Regulation of Insulin-like Growth Factor-I Expression in Mouse Preadipocyte Ob1771 Cells*

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In mouse preadipocyte Ob1771 cells, transcription of the insulin-like growth factor-I (IGF-I) gene was stimulated by growth hormone (GH), and IGF-I protein combined with GH in medium was required for their differentiation to adipocytes. During induction of the differentiation, the intracellular expression of each class of IGF-I mRNA was analyzed by reverse transcriptase-polymerase chain reaction. When the cells were cultured in the presence of GH, the class 1del. IGF-I mRNA was a major molecular species among IGF-I mRNAs. In the presence of both GH and IGF-I, the splicing pattern of IGF-I mRNA changed from class 1del. to class 1. Moreover, as detected by Western blotting, the IGF-I protein was present in cells and in the medium only when the cells were cultured in the presence of both GH and IGF-I. We found that IGF-I secreted from Ob1771 cells could act in an autocrine/paracrine fashion and induce the differentiation of other Ob1771 cells. It was demonstrated that the translation efficiency of class 1 mRNA was higher than that of class 1del. mRNA in vitro. These results suggested that stimulation with exogenous IGF-I in the presence of GH was required for the production of class 1 IGF-I mRNA and that the production of the IGF-I protein was activated by increasing the translation efficiency through shifting the splicing pattern of IGF-I mRNA from class 1del. to class 1. Exogenous IGF-I triggered the differentiation by initiating the synthesis of endogenous IGF-I.

Insulin-like growth factor-I (IGF-I) is a 70-amino acid polypeptide similar to proinsulin (1). Transcription of the IGF-I gene is regulated by growth hormone (GH), and IGF-I is thought to mediate many of the biological effects of GH (2–6). IGF-I has insulin-like activities such as stimulation of glycogen synthesis (7). IGF-I also functions as a mitogen and as a differentiation factor for various cell lines, including preadipo-cytes (8). The biological actions of IGF-I begin by interaction with its cell surface receptor, which is a ligand-activable tyrosine-specific protein kinase similar to the insulin receptor (9).

In mouse and rat (10, 11), the IGF-I genes have two leader exons (exons 1 and 2), resulting in two kinds of mRNAs (classes 1 and 2) (11–13). There is another mRNA species, class 1del., in which a central region of exon 1 is missing (Fig. 1A) (14, 15). Exon 5 is also spliced alternatively, resulting in Ea encoded by exons 4 and 6 and Eb encoded by exons 4, 5, and 6 (10, 16). These diverse IGF-I mRNAs eventually give the same mature protein. The biological significance of the diversity of mRNAs, signal peptides, and E domains is not understood.

Mouse preadipocyte Ob1771 cells (17) can differentiate to adipocytes. GH has a strong adipogenic activity in Ob1771, 3T3-F442A (18–20), and 3T3-L1 (21) preadipocytes. In Ob1771 cells, GH stimulates the formation of diacylglycerol (22), modulates the translation of the lipoprotein lipase gene (23) and transiently increase the expression of the c-fos gene (22). GH also stimulates the transcription of the IGF-I gene (6), and IGF-I is thought to participate in inducing the differentiation. In differentiated Ob1771 cells, enzymes for lipid synthesis, such as glycerophosphate dehydrogenase (GPDH), are activated (24).

Transcription of the IGF-I gene is stimulated by GH, but we observed that IGF-I combined with GH was essential for the differentiation of Ob1771 cells. The following interpretations are possible for these facts. (i) For some reason, IGF-I secreted from Ob1771 cells is not active enough to induce the differentiation. (ii) The IGF-I mRNA is translated when cells are cultured in the presence of both GH and IGF-I, but not when cells are cultured in the presence of GH alone. (iii) The IGF-I protein is not secreted from Ob1771 cells without the signal from the IGF-I receptor. To examine these possibilities, we analyzed the expression of each class of IGF-I mRNA, and the expression and secretion of IGF-I protein during the induction of differentiation. Furthermore, we examined the translation efficiency of class 1 and class 1del. mRNAs in vitro. In this study we show that IGF-I in the medium changed the splicing pattern of IGF-I mRNA and allowed the synthesis of IGF-I protein.

