Localization of sequences for the basal and insulin-like growth factor-I inducible activity of the fatty acid synthase promoter in 3T3-L1 fibroblasts

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Fatty acid synthase (FAS) plays a central role in fatty acid synthesis and its expression is under nutritional and hormonal control. We have investigated insulin-like growth factor-I (IGF-I) regulation of FAS by transfecting into 3T3-L1 fibroblasts chimeric genes comprising the 5′-flanking region of the FAS gene linked to a luciferase (LUC) reporter gene. First, the basal promoter activity of the 5′ serial deletions from nucleotides −318 to −19 of the FAS gene were compared. Deletions of the promoter sequences from −136 to −19 resulted in a step-wise decrease in the promoter activity, with the −67 LUC and −19 LUC plasmids retaining 40% and 16% of the luciferase activity of −136 LUC. Regulatory sequences important for the FAS basal promoter activity in 3T3-L1 fibroblasts are, therefore, located within the −136 to −19 region. Treatment with 10 nM IGF-I also increased luciferase activity 1.8±0.2-, 1.8±0.3- and 2.5±0.1-fold in 3T3-L1 fibroblasts transiently transfected with −136 LUC, −110 LUC and −67 LUC plasmids, respectively. Deletion of sequences from −67 to −19 resulted in the loss of responsiveness to IGF-I. Physiological doses of insulin (10 nM), however, did not increase luciferase activity in 3T3-L1 fibroblasts transfected with any of the above plasmids. Only upon treatment with pharmacological doses of insulin (1 μM), probably through IGF-I receptor, did luciferase activity increase 4.3±0.4-, 3.2±0.4- and 3.5±0.5-fold when transfected with −136 LUC, −110 LUC and −67 LUC plasmids, respectively; there was no increase with −19 LUC. The half-maximal effect of IGF-I on FAS promoter activity was observed at 3 nM and a maximal effect was reached at 10 nM. These results indicate that the increased promoter activities observed are probably mediated through the IGF-I receptor. Furthermore, sequences responsible for IGF-I regulation of the FAS gene are located within the proximal promoter between nucleotides −67 and −19 of the FAS gene.

INTRODUCTION

Fatty acid synthase (FAS) plays a central role in lipogenesis and its expression is regulated by nutritional and hormonal stimuli. When rats are fasted for 1–2 days, FAS synthesis declines whereas refeeding a high carbohydrate diet increases FAS synthesis. Increased circulating insulin and decreased glucagon levels may participate in regulation of FAS synthesis [1]. We have reported previously that, in normal mice, starvation decreases and refeeding increases the FAS mRNA levels [2]. We and others have demonstrated, by nuclear run-on analysis, that the effects of fasting/refeeding on FAS mRNA levels are attributable to the altered transcription of the FAS gene [3,4]. In diabetic animals, transcription of the FAS gene was not increased after refeeding and insulin specifically restored this induction [3]. We have also shown that when mature 3T3-L1 adipocytes, which contain a high number of insulin receptors, were treated with insulin, FAS mRNA levels increased approx. 3-fold [3]. We have located insulin-responsive sequences at the first 332 bp of the FAS promoter region [5]. In cultured adipose tissue, however, insulin may only potentiate glucose-stimulated FAS mRNA levels [6].

Insulin-like growth factor I (IGF-I), which is structurally related to insulin, is also linked to nutrient intake. In fasted rats, as IGF-I concentration in circulation decreases, the steady-state hepatic IGF-I mRNA level decreases [7]. Straus and Takemoto [8] have shown that the fasting-induced decrease in IGF-I level is accompanied by a decrease in IGF-I gene transcription and refeeding produces a prompt increase in the steady-state mRNA level. Many of IGF-I's metabolic effects are also actions attributable to insulin [9]. IGF-I was found to stimulate adipogenesis, glucose uptake, increase fatty acid synthesis and stimulate glycerol-3 phosphate dehydrogenase activity in 3T3-L1 cells [9]. Rubin and co-workers [10] reported that 3T3-L1 fibroblasts do not differentiate into adipocytes in media containing hormone-depleted serum but addition of IGF-I causes the cells to undergo adipose conversion after triggering with dexamethasone and methylisobutylxanthine. Insulin at pharmacological concentrations can bind to the IGF-I receptor site in 3T3-L1 fibroblasts and is equally potent in supporting adipose conversion. 3T3-L1 fibroblasts possess a significant number of IGF-I receptor sites and have the potential for a maximal biological response to IGF-I.

