secondary antibody (Transduction Laboratories) (1:10 000). After final four washes, 100 μl of 1× ECL horseradish peroxidase (HRP) substrate (Cell Signaling Technology) were added to each well and light emission measured three times with a FLUOStar OPTIMA (BMG LABTECH GmbH). Binding to coated STAT oligo and competition with soluble AP-1 oligo were used to check

binding specificity. Results were expressed in arbitrary units of DNA-binding after normalization by values of no template controls (NTC) for each independent experiment.

Luciferase reporter analysis

A total of 0.5 μg PathDetect® cis-Reporting System pAP-1-Luc or pCIS CK (negative control) plasmids (Stratagene Europe, Amsterdam Zuidoost, The Netherlands) were co-transfected with 0.5 μg of Renilla luciferase plasmid (for normalization) (Promega, Luzern, Switzerland) using Lipofectamine 2000 reagent (Invitrogen) according to supplier's instructions. After transfection, cells were maintained for 20 h in culture medium, changed to low glucose medium for additional 20 h, and stimulated for 6 h with 10 mM glucose and 0.2 mM cpt-cAMP (sustained) or stimulated for 1 h followed by 5 h at low glucose (transient). Luciferase activity measurement was performed as described previously (17).

Western blotting

Nuclear extracts (15 μg) were resolved on SDS–PAGE (10% gel) and subject to immunoblot analysis as described earlier (18). Primary antibodies were rabbit anti-c-FOS (1:1000, sc-52) and anti-TFIIB (1:10 000, sc-225) (Santa Cruz Biotechnology, Inc.).

Microarray dataset

The microarray dataset has been submitted to Array Express database (http://www.ebi.ac.uk/arrayexpress) under accession number E-TABM-141.

RESULTS

Global transcriptional output of pancreatic beta cells in response to transient metabolic stimulation is distinct from output obtained by sustained stimulation

In a gene expression profiling screen, we have recently identified several hundred genes for which transcript levels were changed after 4 h stimulation with high glucose and cpt-cAMP (an analogue of cAMP) (Glauser,D. A., Brun,T., Gauthier,B. R. and Schlegel,W. Immediate-early genes as 'third messengers': functional genomics bring a new perspective to an old concept, manuscript submitted). Notably, these included a substantial proportion of IEGs, which are known to respond rapidly to stimuli (normally within 1 h). We wondered whether the persistence of stimulation over the 4 h was necessary to detect IEG transcript accumulation at this time point and, more generally, what would be the global result of reducing the stimuli duration. Further expression profiling analyses using high density oligonucleotide microarrays were thus performed to address these questions. We compared three conditions: (i) a control condition, (ii) a sustained stimulation condition (4 h stimulation with high glucose plus cpt-cAMP) and (iii) a transient stimulation (1 h stimulation followed by 3 h at low glucose). Figure 1A shows a schematic representation of the stimulation protocol; Figure 1B a simple summary of the results.

Sustained stimulation up-regulated 660 genes and down-regulated 1198 genes (1.5-fold change cutoff, see Supplementary Table S2). Transient stimulation up-regulated 169 genes and down-regulated 227 genes. Strikingly, most genes (87%) regulated by sustained stimulation were not regulated by transient stimulation (Figure 1B and Supplementary Table S3); the remaining genes were essentially regulated to a similarly extent (9%) and only few genes (<1%) had improved regulation upon transient stimulation. Both IEGs and their <*targets*> were regulated differentially by transient versus sustained stimulation (Supplementary Table S4).

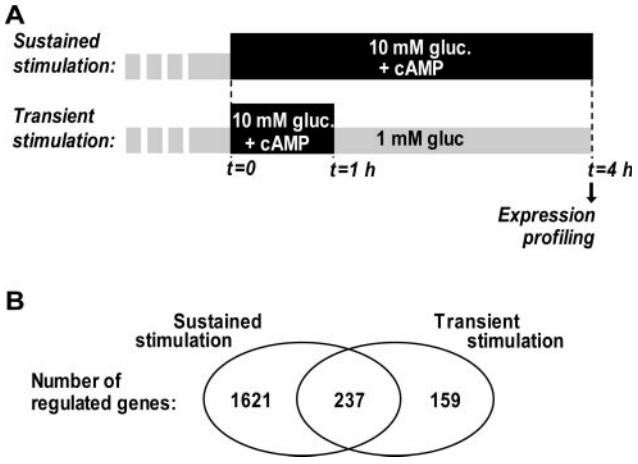

Figure 1. Metabolic stimulations of different durations produce different global transcriptional outputs. (**A**) Schematic representation of the two types of stimulations (sustained and transient) used for the expression profiling experiment. (**B**) Venn diagram representing the number of genes regulated by sustained stimulation, by transient stimulation, and by both. Expression profiles were determined using the Affymetrix Mouse Genome 430 2.0 oligonucleotide array (comprising probes for ~45 000 transcripts). Regulated genes were determined by comparing each stimulated condition with an unstimulated control condition for which cells were maintained in preincubation medium at 1 mM glucose throughout.