EXPERIMENTAL PROCEDURES

Cell Culture—Ob1771 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 200 units of penicillin/ml, 50 μg of streptomycin/ml, 33 μM biotin, 17 μM pantothenate, and 10% (v/v) calf serum (Sigma). This medium was termed the standard medium. At confluence (day 0), cells were exposed to the standard medium supplemented with 2 nM triiodothyronine (Sigma) and 100 μM 3-isobutyl-1-methylxanthine (Sigma), which was termed the differentiation medium. If necessary, cells were cultured from day 0 to day 4 in the differentiation medium that was supplemented with 10% recombinant goat GH purified from Escherichia coli and/or with 10 μM recombinant human IGF-I (Bachem). The differentiation media supplemented with GH, or with both GH and IGF-I, were termed the GH differentiation medium and the GH-IGF-I differentiation medium, respectively. Cells were cultured in the standard medium for 4 additional days (~day 8) and used for the GPDH assay. Both the standard medium and the differentiation medium were changed every 2 days.

To obtain conditioned media and cell lysates for Western blotting, cells were incubated in the serum-free medium, ASF 104 (Ajinomoto), for 24 h. After the medium was removed, the cells were harvested in the lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 0.5 mM (p-aminophenyl)mercuranisulfon fluoride hydrochloride, 2 mM benzamidine, 165 KIU/ml aprotinin, and 1% Nonidet P-40. GPDH Assay—After induction of the differentiation (day 8), the cells...
IGF-I Regulates Its Own Expression Post-transcriptionally

were harvested and the GPDH activity was assayed as described previously (25-28) by measurement of oxidation of NADH based on the absorbance at 340 nm. Two independent experiments were performed.

RT-PCR and Southern Hybridization—For detection of both class 1 and class 1del. mRNAs, we prepared primer 1-1del. (ATGGGGAAAATT-CAGCAGTCT), the nucleotide sequence of which appears in exon 1. For exclusive detection of class 1 mRNA, we prepared primer 1 (TGAAATGTGAC), the nucleotide sequence of which appears in the region of exon 1 that is to be removed upon splicing to form class 1del. mRNA. For detection of class 2 mRNA, we prepared primer 2 (TGCTGTTGAAACGACCCGG), the nucleotide sequence of which appears in exon 2. We also prepared primer 6 (AGGTCTTGTTCTCTGCAC), the nucleotide sequence of which appears in the complementary strand in exon 6 (Fig. 1A). Total RNA was prepared from cells by acid-guanidine thiocyanate-phenol-chloroform methods (29). One microgram of the total RNA was transcribed into cDNA with 200 units of reverse transcriptase from murine leukemia virus (Life Technologies, Inc.) in 20 μl of a reaction mixture containing 50 mM KCl, 20 mM Tris-Cl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 1 mM each dNTP, 5 mM random hexamer, and 20 units of the ribonuclease inhibitor RNasin (Promega). The cDNA synthesized during incubation for 1 h at 42 °C was used as a template for PCR in a reaction mixture containing 5 units of Taq DNA polymerase, 50 mM KCl, 20 mM Tris-Cl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 0.2 μM each primer, and 0.2 mM each of the two primers. After 25 cycles (1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C) of the PCR, DNA fragments were electrophoresed on a 1% agarose gel and transferred to GeneScreen Plus. Hybridization was done using the mouse IGF-I cDNA as a probe, and positive signals were detected with an ECL labeling and detection system (Amersham Corp.).

