Amino acid regulation of gene transcription of rat insulin-like growth factor-binding protein-1

A Takenaka, K Komori, T Morishita, S-I Takahashi, T Hidaka and T Noguchi

Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

(Requests for offprints should be addressed to T Noguchi)

(A Takenaka is now at Department of Bioresource Engineering, Section of Bioresource Utilization, Yamagata University, Tsuruoka-shi, Yamagata 997-8555, Japan)

(T Hidaka is now at Center for Gene Research, Ehime University, Matsuyama, Ehime 790-8566, Japan)

(S-I Takahashi is now at Department of Applied Animal Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan)

Abstract

To investigate the molecular mechanisms of increased transcription of the insulin-like growth factor-binding protein-1 (IGFBP-1) gene in dietary protein-deprived animals, the *cis*-acting sequence that is involved in this regulation was analyzed. We first showed that IGFBP-1 gene transcription was up-regulated by amino acid deprivation in cultured liver cell lines: H4IIE and HuH-7. Since HuH-7 cells showed a greater increase in IGFBP-1 mRNA in response to amino acid deprivation, this cell line was used in further experiments. Using a promoter function assay, we found that up-regulation of promoter activity responding to amino acid deprivation was abolished by deleting the region between -112 and -81 bp from the cap site from the gene construct. This *cis*-acting region includes the insulin-responsive element (IRE) and glucocorticoid responsive element (GRE) of IGFBP-1. In summary, the present observation suggests that the 32-bp (-112 to -81) in the IGFBP-1 gene 5' promoter region is involved in the induction of the IGFBP-1 gene in response to amino acid deprivation.

Journal of Endocrinology (2000) **164** R11-R16

Introduction

Insulin-like growth factor-I (IGF-I) is a polypeptide growth factor which has wide-ranging biological effects including the stimulation of DNA synthesis and protein synthesis (Jones & Clemmons 1995). From the viewpoint of body protein metabolism, IGF-I is a key factor whose plasma concentration is regulated by the quality and quantity of dietary proteins (Takahashi *et al.* 1990). IGF-I is complexed with several types of IGF-binding proteins (IGFBP) in plasma and other body fluids (Jones & Clemmons 1995), and these have been shown to play an important role in the regulation of IGF bioactivities (Jones & Clemmons 1995). Plasma concentrations of each IGFBP as well as IGF-I are affected by dietary proteins in different ways (Takahashi *et al.* 1990, Umezawa *et al.* 1991, Thissen *et al.* 1994). Among the IGFBPs, the plasma concentration of IGFBP-1 was increased most strongly under protein deprivation. This increase in IGFBP-1 concentration was accompanied by an increase in IGFBP-1 mRNA in liver (Takenaka *et al.* 1993), which is caused by an increased transcription rate of the IGFBP-1 gene (Miura *et al.* 1992, Takenaka *et al.* 1996).

The structure of the IGFBP-1 gene 5' region has been studied extensively, and a series of results reveal that a TATA element is located at 28 bp 5' to the cap site and a CCAAT element is located at 72-68 bp 5' to the cap site. Furthermore, the first 1205 bp 5' to the cap site can direct an efficient expression of the reporter genes (Suwanichkul *et al.* 1990, Powell *et al.* 1989, Powell *et al.* 1991), suggesting that the IGFBP-1 promoter is located within this 1205-bp region. Several studies have demonstrated that the first 470 bp 5' to the cap site are highly conserved in human and rat sequences (Unterman *et al.* 1992) and several elements in this region, such as the insulin-responsive element (IRE), the glucocorticoid-responsive element (GRE), the cAMP-responsive element (CRE), and the hepatocyte nuclear factor-1 (HNF-1) binding site are responsible for the regulation of IGFBP-1 transcription under various conditions (Lee *et al.* 1993).

To elucidate the mechanism by which IGFBP-1 gene expression was activated in liver under protein deprivation, we first demonstrated that the IGFBP-1 mRNA content of hepatoma cells increases when the cell-culture medium is deprived of amino acids. We then characterized *cis*-acting sequences that up-regulate IGFBP-1 gene transcription in response to amino acid deprivation, using 5'- and internal deletions of the rat IGFBP-1 promoter in reporter gene constructs.