These gene array hybridization results were verified with quantitative RT-PCR analysis, which confirmed for a series of induced genes, both <*targets*> and IEGs, a significant decrease in transcript levels following transient stimulation compared to sustained stimulation (Figure 2). Irrespective of the various induction levels reached for different genes by a sustained 4 h stimulation (note the various scales in Figure 2), transient 1 h stimulation was mostly insufficient to cause elevated transcript levels at 4 h. i.e. after a further 3 h at low glucose.

In addition, the microarray data showed that a significant number of genes are exclusively regulated by transient but not by sustained stimulation (159 genes; 74 up-regulated, 85 down-regulated; Figure 1B). Altogether, these results demonstrate that beta cell produces very distinct gene regulatory outputs at a given time when stimulated for various durations within the total incubation period.

Sustained expression of IEGs due to sustained metabolic stimulation

IEG induction is most often studied after a transient stimulus of resting cells with growth factors or following exposure to cellular stress. IEGs are thus viewed in general as transiently arising mediators that will drive a cell into a new program, be it division, differentiation or programmed cell death. Much

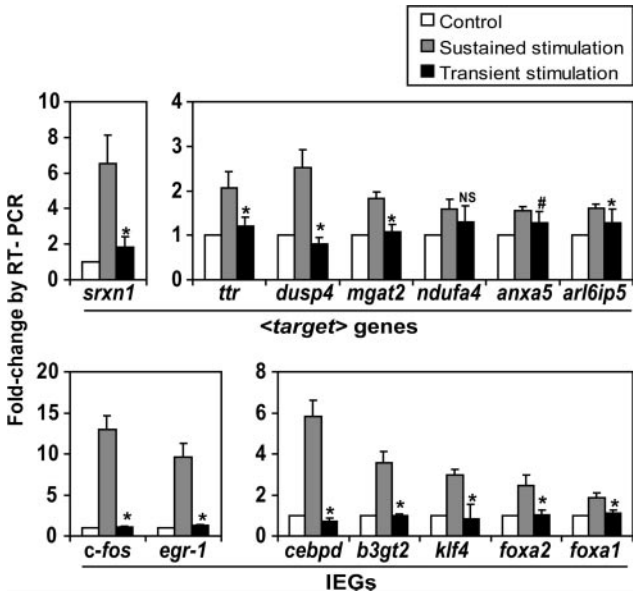

Figure 2. Validation of gene expression profiling by RT-PCR. mRNA levels for indicated genes were assessed by quantitative real-time RT-PCR and normalized with 18S rRNA. Results are expressed as mean of fold change compared to control condition (SD as error bars, n = 5). Comparison with sustained stimulation by Student *t*-test: *, $P < 0.01$; #, $P < 0.05$; NS, non significant.

overlooked is the fact the many IEGs are continuously being expressed, also in a <resting state>. Furthermore, altered expression levels are detected after 4 h of stimulation for a very large number of IEGs (1278 IEGs, Supplementary Table S2).

In Figure 3 we show the kinetics of mRNA levels for 4 IEGs during the sustained 6 h metabolic stimulation of beta cells (solid dots) in comparison with the evolution of mRNA levels following a transient 1 h stimulation (open circles). These IEG examples were chosen to cover a large range of activation (with fold-changes from 25 to 2), and to represent different functions [two transcription factors (c-*fos*, *kfl4*), a signaling enzyme (*sgk1*), and a metabolic enzyme (*b3gt2*)]. The strongly and rapidly induced IEGs c-*fos* and *klf4* reach a peak after 1 h of stimulation. Subsequently, mRNA levels decline differentially: after transient stimulation, prestimulatory levels are observed already at 2 h; in contrast, sustained stimulation leads to an elevated steady-state, which is maintained over at least 6 h. IEGs, such as *sgk1* and *b3gt2* which are induced to a lesser extent, clearly show that only sustained stimulation leads to a sustained elevated steady-state mRNA level, whereas transient stimulation produces a peak of induction, which is lost at 2 h, when prestimulatory mRNA levels are re-established.