Western Blotting—Cell lysates and conditioned media were separated on a 13% SDS-polyacrylamide gel and transferred to cellulose nitrate membranes (Schleicher & Schuell). The membrane was blocked for 2 h in PBS containing 0.1% Tween 20 (PBS-T) with 5% skim milk (Difco) and incubated for 1 h with monoclonal anti-human insulin-like growth factor-I antibody (Upstate Biotechnology, Inc.) in PBS-T. The membrane was washed twice for 15 min with PBS-T and incubated for 1 h with goat anti-mouse IgG antibody horseradish peroxidase conjugate (Bio-Rad) in PBS-T. After washing, the bound antibody was made visible using an ECL detection system (Amersham).

In Vitro Transcription and in Vitro Translation—Plasmid constructs that contained the cDNAs for mouse IGF-I class 1 and class 1del. mRNAs at the BamHI site of pBluescript II KS were linearized and transcribed in vitro using T7 RNA polymerase. Messenger RNAs were synthesized in the presence of 0.3 μM of the cap analogue m7GpppG (New England Biolabs). In vitro transcription of four micrograms each of in vitro transcribed IGF-I mRNAs was done according to the manufacturer's instructions (Amersham) in rabbit reticulocyte lysates containing biotin-Lys-tRNA. The decay of individual mRNAs during the translation was examined by Northern blotting. Newly synthesized biotinylated RNA was separated on a 13% SDS-polyacrylamide gel and transferred to cellulose nitrate membranes. The membrane was probed with horseradish peroxidase-labeled streptavidin (Amersham), and the bound streptavidin was made visible using an ECL detection system.

RESULTS AND DISCUSSION

Requirement of GH and IGF-I for the Differentiation of Ob1771 Cells—Based on the activation of GPDH, we analyzed the differentiation of post-confluent Ob1771 cells that had been cultured in the GH differentiation medium and in the GH-IGF-I differentiation medium. In the differentiation medium with no supplement, Ob1771 cells did not differentiate and GPDH was not activated. Cells that had been cultured in the GH-IGF-I differentiation medium differentiated to adipocytes; exclusive detection of class 1 mRNA (data not shown). GPDH was markedly activated and many intracellular oil droplets were observed. With the cells cultured in the GH differentiation medium, the GPDH activity was only 5.8% of that of the cells cultured in the GH-IGF-I differentiation medium (data not shown). This showed that exogenous IGF-I combined with GH was essential for the differentiation of Ob1771 cells.

Formation of Each Class of IGF-I mRNA during Induction of the Differentiation—In Ob1771 cells, it was reported that transcription of the IGF-I gene was stimulated by GH (6). Here we analyzed the formation of each class of mRNA when post-confluent Ob1771 cells were cultured in the GH differentiation medium. In the GH-IGF-I differentiation medium, and the synthesis and secretion of the IGF-I protein were analyzed. Cell lysates and conditioned media were separated on a 13% SDS-polyacrylamide gel electrophoresis and the IGF-I protein was detected by Western blotting using anti-IGF-I antibody (Fig. 2). Neither the cells nor the conditioned medium contained the IGF-I protein when the cells had been cultured in the GH differentiation medium. The IGF-I protein was found in the cells from day 2 to day 4 when the cells had been cultured in the GH-IGF-I differentiation medium. In this case, IGF-I was found to be secreted from the cells. On day 4, the medium was changed to the standard medium that did
IGF-I Regulates Its Own Expression Post-transcriptionally

Fig. 2. Secretion and production of IGF-I. Ob1771 cells were cultured to confluence in the standard medium (day 0) and cultured up to day 4 in the differentiation medium supplemented with 10 nM GH (GH) or with 10 nM GH and 10 nM IGF-I (GH + IGF-I). The medium was changed to the standard medium, and the cells were cultured for 2 days. On the day indicated on top of the panel, the medium was changed to the serum-free medium, and the cells were cultured for 24 h. The cells and the media were collected separately and the IGF-I proteins in them were analyzed by Western blotting using the mouse anti-human IGF-I monoclonal antibody as described under “Experimental Procedures.” Similar results were obtained in three independent experiments.