Journal of Endocrinology (2000) **164,** R11–R16 Accepted 20 January 2000
0022-0795/00/0164-00R11 ©2000 Society for Endocrinology *Printed in Great Britain*

Online version via http://www.endocrinology.org

Materials and Methods

Materials

H4IIE-C3 (ATCC No. CRL-1600) was obtained from Dainippon Pharmaceutical Co. (Osaka, Japan), and HuH-7 was obtained from Health Science Research Resource Bank (Osaka, Japan). Dulbecco's modified Eagle's minimum essential medium (DMEM), Eagle's minimum essential medium (MEM), Earle's balanced salt solution (EBSS), and Dulbecco's phosphate buffered saline (PBS) were purchased from Nissui (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Irvine Scientific (Santa Ana, CA, USA). 100x MEM vitamin mixture was obtained from Flow Laboratories (Irvine, Scotland). Cell-culture dishes were purchased from Corning (Corning, NY, USA). Other chemicals were of the reagent grade available commercially.

Cell culture and cell treatment

The H4IIE-C3 cell line and the HuH-7 cell line were grown in DMEM supplemented with 10% FBS under 95% air-5% CO_2 at 37°C. All media contained the following antibiotics: penicillin (1×10^5 units/liter), streptomycin (10 mg/liter) and fungisone (100 µg/liter). Cultures were passaged every 4 days at a ratio of 1:5 for H4IIE and 1:4 for HuH-7 using 0.2% trypsin-0.02% EDTA by the usual method. For preparation of total RNA or nuclear extracts, the cells were plated onto 100 mm culture dishes and grown to confluence in DMEM supplemented with 10% FBS. Confluent cultures were incubated with serum-free MEM containing 0.1% BSA (AA+) or EBSS containing 0.1% BSA and 1x MEM vitamin mixture (AA-) for 24 h.

RNA preparation from cells and Northern-blot analysis

Total RNA was prepared from H4IIE cells and HuH-7 cells and Northern-blot analysis was performed in the same way as previously reported (Takenaka *et al.* 1996). After electrophoresis, the RNA was stained with ethidium bromide to verify the quality and quantity of RNA loaded in each lane.

Preparation of plasmids for transfection

The fragments -913 to +70, -564 to +70, -285 to +70, -140 to +70, and -81 to +70 of the 5'-flanking region of the rat IGFBP-1 gene promoter were prepared by PCR using the -913 to +93 fragment as a template, which in turn had been cloned by PCR using rat genomic DNA as a template. Amplified products were blunted by T4 DNA polymerase (DNA blunting kit; Takara, Osaka, Japan), purified by electrophoresis on agarose gels, and ligated into the pGL3 Basic vector (Promega, Madison, WI, USA) to prepare plasmids p913Luc, p564Luc, p285Luc, p140Luc, and p81Luc, with each plasmid named for the number of remaining IGFBP-1 promoter bp to the cap site. The plasmids pdGRE/IRE Luc and pdHNF-1 Luc were prepared utilizing SacI and BamHI sites. The plasmids for promoter assay were sequenced and no new sites were created by nucleotide misincorporation by Taq polymerase. All plasmids used for transfection studies were prepared using the Qiagen plasmid extraction kit (Qiagen, Chatsworth, CA, USA) and were quantitated by absorbance measurement at 260 nm.

Transfection of HuH-7 cells

Before transfection, 1×10^5 HuH-7 cells were plated onto each 35 mm dish. After 24 h, the cultures were washed once with serum-free MEM and transfected using the cationic liposome-mediated procedure, as modified for LipofectAMINE (Gibco-BRL, Gaithersburg, MD, USA) by the manufacturer. Each culture dish received 20 µg of LipofectAMINE complexed to the appropriate plasmid DNAs: 1 µg of luciferase plasmid DNA containing rat IGFBP-1 fragments and 2 µg of pSV β-galactosidase plasmid (Promega, Madison, WI, USA). Plasmid DNA and LipofectAMINE were each diluted in Opti-MEM I medium (Gibco-BRL, Gaithersburg, MD, USA), combined, mixed gently, and incubated at room temperature for 15 min

Figure 1 A schematic representation of the IGFBP-1 gene promoter fragment from bp -126 to -31 from the transcription initiation site. Putative *cis*-regulatory elements; IRE (insulin responsive element), GRE (glucocorticoid responsive element), and HNF-1 binding site are underlined. The deleted sequences of the transfected plasmid pdIRE/GRE Luc and pdHNF-1 Luc are shown as dashed lines.