Taken together, these results indicate that IEG mRNA accumulation at late time points (4 h for the microarray data) is not only residual to an initial induction, but that further regulatory mechanisms occur afterward.

Steady-state IEG expression levels are adjusted according to beta cell stimulatory state

Establishment of a new expression steady state upon sustained stimulation suggests that IEG induction is not solely a response to a sudden metabolic change, but rather that the levels of IEG expression are continuously adjusted according to metabolic status of the cell. We wondered whether this effect would occur on a longer time scale and whether this system will also sense subtle variations in the intensity of a metabolic stimulation. Thus, we determined the expression levels of two IEGs (c-*fos* and *sgk1*) after long-term culture (20 h) at various glucose concentrations (from 1 to 25 mM, but without co-stimulant). The results in Figure 4 show that the expression levels of both IEGs are gradually adjusted with increasing glucose levels. Notably, maximal variations occur within a physiological range of glucose concentrations between 5 and 10 mM. With these mild stimulations (as cpt-cAMP co-stimulant was not used), mRNA levels for the moderately-responding *b3gt2* gene were not significantly affected (data not shown). However, after 20 h of co-stimulation with high glucose plus cpt-cAMP, we observed mRNA levels for *b3gt2* and c-*fos* that were similar to those observed at 6 h (Figure 3 and data not shown).

These results indicate that IEG expression levels are continuously adjusted according to the stimulatory level of the cell. This effect is gradual such that steady state levels of mRNA reflect the graded intensity of metabolic stimulation. As IEG transcript levels respond within minutes to either increased or decreased signal intensity, the system has the capacity to integrate the temporal pattern of stimulation.

Stimuli interruption, which shortens IEG expression, impairs the delayed impact on <*target*> transcription

We also analyzed the kinetics of <*target*> gene responses to transient (1 h) versus sustained metabolic stimulation. Upon sustained stimulation, significant changes in <*target*> gene expression were observed after 1 or 2 h (Figure 5). Thus, <*target*> induction is delayed compared to IEG induction (compare Figures 5 and 3). These findings are in agreement with the notion that IEG transcription factors relay intracellular signals by acting upstream of <*target*> gene expression. Indeed, the time required to synthesize IEG products is expected to delay <*target*> induction following stimulation. As a consequence of this delay, shortening the stimuli (transient stimulation) abrogated the regulation of most <*target*> genes examined (confirming results in Figures 1 and 2). This suggests that the temporal pattern of metabolic signals, which is integrated in IEG expression, will markedly affect the expression of downstream <*target*> genes. Thus, by using the IEGs as relay between signaling and <*target*> gene expression, pancreatic beta cells would discriminate between transient and prolonged stimulation.

Upon stimuli removal, reduction of c-*fos* mRNA is followed by reduced c-FOS protein level in AP-1 complex and decreased transcription of AP-1 <*targets*>

Further experiments were undertaken to address whether the shortening of IEG mRNA accumulation could really explain the impaired regulation of <*targets*> upon transient stimulation. We chose to study the AP-1 transcription factor, since previous results had shown a predominant role for this IEG-encoded transcription factor in the regulation of glucose and cAMP-responsive <*target*> genes, (such as *srxn1*) (Glauser,D. A., Brun,T., Gauthier,B. R. and Schlegel,W.

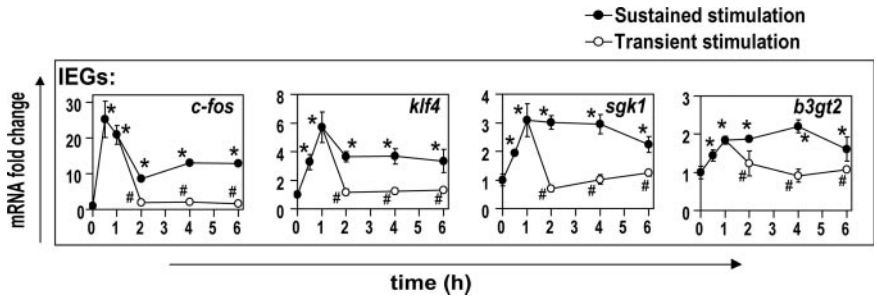

Figure 3. Kinetics of IEG expression during sustained or transient metabolic stimulation. Min6 cells cultured at low glucose were stimulated with high glucose (10 mM) and cpt-cAMP (0.2 mM) for the indicated period of time. For transient stimulation, medium was replaced with low glucose medium after 1 h of stimulation. mRNA levels for indicated genes were quantified by real-time RT-PCR and results expressed as mean (±SD) of fold change values relative to basal condition (n = 3). *, $P < 0.05$ versus basal condition; #, $P < 0.05$ versus sustained stimulation at the corresponding time point, by Student *t*-test.