Regulation of the IGF-I Gene Expression and the Differentiation of Ob1771 Cells—The results in this paper demonstrated that the IGF-I gene expression was regulated post-transcriptionally by IGF-I itself. In the case of insulin, it was reported that insulin regulated the expression of some genes post-transcriptionally. For example, insulin combined with thrombin stabilizes c-myc mRNA (31), and insulin regulates the relative level of expression of the two mRNA variants of the highly insulin-induced delayed early gene, hrs, in differentiating H35 cells (32). Insulin also regulates the alternative splicing of exon 11 of the insulin receptor gene in FAO cells (33) and of the protein kinase Cβ in BC3H-1 myocytes (34). Here we demonstrated that exogenous IGF-I changed the splicing pattern of IGF-I mRNA from class 1del. to class 1 in Ob1771 cells. In addition, when the cells were cultured in the GH differentiation medium, class 1del. mRNA appeared in two bands in the medium. However, we cannot rule out the possibility that the activity was attributed to IGF-I in vivo. It is suggested that the upper and the lower bands reflect the mRNA species, including and excluding exon 5, respectively (Fig. 1A). Thus, the alternative splicing of exon 5 is also regulated by IGF-I. However, the significance of this regulation is not clear. Although the growth factor-dependent regulation of the alternative splicing has not been reported, it is likely that IGF-I regulates factors involved in the alternative pre-mRNA splicing.

In our studies, class 1 mRNA was proved to be more efficiently translated into protein than class 1del. mRNA in vitro. This suggests that exogenous IGF-I activates the intracellular production of IGF-I protein by shifting the splicing pattern of IGF-I mRNA from class 1del. to class 1, which is much more active in translation. The present result also suggests the presence of specific cis-elements involved in the translational control. It is reported that the efficiency of translation initiation is affected by the sequence context near the 5′ cap (35), by the upstream AUG codons and by the length and secondary structure of the mRNA leader (36). Further studies are necessary to identify specific elements involved in the translational regulation of IGF-I mRNA. However, we cannot rule out the possibility that exogenous IGF-I also activates cellular translation machinery in vivo.

In Ob1771 cells, class 2 mRNA may be translationally inactive, since the time of its appearance and requirement of GH and IGF-I do not coincide with those for the IGF-I production. However, it is possible that class 2 mRNA is translationally regulated in different ways specified by cell types and growth stages.

We found that the GH-IGF-I conditioned medium, which contained IGF-I secreted from Ob1771 cells, had an activity that induced the differentiation of other Ob1771 cells. It is strongly suggested that the activity was attributed to IGF-I in the medium. However, we cannot rule out the possibility that another adipogenic factor is secreted from Ob1771 cells in the presence of GH combined with IGF-I and regulates the differ-
IGF-I Regulates Its Own Expression Post-transcriptionally

orientation. Thus, we propose the following hypothesis. GH stimulates the transcription of the IGF-I gene, but the produced mRNA is mainly class 1d. mRNA, which is not efficiently translated into protein. Exogenous IGF-I acts on preadipocytes in an endocrine fashion to initiate the differentiation and also to initiate the synthesis and secretion of endogenous IGF-I, which then acts in an autocrine fashion and stimulates the next round of the production and secretion of endogenous IGF-I. Therefore, once endogenous IGF-I was produced and secreted, exogenous IGF-I may not be needed any more in the medium. An Ob1771 cell stimulated by IGF-I combined with GH synthesizes and secretes many IGF-I molecules, which act on other Ob1771 cells in a paracrine fashion. Amplified IGF-I acts on a large number of cells and induces the differentiation and synthesis of IGF-I to a progressively greater extent. In this way, many cells can differentiate into adipocytes in response to the initial stimulation with IGF-I. Although it is not clear whether a similar mechanism regulates the expression of the IGF-I gene in other cell types, it is possible that one of the post-transcriptional control mechanisms of the IGF-I gene expression is the exogenous IGF-I-dependent regulation of alternative splicing of pre-mRNA.

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