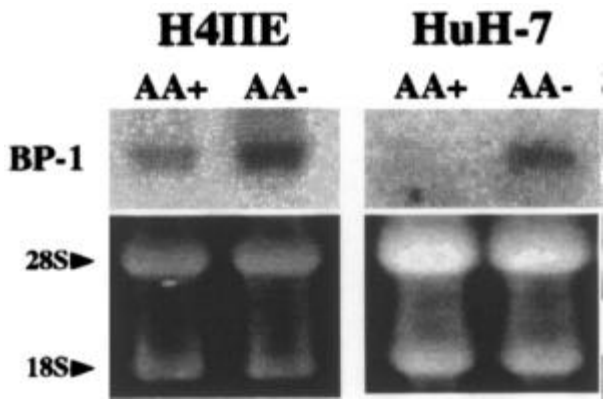

Figure 2 Northern-blot analysis of IGFBP-1 mRNA in which 10 µg of total RNA from H4IIE cells and 40 µg of total RNA from HuH-7 cells were analyzed. Autoradiogram of Northern blots of IGFBP-1 mRNA (upper panel) and ribosomal RNA visualized with ethidium bromide (lower panel) are shown. The confluent cell cultures were maintained in serum-free MEM containing 0.1% BSA (AA+) or Earle's balanced salt solution (EBSS) containing 0.1% BSA and 1x MEM vitamin mixture (AA-) for 24 h before RNA preparation. The experiments were performed at least three times independently and each representative result is shown.

before being added to the cell cultures. The cultures were incubated with the LipofectAMINE-DNA complex for 5 h, and the medium was replaced with DMEM containing 10% FBS. After a further 12 to 16 h of incubation, the medium was replaced again with the experimental medium: serum-free MEM containing 0.1% BSA (AA+) or Earle's balanced salt solution containing 0.1% BSA and 1x MEM vitamin mixture (AA-). After 24 h of incubation, the cells were harvested for the assays as described below.

Luciferase and b-galactosidase assay

Transfected cells were washed three times with PBS and harvested in TEN buffer (40 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl). The cells were centrifuged at 10 000x ***g*** for 1 min at 4°C, and the pellet was resuspended in 150 µl of 0.25 M Tris–HCl, pH 8.0. The cells were then subjected to three rapid freeze/thaw cycles, vortexing after each thaw cycle, and centrifuged at 10 000x ***g*** for 2 min at 4°C. The resulting supernatant was assayed directly or stored at -70°C. The protein content of each sample was determined using a Protein Assay Kit (BioRad Laboratories, Richmond, CA, USA). The β-galactosidase assay was performed employing the β-Galactosidase Assay System with Reporter Lysis Buffer (Promega, Madison, WI, USA) using 50 µl of the extract. Luciferase activity in 20 µl of the cell extract was measured by a PicaGene instrument (Toyo Inki, Tokyo, Japan), using an automated luminometer (Berthold Clini-Lumat, Berthold Systems, Pittsburgh, PA, USA). Luciferase activity was normalized by β-galactosidase activity or the protein contents of the extracts, and the data thus normalized by β-galactosidase activity are presented in Results (Fig. 3). Light emission from 390-620 nm was quantitated for 5 sec at room temperature. Each promoter construct was transfected into three separate dishes, and the results were confirmed in three to five independent experiments.

Statistics

The results were analyzed statistically according to Duncan's multiple range test (Duncan 1955).

Results

Northern-blot analysis of IGFBP-1 mRNA in hepatoma cells under amino acid deprivation

We previously demonstrated that liver IGFBP-1 gene transcription and mRNA content increased when rats were deprived of dietary protein (Takenaka *et al.* 1993, Miura *et al.* 1993). In the present study, the effect of amino acid deprivation from the cell culture medium on IGFBP-1 mRNA in the rat hepatoma cell line H4IIE and in the human hepatoma cell line HuH-7 was examined. In H4IIE cells, IGFBP-1 mRNA