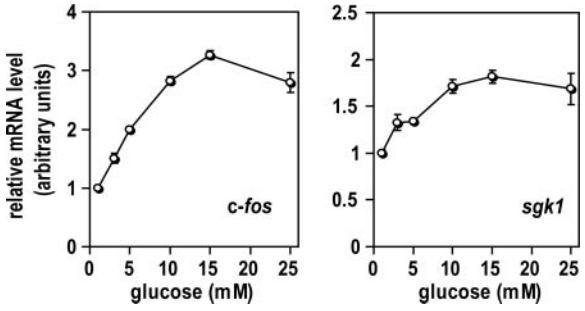

Figure 4. Steady-state expression levels of IEGs are adjusted according to glucose concentration. Min6 cells were cultured at indicated glucose concentrations for 20 h. mRNA levels for indicated genes were assessed by quantitative real-time RT-PCR and normalized with 18S rRNA. Results are expressed as mean of fold change compared to control condition (±SD, n = 3).

Immediate-early genes as 'third messengers': functional genomics bring a new perspective to an old concept, manuscript submitted).

A 4 h co-stimulation with glucose and cAMP alters the expression pattern of genes coding for constituents of the AP-1 complex, i.e. of *fos* and *jun* genes. Our microarray data presented in Figure 6 show the induction of *fosB*, *fra-1*, *fra*-2, *junB* and most predominantly, c-*fos*. This pattern of alteration is completely abolished under a protocol of transient stimulation, i.e. 1 h of elevated glucose followed by 3 h of low glucose (as above in Figures 1–3 and 5). The microarray data were confirmed by quantitative RT-PCR (Figure 2 and D. A. Glauser and W. Schlegel, unpublished data).

To be the cause of the negative impact on AP-1 <*targets*>, the rapid decrease in the mRNA coding for an AP-1 component must be effectively followed by a rapid decrease in the corresponding protein. We thus investigated the kinetics of c-FOS protein levels by immunoblotting. During sustained stimulation c-FOS protein levels were increased within the first hour and maintained thereafter. Conversely, in the transient stimulation protocol, c-FOS level was rapidly and markedly reduced after stimuli removal (within 2 h, Figure 7A). We obtained similar results for JUNB (data not shown). Thus, the turn-over of the proteins which constitute AP-1 is sufficiently rapid for the changes in mRNA levels to be readily translated into changes in AP-1 proteins.

c-FOS activates transcription when binding to DNA as part of the AP-1 complex. We quantified *in vitro* c-FOS binding to solid phase tethered dsDNA with the AP-1 consensus sequence. This constitutes a measure of how much c-FOS is present in actively DNA-binding AP-1 complexes in nuclear extracts obtained from cells after various stimulation protocols (Figure 7B). We stimulated cells with glucose and GLP-1, a gut hormone that physiologically raises cAMP in pancreatic beta cells (8). c-FOS was recruited to AP-1 within the first hour of stimulation. While c-FOS DNA-binding activity was maintained after 3 h of sustained stimulation, it was drastically reduced upon removal of the stimuli (transient stimulation). In parallel, JUND was constantly present in AP-1 complexes, consistent with *junD* expression levels, which were unchanged by the treatments (see Figure 6).

Expression of c-FOS and its recruitment to AP-1 complex can induce AP-1 <*target*> gene transcription. Thus, as c-FOS rapidly disappears from AP-1 after stimuli removal, this treatment should reduce transcriptional activation of AP-1 <*target*> genes. In order to test this, we transfected Min6 cells with the pAP-1-luc reporter vector. In this construct, a luciferase reporter gene is under the control of an artificial promoter harboring solely AP-1 sites as enhancer sequences (see scheme in Figure 7C). The effects of sustained versus transient stimulation on the transcription of this reporter were compared. The transcriptional activation observed upon sustained stimulation was drastically reduced under transient stimulation (Figure 7D). Similar results (data not shown) were obtained with a reporter under the control of the promoter of *srxn1* gene (a known AP-1 direct <*target*>). In addition, accumulation of endogenous *srxn1* mRNA levels was also dependent on stimuli duration (Figure 2). Thus, a prolonged recruitment of c-FOS to AP-1 is necessary to produce a full induction of AP-1 <*target*> genes.