In the present study, we examined whether IGF-I regulates FAS promoter activity in 3T3-L1 fibroblasts. We report here that physiological doses of IGF-I (or pharmacological doses of insulin) increase FAS promoter activity in 3T3-L1 fibroblasts. Insulin at physiological concentrations did not affect the FAS promoter activity in fibroblasts. We found that sequences responsible for IGF-I regulation of FAS expression in 3T3-L1 fibroblasts are located within −67 to −19 bp of the transcription start site.

MATERIALS AND METHODS

Cell culture

3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum [10]. Cells at confluence were utilized for transfection as fibroblasts (preadipocytes).

Abbreviations used: FAS, fatty acid synthase; IGF-I, insulin-like growth factor-I; LUC, luciferase.
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Transient and stable transfections

For transient transfections, each 60 mm dish of 3T3-L1 fibroblasts was transfected with 10 μg of FAS–LUC constructs [3T3-L1 fibroblasts transfected with chimeric genes comprising the 5′-flanking region of the FAS gene linked to a luciferase (LUC)] by the calcium phosphate DNA co-precipitation method [11]. To normalize for efficiency of transfection, 7 μg of pRSV-βgal or 2 μg of pCMV-βgal was co-transfected with the test plasmids. The precipitate was allowed to cover the cells overnight. The transfected cells were maintained in serum-free medium before addition of IGF-I or insulin, and were harvested after 48 h of hormone treatment. At least two different plasmid preparations were used in each experiment.

For stable transfections, a 1 μg portion of pSV2-Neo was co-transfected with 10 μg of −2100 LUC construct by calcium phosphate precipitation into exponentially growing 3T3-L1 fibroblasts. Cells were incubated in serum-containing media for 2 days before the antibiotic geneticin was added at 0.4 mg/ml. After 2 weeks of selection in the presence of the antibiotic, colonies (approx. 100 per 100 mm plate) were pooled. Pooled colonies were grown without geneticin and assayed for luciferase expression after 48 h of treatment with various concentrations of IGF-I in serum-free media.

Luciferase and β-galactosidase assays

The cells were lysed in 200 μl of a buffer containing 1% (v/v) Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA and 1 mM dithiothreitol. The enzyme activities were assayed in cytosolic extracts. Luciferase activity was assayed as previously reported [5], using a luminometer (Lumat 950I, Berthold, Nashua, NH, U.S.A.). The reaction mixture contained 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate (pH 7.8), 1 mM dithiothreitol and 2 mM ATP. β-Galactosidase activity was assayed spectrophotometrically as previously described [5]. The protein concentration of extracts was determined by the Bio-Rad (San Diego, CA, U.S.A.) Coomassie Blue dye-binding assay using BSA as standard [12]. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency.

Plasmid constructions

The −2100 LUC plasmid was constructed by ligating the *Bst*Y1 restriction fragment containing nucleotides −2100 to +67 of the rat FAS gene into the *Bam*H1 cloning site of the promoterless pOLUC vector obtained from Dr. J. Habener (Massachusetts General Hospital, Boston, MA, U.S.A.) [13]. The *Eag*I site at position −318 in the FAS gene was used to generate 5′ deletion constructs from −2100 LUC plasmid, using an *Exo*III/Mung bean nuclease deletion kit (Stratagene, La Jolla, CA). The sequence of the inserts was verified by dideoxy sequencing using Sequenase [14].