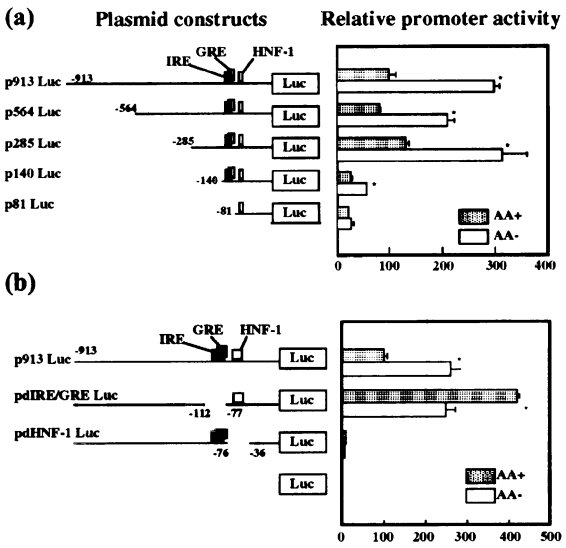

Figure 3 Luciferase assays of reporter gene constructs which contain (a) different 5' ends and (b) different deleted sequences, the constructs of which are shown schematically on the left. Transfected cells were cultured in serum-free MEM containing 0.1% BSA (AA+) or Earle's balanced salt solution (EBSS) containing 0.1% BSA and 1x MEM vitamin mixture (AA-) for 24 h. Luciferase activity was normalized by β-galactosidase activity, and the data represent triplicate determinations (mean+S.E.M.) from three separate dishes and are expressed relative to p913 Luc / AA+ which was assigned a value of 100. * indicate significant difference between AA+ and AA- (*P<0.05).

increased 2- to 3-fold by amino acid deprivation for 24 h (Fig. 2). At the same time, the result of nuclear run-on assay (performed as described in Miura *et al.* 1993) showed that the transcription rate of the IGFBP-1 gene also increased 2- to 3-fold in H4IIE cells (data not shown). On the other hand, in HuH-7 cells, IGFBP-1 mRNA could not be detected in a normal culture medium, but was markedly increased by amino acid deprivation (Fig. 2). The increase of IGFBP-1 mRNA was not observed under serum deprived condition but was observed when only essential amino acids or all amino acids were deprived from the culture medium (data not shown). Since the response of IGFBP-1 to amino acid deprivation in HuH-7 cells appeared to be similar to that *in vivo* in rat liver (Takenaka *et al.* 1993), we decided to use HuH-7 cells to measure the promoter activity of the IGFBP-1 gene.

Regulation of promoter activity of the 5'-flanking region of the IGFBP-1 gene in HuH-7 cells under amino acid deprivation

The fragments -913 to +70, -564 to +70, -285 to +70, -140 to +70, and -81 to +70 of the 5'-flanking region of the rat IGFBP-1 gene were inserted into promoterless pGL-3 vector (p913Luc, p564Luc, p285Luc, p140Luc, and p81Luc), and after transfection of each plasmid to HuH-7 cells, the luciferase activity was measured. The luciferase activity was normalized by the β-galactosidase activity, and the results were expressed relative to p913 Luc / AA+ which was assigned a value of 100. The luciferase activity in the cells transfected with p913Luc, p564Luc, p285Luc, and p140Luc was significantly stimulated 2- to 3-fold by culturing the cells in the medium without amino acids, but was not stimulated in the cells with p81Luc (Fig. 3(a)). These results suggest that the sequence -140/-81 contains the *cis*-element which responds to amino acid deprivation from the cell culture medium to induce IGFBP-1 gene transcription. To investigate the role of GRE and IRE in this region of IGFBP-1, we transfected pdGRE/IRE Luc and pdHNF-1 Luc and measured the luciferase activity (Fig. 3(b)). As a result, the response to amino acid deprivation disappeared in the cells transfected with pdGRE/IRE Luc. Luciferase activity itself decreased to the basal level in cells transfected with pdHNF-1 Luc, as others have also reported (Babajko & Groyer 1993) (Fig. 3(b)). This is the first report demonstrating that the amino acid-responsive region of the IGFBP-1 gene promoter is located in the bp region from -112 to -81, which includes GRE and IRE.

Discussion

The molecular mechanism by which amino acid deprivation regulates IGFBP-1 gene transcription is presently not understood. We previously showed that dietary amino acid deprivation increased IGFBP-1 mRNA in rat liver, due to an increase in the transcription rate of the IGFBP-1 gene (Takenaka *et al.* 1993, Miura *et al.* 1993). In this report, we demonstrate that the IGFBP-1 mRNA level increased in cultured liver cells due to amino acid deprivation from the medium and reproduced the *in vivo* effect of amino acid deprivation on IGFBP-1 gene transcription in the cell-culture system as well. These results show that the amino acid concentration outside of the liver cells can provide the signals to regulate IGFBP-1 gene transcription. In this simple system, we can exclude the possibilities that endocrine factors such as insulin or glucocorticoid are also effective under amino acid deprivation.