In summary, the data presented in Figures 6 and 7 show the differential effects of sustained versus transient stimulation on AP-1 composition and function. A sustained stimulation produced a prolonged accumulation of c-FOS, leading to its persistent recruitment to AP-1 and strong transactivation of <*targets*>. To the opposite, a transient stimulation produced only short expression of c-FOS, which rapidly disappeared from AP-1, leading to weak transactivation of <*targets*>. This constitutes a specific mechanistic example by which IEG expression integrates the temporal pattern of stimulation to convert it into differential output in <*target*> gene transcription.

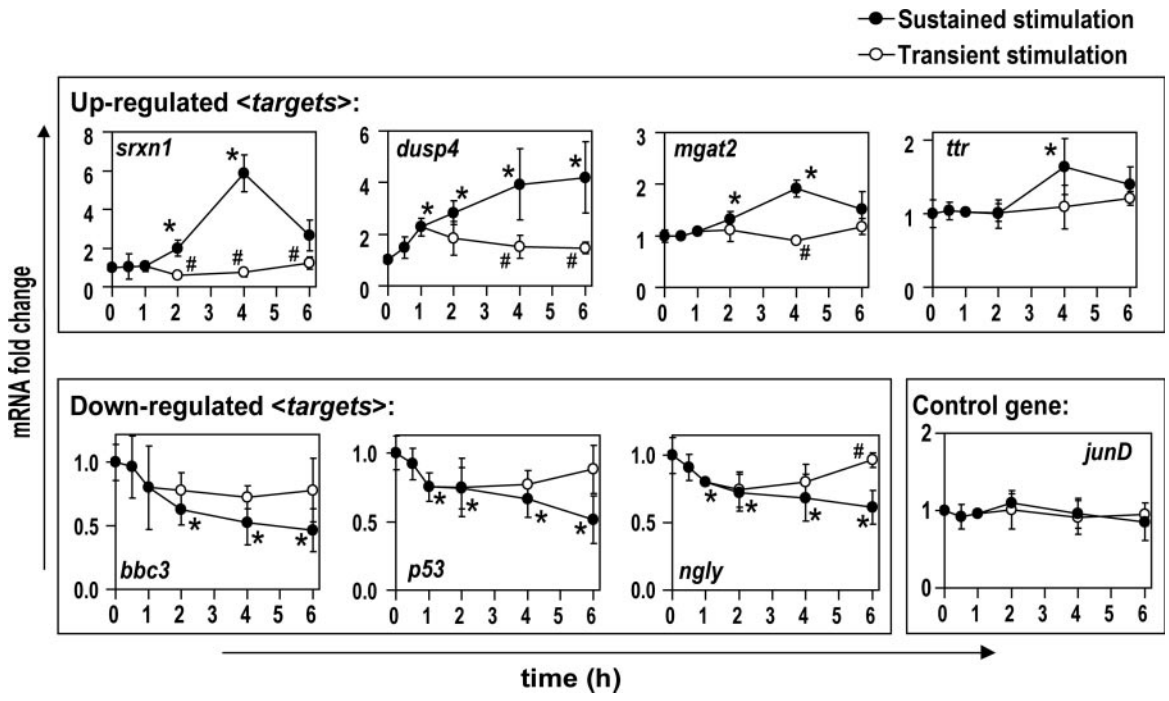

Figure 5. Kinetics of <*target*> gene expression during sustained or transient metabolic stimulation. Min6 cells cultured at low glucose were stimulated with high glucose (10 mM) and cpt-cAMP (0.2 mM) for the indicated period of time. For transient stimulation, medium was replaced with low glucose medium after 1 h of stimulation. mRNA levels for indicated genes were quantified by real-time RT-PCR and results expressed as mean (±SD) of fold change values relative to basal condition (n = 3). *, $P < 0.05$ versus basal condition; #, $P < 0.05$ versus sustained stimulation at the corresponding time point, by Student *t*-test.

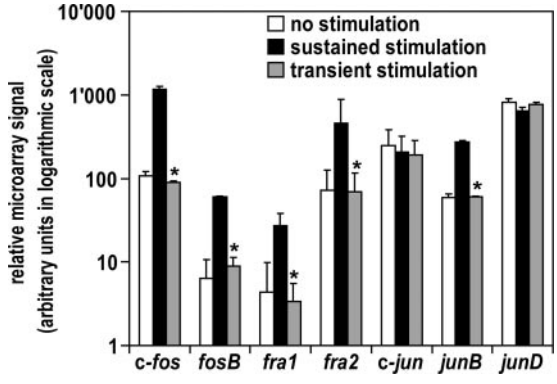

Figure 6. Expression pattern of AP-1 component genes is modulated according to the length of stimulation. A schematic representation of the two stimulation protocols—sustained or transient—is shown in Figure 1A. Data shown are selected from the expression profiling experiment summarized in Figure 1B. Expression profiles were determined using the Affymetrix Mouse Genome 430 2.0 oligonucleotide array. Shown are the means of gene-expression values obtained in the indicated condition, represented on a logarithmic scale (with SD as error bars, n = 3). *, $P < 0.01$ versus sustained stimulation, by Student *t*-test.