RESULTS AND DISCUSSION

Basal expression of FAS–LUC fusion genes by transient transfection into 3T3-L1 fibroblasts

To determine the regulatory region important for the basal expression of the FAS gene in 3T3-L1 fibroblasts, a series of deletions within the first 318 bp of the FAS promoter ligated to the luciferase reporter gene were used. These deletions lie between base pairs −318 and −19 and have a common 3′ end at +67. To compare basal promoter activity between the serial deletions, the constructs were introduced into 3T3-L1 fibroblasts. Luciferase activity observed for different deletion constructs was corrected for transfection efficiency by co-transfecting the test plasmids with 7 μg of pRSV-βgal and normalization to that of −318 LUC. As shown in Table 1, deletion of the promoter sequence from −318 to −248 resulted in an 18% decrease in luciferase activity. The constructs −184 LUC and −136 LUC exhibited similar luciferase activity to that of −248 LUC but deletions of the promoter sequences from −136 to −19 resulted in a stepwise decrease in the reporter gene activity. The construct −67 LUC retained 40% of the activity of −136 LUC. The decrease in promoter activity associated with deletion of the region between −136 to −67 might be attributable to the two potential binding sites for Sp-1 located in this region. The role of Sp-1 in regulating gene expression has been well described [15]. The −19 LUC construct, which lacks a TATA box, retained 41% of the activity of the −67 LUC, which contains the TATA box. These results suggest that regulatory sequences important for the basal promoter activity of the FAS gene in 3T3-L1 fibroblasts are located in the region −136 to −19.

Localization of IGF-I-responsive sequences in the FAS promoter in 3T3-L1 fibroblasts

3T3-L1 fibroblasts provide an excellent system with which to study IGF-I regulation of the FAS gene. In undifferentiated cells, the concentration of IGF-I receptor (13000 sites/cell) is twice that of the insulin receptor, and preadipocytes have the potential for a maximum response to IGF-I [10]. To define DNA sequences required for up-regulation of the FAS gene, 3T3-L1 fibroblasts were transiently transfected with FAS–LUC deletion constructs containing nucleotides −136 to −19 of the FAS gene. The transfected cells were then kept in serum-free media and subsequently treated with 10 nM IGF-I. The cells were not treated with dexamethasone and methylisobutylxanthine, agents for triggering adipose conversion [10]. Without this treatment, the cells in serum-free media retain a fibroblastic morphology and do not undergo differentiation [16]. Upon treatment with IGF-I, the FAS promoter activity, as determined by luciferase reporter gene

Table 1 Basal activity of the FAS promoter in 3T3-L1 preadipocytes transiently transfected with FAS–LUC fusion constructs

3T3-L1 preadipocytes were cotransfected with 10 μg of fusion constructs containing various deletions of the FAS promoter ligated to the luciferase reporter gene, and 2 μg of pCMV-βgal. Cells were then harvested 48 h after transfection and assayed for luciferase and β-galactosidase activities. Luciferase/β-galactosidase served as a measure of normalized luciferase activity for transfection efficiency, and is presented as percentage of the luciferase activity recorded for the −318 LUC construct. Data are presented as means ± S.E.M. of three experiments, where triplicate dishes were transfected. *, differences statistically significant ($P < 0.05$) when compared with the next-longer construct.

Plasmids	Luciferase activity (%)
−318 LUC	100
−248 LUC	81.6 ± 6.2*
−184 LUC	79.9 ± 8.1
−136 LUC	75.0 ± 9.8
−110 LUC	54.3 ± 4.7*
−67 LUC	30.0 ± 4.0*
−19 LUC	12.2 ± 2.9*

Table 2 IGF-I-stimulated expression of the FAS–LUC fusion genes in transient transfection of 3T3-L1 preadipocytes

The effects of physiological doses of IGF-I and insulin on FAS promoter activity in 3T3-L1 fibroblasts were compared. 3T3-L1 fibroblasts at confluence were cotransfected with 7 μg of RSV-βgal plasmid and 10 μg of FAS–LUC fusion gene constructs. Following transfection, cells were preincubated overnight in serum-free medium, then treated with 5 nM insulin and 10 nM IGF-I and one set of plates were treated identically without insulin or IGF-I. After 48 h of hormone treatment, the cells were harvested and assayed for luciferase and β-galactosidase activities. The fold increase in luciferase activity, normalized to β-galactosidase activity, is indicated for three separate experiments (mean ± S.E.M.), where triplicate dishes were transfected. SV40, simian virus 40. *, **, differences statistically significant for $P < 0.05$ and $P < 0.01$, respectively.