In addition, a 32-bp sequence between -112 and -81 bp from the cap site of IGFBP-1 gene is involved in amino acid-dependent regulation of IGFBP-1 gene transcription. This region contains both IRE and GRE. A similar transcriptional regulatory unit is also found in the 5'-flanking region of PEPCK (phosphoenol-pyruvate carboxykinase) gene and this unit is presumed to have some roles in glucose homeostasis (O'Brien *et al.* 1995). Molecular mechanisms of transcriptional regulation by insulin and glucocorticoid were also discussed recently in the case of the PFK-2 (6-phosphofructo-2-kinase) gene (Pierreux *et al.* 1998).

It is well known that the expression of many genes is regulated differently by the quantity of dietary proteins (Marten *et al.* 1994). The effects of amino acids in the culture medium on IGFBP-1 mRNA in hepatocyte primary culture have also been reported (Pao *et al.* 1993). However, the molecular mechanism of amino acid-dependent transcriptional regulation has only been studied in the asparagine synthase gene (Guerrini *et al.* 1993). Although the nucleotide sequence of an amino acid-responsive element (AARE) has been reported (Guerrini *et al.* 1993), the AARE sequence is not homologous with the 32-bp sequence in the present study. Accordingly, there may be multiple mechanisms by which the genes are transcriptionally regulated by amino acids. Although the role of the AARE is unknown at present, negative *trans*-acting factor(s) may interact with the -112 to -77 region of the IGFBP-1 gene because deletion of this region increases the luciferase activity about 4-fold under an amino acid-supplemented (AA+) condition (Fig.3 (b)). *Trans*-acting factor(s) which can bind to this region of the IGFBP-1 gene is (are) under investigation.

It has been known that the plasma level and the liver mRNA content of IGFBP-1 increase in fasted or diabetic animals (Murphy *et al.* 1990, Ooi *et al.* 1990). These changes have been well explained by the inhibition of IGFBP-1 gene transcription in response to insulin, because the plasma insulin level is also low in fasted animals or streptozotocin-induced diabetic animals (Powell *et al.* 1991, Orlowski *et al.* 1990). The mechanism of the insulin-dependent inhibition of IGFBP-1 gene transcription has been studied extensively and some groups have shown that a specific sequence termed IRE is the *cis*-acting regulatory element (Suwanichkul *et al.* 1993). A recent study shows that the insulin signal is transduced through activation of PI3K (phosphatidylinositol 3'-kinase) and PKB/Akt (protein kinase B/Akt) to transcription factors which bind to the IRE of the IGFBP-1 gene (Cichy *et al.* 1998, Guo *et al.* 1999). HNF-3 is reported to interact with the IRE sequence in

several genes including IGFBP-1, apoCIII and tyrosine aminotransferase (O'Brien *et al.* 1995, Unterman *et al.* 1994, Li *et al.* 1995, Ganns *et al.* 1994). However, HNF-3 binding did not correlate with the insulin effect on the inhibition of IGFBP-1 gene transcription (Allander *et al.* 1997).

Because it takes at least 12 h for up-regulation of the IGFBP-1 gene expression by amino acid deprivation from the cell-culture medium (data not shown), a signal of amino acid deprivation may be indirectly transduced to the -112 to -77 region of the IGFBP-1 gene. We could not exclude the possibility that the effects of amino acid depletion on the increase in the IGFBP-1 transcription rate are secondary such as inhibition of protein synthesis or reflection of a general stress.

Reduced protein synthesis rate under amino acid deprived condition could affect hepatic IGFBP-1 mRNA contents as reported previously (Ooi *et al.* 1993, Straus *et al.* 1993). Whether the signals of amino acid deprivation affect the insulin signal mediated by the tyrosine phosphorylation cascade or the glucocorticoid signal mediated by a nuclear receptor, is unknown at present. The present study adds new insight to our knowledge of nutritional and hormonal regulation of gene expression.

In summary, we showed that IGFBP-1 gene transcription was up-regulated by amino acid deprivation from the culture medium in liver cell lines. The *cis*-acting sequence which is involved in the amino acid signal was determined to be between -112 and -81 bp from the cap site, which includes the IRE/GRE region of this gene. These results suggest that IGFBP-1 gene transcription may be regulated by a new cross-talk mechanism between nutrients such as amino acids and endocrine factors such as insulin and glucocorticoid through a novel AARE.

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