Gradual stimuli durations, which produce distinct kinetics of c-*fos* induction, result in graded expression levels of the c-*fos* target gene *srxn1*

As a final test for our proposal, we investigated the effects of graded stimuli durations on the expression of c-*fos* and on its target *srxn1*. To that purpose we used three stimuli durations: (i) a short stimulation (transient stimulation of 1 h followed by 3 h at low glucose), (ii) an intermediate stimulation (transient stimulation of 160 min followed by 80 min at low glucose) and (iii) a long stimulation (sustained stimulation maintained throughout the 4 h of the experiment). As expected, these stimulations of various durations resulted in distinct kinetic patterns of c-*fos* expression (Figure 8A). *srxn1* transcript levels measured at the end of the stimulation period (4 h) varied quantitatively (Figure 8C). Indeed, an ANOVA statistical test showed a significant effect of stimuli duration on *srxn1* mRNA levels [$F(2,11) = 103.5$, $P < 0.001$] and gradation of the effect was confirmed by Tukey Post Hoc tests ($P < 0.01$). The final *srxn1* expression levels correlate with the calculated values for the Area under the curve (AUC) of c-*fos* kinetic (compare Figure 8B and C), as both parameters reflect the duration of stimulation. These findings are consistent with our proposal that temporal integration of metabolic signals (leading to adaptation of gene expression) results from the dynamic properties of the IEG-<*target*> system. It illustrates how this system can produce finely graded mRNA outputs for <*target*> genes according to the duration of metabolic activation.

In summary, we show here that pancreatic beta cells produce different transcriptional outputs upon metabolic stimulations of various durations. A sustained activation, which encompasses sustained elevations of steady state expression levels of IEGs, results in strong induction of <*target*> genes, presumably since the mechanisms of <*target*> gene induction require a lasting action of IEG derived transcription factors. In contrast, short transient metabolic activation, which induces strong but transient IEG expression, has little effect on <*target*> gene expression; intermediary protocols produce an intermediate transcriptional output. In this manner, IEG expression, widely known as a correlate of single important changes in cell fate, becomes a paradigm for sustained finely tuned gene