	Change in luciferase activity (fold)	
Plasmids	+ Insulin (5 nM)	+ IGF-I (10 nM)
−136 LUC	0.90 ± 0.24	1.8 ± 0.22*
−114 LUC	0.65 ± 0.12	1.8 ± 0.29*
−67 LUC	0.70 ± 0.14	2.5 ± 0.08**
−19 LUC	0.50 ± 0.12	0.50 ± 0.13
SV40 LUC	0.55 ± 0.15	0.45 ± 0.17

Table 3 Stimulation of the FAS–LUC activity by a pharmacological concentration (1 μM) of insulin in transient transfection of 3T3-L1 fibroblasts

The effects of pharmacological doses of insulin (1 μM) on FAS promoter activity in 3T3-L1 fibroblasts were determined according to the procedures described in the Materials and methods section and in Table 2. The fold increase in luciferase activity, normalized to β-galactosidase activity, is indicated for three separate experiments (mean ± S.E.M.), where triplicate dishes were transfected. **, differences statistically significant for $P < 0.01$.

Plasmids	Change in luciferase activity (fold)
−136 LUC	4.3 ± 0.4**
−114 LUC	3.2 ± 0.4**
−67 LUC	3.5 ± 0.5**
−19 LUC	0.70 ± 0.14
SV40 LUC	0.75 ± 0.16

activity, increased 1.8 ± 0.2 and 1.8 ± 0.3-fold (mean ± S.E.M.), respectively, in cells transfected with −136 LUC and −110 LUC constructs and 2.5 ± 0.1-fold with −67 LUC (Table 2). The deletion of the region between nucleotides −67 and −19 resulted in the loss of activation of the reporter gene by IGF-I. The control plasmid, simian virus 40, showed no effect on luciferase activity when the cells were treated with IGF-I. These results indicate that sequences for IGF-I responsiveness in 3T3-L1 fibroblasts may be located in the proximal promoter region between nucleotides −67 and −19 of the FAS gene. To further confirm that the responsiveness of the FAS promoter observed above is mediated through the IGF-I receptor, we also examined the effects of 5 nM insulin in 3T3-L1 preadipocytes transiently transfected with −136 LUC, −110 LUC, −67 LUC and −19 LUC. As shown in Table 2, when the transfected cells were treated with physiological concentrations of insulin, the promoter activity was not affected significantly. Only upon treatment with a pharmacological dose of insulin (1 μM) did the luciferase

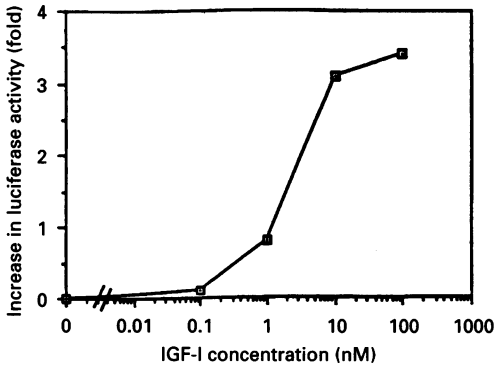

Figure 1 Dose–response characteristics of IGF-I-stimulated FAS–LUC fusion gene expression

3T3-L1 cells were co-transfected with −2100 FAS–LUC and pSV2-Neo (whose expression confers resistance to the antibiotic geneticin). After 2 weeks of selection in geneticin-supplemented medium, resistant clones were isolated as pooled clones. Cells were treated with various concentrations of IGF-I for 48 h before harvesting. Luciferase activity was measured using cell extracts. The figure is representative of two separate experiments.