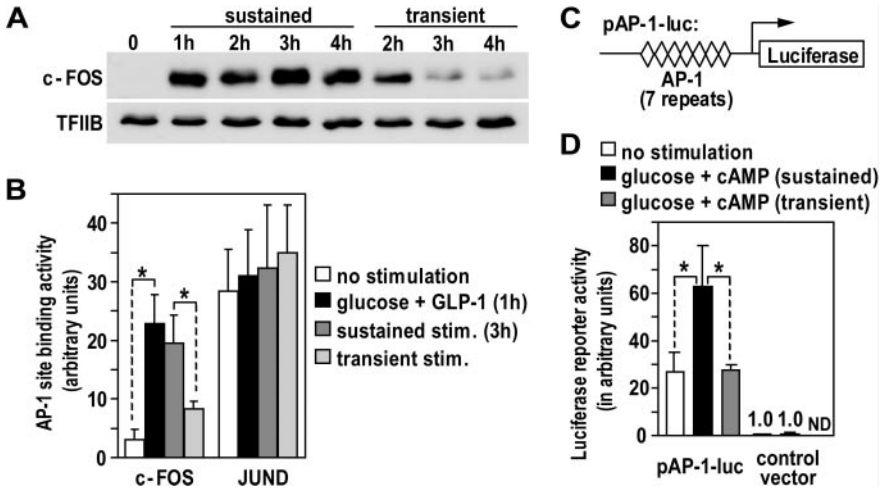

Figure 7. Upon the withdrawal of metabolic stimuli c-FOS protein rapidly disappears from AP-1 complexes, reducing AP-1-dependent reporter gene transcription. (**A**) Min6 cells cultured at low glucose were stimulated with high glucose (10 mM) and cpt-cAMP (0.2 mM) and harvested after indicated period of time. Nuclear extracts were analyzed by immuno-blotting for c-FOS protein or TFIIB (loading control). (**B**) Cells cultured at low glucose were stimulated with 10 mM glucose and 10 nM GLP-1 and collected at 1 or 3 h for the preparation of nuclear extracts. c-FOS and JUND protein in AP-1 complexes bound to solid phase tethered dsDNA with AP-1 consensus sequence were quantified by ELISA. For transient stimulation, cells were stimulated 1 h followed by 2 h in low glucose medium. (**C**) Schematic representation of pAP-1-luc reporter gene. (**D**) Min6 cells were transfected with pAP-1-luc (or control vector) and maintained at low glucose before stimulation with 10 mM glucose and 0.2 mM cpt-cAMP for 6 h (sustained), or for 1 h followed by 5 h at low glucose (transient). *, $P < 0.01$ ($n = 4$), by Student *t*-test. Error bars: SD.

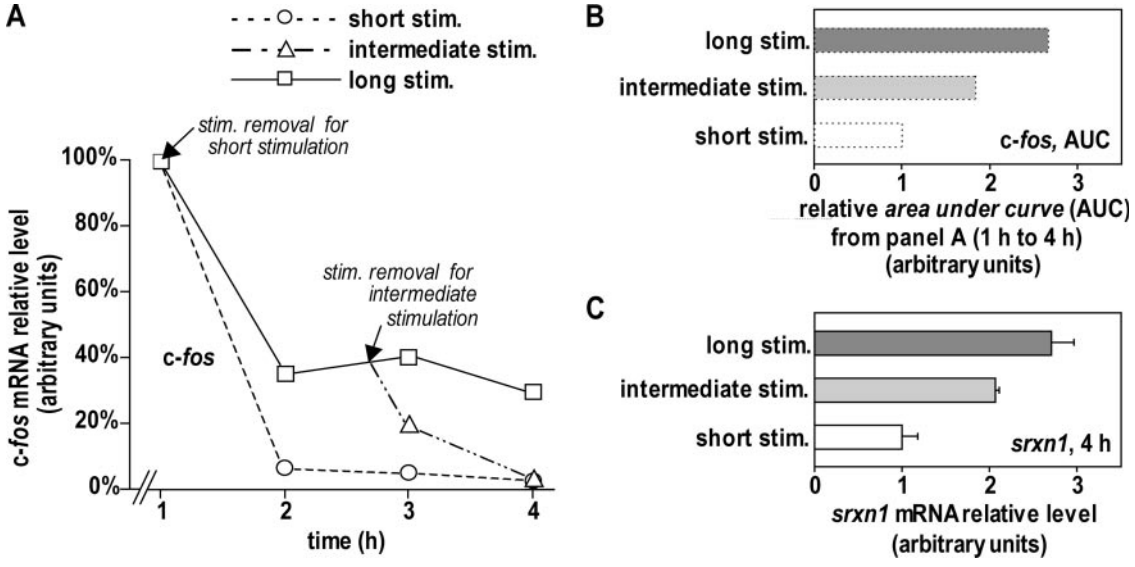

Figure 8. Gradual differences in stimuli duration result in distinct kinetic patterns of c-*fos* expression and produce gradual differences in the transcript level of the c-*fos* target gene *srxn1*. Min6 cells were co-stimulated with 10 mM glucose and 0.2 mM cpt-cAMP (at time 0), and gene expression was measured every hour over a period of 4 h. c-*fos* and *srxn1* mRNA levels were quantified by RT-PCR and results were normalized with 18S rRNA. Three types of stimulations of graded durations were used: first, a short stimulation (a transient stimulation of 1 h followed by 3 h without stimulus); second, an intermediate stimulation (a transient stimulation of 160 min followed by 80 min without stimulus) third, a long stimulation (sustained stimulation throughout the 4 h). (**A**) Kinetic of c-*fos* induction with the different stimulation protocol, presented as mean values ($n = 3$; SD below 24% of the mean values). (**B**) Area Under Curve (AUC) calculated between 1 and 4 h from (A). (**C**) Relative *srxn1* mRNA levels at the end of experiment (4 h), shown as mean values ($n = 3$; ±SD).

expression needed for cell adaptation. The relay role of IEG expression thereby serves as a mechanism of temporal signal integration.

DISCUSSION

Gene regulatory mechanisms underlying adaptive processes in bacteria and yeast have historically been the first examples to show the essential elements of the control of gene expression (19). More recent studies in these single cell model organisms helped defining the structure of gene regulatory networks. However so far, few studies have addressed the dynamic properties of these systems (20–22). The present study provides evidences in a mammalian model that a transcriptional network system composed of IEGs and their downstream <*target*> gene functions

dynamically to integrate the temporal pattern of signal and to convert it into differential gene output.

Rapid changes in glucose levels (both increases and decreases) imposed by our experimental protocol were followed by rapid adjustments in IEG expression levels. This was observed for mRNA levels and also, in the case of AP-1, for corresponding protein levels. The rapidity with which expression levels are reduced following stimuli removal is in agreement with the generally rapid turn-over characterizing IEG transcripts and proteins. Thus, quantitative regulation of their transcriptional rates may condition the steady-state expression levels of IEGs. These properties imply that signal transduction systems acting upstream of IEG transcription are reflecting continuously the activation state of the cell. Furthermore, our data (Figure 4) have shown that these steady-state IEG expression levels are modulated by the intensity of metabolic activation of the cell. It should be mentioned that transcriptional activation is not the only possible mechanism able to explain changes in mRNA levels; alternatively, stability of mRNA molecules may be regulated.

Ability of signaling system to conserve and transmit the intensity and kinetic information of cell activation to IEG expression has been elegantly demonstrated in neurons. Indeed, the temporal pattern of action potentials in these cells determines the quantitative output in IEG transcription (23–25). Our results show that, in neuroendocrine cells, a similar coupling exists between IEG expression and the signaling network which is activated by increased glucose metabolism of beta cell. Changes in frequency and pattern of Ca^{2+} action potentials are among the signaling correlates of metabolic activation of the pancreatic beta cells (26). Induction of IEGs by metabolic fuels is also Ca^{2+} dependent (6,27,28). The coupling between Ca^{2+} signaling and IEG expression may therefore be common to neurons and neuroendocrine cells.

However, the major finding provided by the present study is that the temporal pattern of IEG expression is itself relayed to modulate <*target*> gene outputs in a manner that converts input duration into graded quantitative output. This integration property emerges from the combination of two main features of the system. First, the very high temporal resolution with which IEG expression follows the temporal pattern of signal; second, the delay necessary for <*target*> gene transcription to be regulated. Indeed, a long signal will produce long-lasting accumulation of IEG products, which will then produce an important impact on <*target*> transcription; whereas a short signal will only produce a transient IEG expression, of insufficient duration to markedly alter the delayed <*target*> transcription (Figure 9). So the system translates not only the amplitude, but also the duration of signal input into quantitative transcriptional outputs of <*target*> genes.

In vivo handling of a glucose load will mainly affect the duration rather than the amplitude of the ensuing transient hyperglycemia. Based upon IEG expression capable to recognize kinetic changes of glucose levels, <*target*> gene expression in pancreatic beta cell will be able to reflect physiological or pathological kinetic features of glycemia, such as in type II diabetes. Decrypting these mechanisms may consequently be important to understand the progression of beta cell dysfunction (29,30).

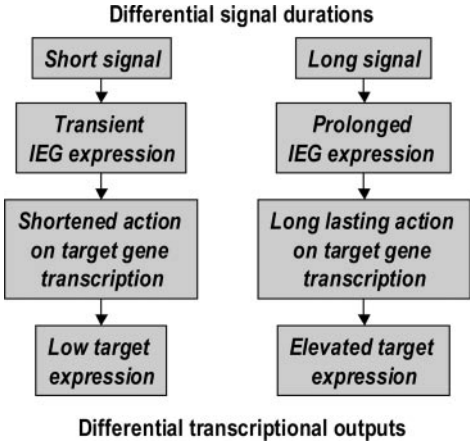

Figure 9. Model for signal integration by the IEG-<*target*> transcriptional system.

IEG induction has been observed in many mammalian tissues upon cellular activation or stress (31–34). Their role in integration of stimuli duration is thus not restricted to pancreatic beta cells. One can anticipate that many diverse adaptive processes requiring regulation of gene expression are based upon transcription networks linking IEGs and their <*targets*> like in the beta cell. To detect and characterize such systems, expression of IEGs has to be considered not only as a punctual event leading to dramatic changes in cell fate, but rather as being continuously modulated in response to subtle changes in intracellular signaling. Considerable technical progress in the quantification of small changes in IEG mRNA and protein expression will be a prerequisite to carry out further successful studies in the pancreas or other tissues under physiological conditions *in vivo*.

In conclusion, our findings show that a system, composed of IEGs and of their downstream <*targets*>, functions in temporal integration of stimuli, allowing cells to produce very different gene expression outputs in response to varying signal duration.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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