activity increase, 4.3 ± 0.4-, 3.2 ± 0.4- and 3.5 ± 0.5-fold, respectively, when transfected with −136 LUC, −110 LUC and −67 LUC, probably by binding to IGF-I receptor; no increase was observed for −19 LUC (Table 3). The longer constructs of −2100 LUC, −318 LUC, −248 LUC and −184 LUC all showed similar 3-fold increase in luciferase activity by high concentration of insulin (results not shown). These results further confirm that the FAS promoter is specifically activated by IGF-I and the responsive sequences are present within the −67 to −19 region of FAS promoter in 3T3-L1 fibroblasts.

Next, the dose dependence for the stimulation of FAS promoter activity by IGF-I was examined in 3T3-L1 fibroblasts stably transfected with the −2100 LUC plasmid. The results are presented in Figure 1. The maximal stimulation was obtained at 10 nM IGF-I concentration and approx. 50% of the maximal IGF-I stimulation was observed at 3 nM. These data further suggest that the IGF-I effect on FAS promoter activity in 3T3-L1 fibroblasts is probably mediated through binding to IGF-I receptor. In contrast to our present observation of the independent effect of IGF-I on FAS promoter activity, Goodridge and coworkers [17] could not detect a direct effect of IGF-I in chick embryo hepatocytes. The investigators reported that IGF-I or insulin at high concentrations only amplified the stimulation of transcription caused by tri-iodothyronine. Further studies are needed to verify this discrepancy.

In conclusion, (i) physiological concentrations of insulin have no effect on the FAS promoter activity in 3T3-L1 fibroblasts, whereas physiological concentrations of IGF-I (or pharmacological doses of insulin) increase FAS promoter activity; (ii) *cis*-acting DNA sequences that mediate IGF-I responsiveness in 3T3-L1 fibroblasts are located in the proximal promoter region between nucleotides −67 and −19 of the FAS gene. Blake and Clarke [18] have shown that IGF-I stimulates FAS expression in 3T3-L1 cells during adipose conversion triggered by dexamethasone and methylisobutylxanthine treatment, and that the expression is under the control of IGF-I and not insulin. Recently, in TA1 cells [19] and in muscle cells [20,21], it has been reported that insulin-like growth factors are the primary regulators of undifferentiated cells and insulin primarily regulates differentiated cells. Recent studies from our laboratory indicate that the insulin-responsive sequences in the FAS promoter, as determined

by transient transfection into 3T3-L1 adipocytes, are located between nucleotides −67 and −25 [22]. This region in FAS promoter contains, in addition to a TATA box, a region for nuclear protein binding that shares no sequence identity with consensus sequences for known transcription factors. The transcription factor Sp-1 has been implicated in mediating IGF-I effects on the δ1-crystallin gene [23]. Although there are several copies of putative Sp-1 sites in the 5′-flanking region of the FAS gene, none of them are present between −67 and −19. We therefore postulate the involvement of different transcription factor(s) in the IGF-I regulation of the FAS gene. Interestingly, this region contains information for responsiveness for IGF-I in preadipocytes as well as for insulin in 3T3-L1 adipocytes. Further studies are necessary to elucidate whether or not the IGF-I responsiveness in preadipocytes and the insulin responsiveness in adipocytes are mediated by the same element. Although both insulin and IGF-I receptors show intrinsic tyrosine kinase activities, the post-receptor signalling pathways or the effector proteins that transmit signalling are not well understood. Ras proteins and insulin receptor substrate 1 (IRS-1) have been implicated in mediating some of the actions of both insulin and IGF-I [24–26]. Furthermore, a recent report indicates that both insulin and IGF-I induce c-*fos* expression in postmitotic neurons by a protein kinase C-dependent pathway [27]. It would be of interest to elucidate whether insulin and IGF-I act through a common intracellular signalling system in regulating FAS gene expression in 3T3-L1 cells.

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