Differential Regulation of Insulin-like Growth Factor-I (IGF-I) Receptor Gene Expression by IGF-I and Basic Fibroblastic Growth Factor*

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Insulin-like growth factor-I receptor (IGF-IR) gene expression is regulated by various stimuli, including hormones, growth factors, and nutritional status. We have investigated the molecular mechanism by which two growth factors, insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF) regulate IGF-IR gene expression. bFGF increases the endogenous IGF-IR mRNA levels and IGF-IR promoter activity. This effect is mediated by a region of the IGF-IR promoter located between nucleotides −476 and −188 in the 5′-flanking region. In contrast, IGF-I decreases the IGF-IR mRNA levels. IGF-I down-regulates IGF-IR transcriptional activity as deduced from experiments in which the levels of pre-mRNA and mRNA were measured. IGF-I reduced pre-mRNA and mRNA levels in parallel, while the mRNA stability was found to be unchanged by IGF-I treatment. While these results strongly suggest an effect of IGF-I on IGF-IR transcriptional activity, no specific IGF-I response element was demonstrated in the 5′-untranslated region or 5′-flanking region studied. Thus, bFGF and IGF-I have differential effects on IGF-IR gene transcription, with the IGF-I response region as yet unidentified.

The insulin-like growth factors (IGFs)1 and the IGF-I receptor (IGF-IR) are important modulators of cell growth and differentiation in many tissues (1). IGF-IR gene expression is highly regulated under many physiological and pathological conditions (for review, see Ref. 2). For instance, IGF-IR mRNA levels and IGF-I binding are tightly regulated by the nutritional status of the animal. In vivo, fasting was shown to increase IGF-I-specific binding in several tissues, and these changes in IGF-I binding were accompanied by a 2-fold increase in IGF-IR mRNA abundance (3). Since, in some of the tissues, the local levels of IGF-I are decreased after a reduction in caloric intake, it is possible that the increases in IGF-IR expression are secondary to the decrease in local tissue IGF-I concentrations (4). In contrast, some growth disorders such as Laron-type dwarfism are associated with long-term reductions of circulating levels of IGF-I. In these disorders the expression of the IGF-IR gene in mononuclear cells is enhanced several-fold, suggesting an effect of circulating IGF-I (5). Drug-induced diabetes in rats is an additional animal model in which the regulation of the IGF-IR gene has been studied. Streptozotocin-treated rats show a reduction in circulating IGF-I levels and increased expression of the IGF-IR in the kidney. Both circulating IGF-I levels and IGF-IR expression returned to control values following insulin treatment (6). Similarly, the levels of IGF-IR in cultured cells are affected by the concentration of IGF-I in the medium. For instance, in cultured IM-9 lymphoid, FRTL-5 thyroid, and endothelial cells, IGF-IR expression is regulated by the concentration of IGF-I in the culture medium (7, 8), with increased IGF-I levels causing a decrease in receptor number. Under certain circumstances this down-regulation involves a translocation of cell-surface receptors to an intracellular pool (9), whereas in other cell types the effect is due to decreased IGF-IR gene expression (10).

A possible mechanism by which competence factors like bFGF and platelet-derived growth factor (PDGF) stimulate entry of cells into the G1 phase of the cell cycle (11, 12) is their ability to increase the expression of the IGF-IR, thus enhancing the progression factor activity of the IGF-1 ligand. For instance, bFGF increases IGF-I binding and IGF-IR mRNA levels in the BC3H-1 muscle cell line (13), and this change is associated with a decrease in IGF-II expression in these cells. Similarly, PDGF increases IGF-IR gene expression and ligand binding in cells in culture (14), and this effect is due, at least in part, to PDGF stimulation of IGF-IR gene transcriptional activity. This transcriptional effect is mediated by a 200-base pair (bp) region located immediately upstream of the transcription start site (15).

In the present report, we studied the molecular mechanisms involved in the regulation of IGF-IR gene expression by bFGF and IGF-I. We found that bFGF and IGF-I regulate transcriptional activity of the IGF-IR gene positively and negatively, respectively. The region responsible for the response to bFGF is located in the proximal 476 bp of the 5′-flanking region, whereas the region responsible for the response to IGF-I is apparently located outside of the −2350-bp to +640-bp region of the IGF-IR gene promoter.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and reagents were purchased from Biofluids, Inc. (Rockville, MD) and Advanced Biotechnologies (Columbia, MD). Insulin-free bovine serum albumin (fraction V) was obtained from Armour (Kankakee, IL). Human recombinant IGF-I was a kind gift from Genentech (San Francisco, CA). Recombinant human bFGF, Lipofectin and LipofectAMINE reagents were purchased from Life Technologies, Inc. 5,6-Dichlorobenzimidazole riboside (DRB) was purchased from Sigma. The ECL detection kit and horseradish peroxidase-

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§ The abbreviations used are: IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor-I receptor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; DRB, 5,6-dichlorobenzimidazole riboside; IRS-1, insulin receptor substrate-1; IGFBP, IGF binding proteins; UTR, untranslated region; bp, base pair(s).
Cells were cultured in 6-well plates, and each well received 3 promoter activity of these fragments has been previously described (16). Reporter plasmids were used for transient transfections. The basal (pSV2-LUC) containing the SV40 enhancer/promoter was used. The erase (pOLUC) reporter gene. As a control the expression plasmid

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reporter plasmid and 3 promote luciferase mRNA was measured) or 10 µg of reporter plasmid plus 10 µg of pGEM-ZZ (Promega, Madison, WI) (in experiments in which luciferase activity was measured). Eighteen hours after transfection, the DNA-containing medium was changed to serum-containing medium for approximately 30 h. Cells were then serum-starved overnight and incubated with the indicated concentrations of IGF-I and for various times. Cells were harvested either for luciferase activity measurements or for RNA extraction. Protein content was determined using the Bio-Rad reagent according to manufacturer’s directions.

Western Blot Analysis—C2C12 cells were serum-starved overnight and then incubated with 1 nM bFGF for 24 h. Cells were lysed in the presence of 50 mM Hepes, pH 7.9, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 2 µg/ml leupeptin, and 2 µg/ml aprotinin. Cell lysates were clarified by centrifugation. Protein content was determined by the method of Bradford using a protein assay kit (Bio-Rad). Eighty micrograms of protein were reduced by β-mercaptoethanol and fractionated by 7.5% SDS-polyacrylamide gel electrophoresis. Resolved proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell). The upper part of the membrane was incubated with anti-IRS-1 antibody (1 µg/ml) while the bottom part was incubated with anti-IGF-I receptor β-subunit antibody (1:1000 dilution) and detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:2500 dilution) using the ECL reagent. The digitalized signal from the x-ray film and analyzing the signal using NIH image version 1.6.5 software.

Solution Hybridization-RNase Protection Assay of IGF-IIR mRNA and Pre-mRNA—C2C12 and SH-SYSY cells were lysed in 4 M guanidinium isothiocyanate containing 0.01% β-mercaptoethanol, and RNA was isolated by ultracentrifugation through a cesium chloride gradient as described previously (17). Total RNA (20 µg) from C2C12 cells was hybridized with a 32P-labeled mouse antisense IGF-IIR RNA probe (2 × 106 dpm). The mouse antisense RNA probe was generated by transcription of a mouse genomic IGF-IIR 417-bp EcoRI–BamHI fragment, subcloned into a pBluescript SK+ vector (construct provided by A. Efstathios).
under “Experimental Procedures.” bFGF increased endogenous IGF-IR mRNA levels by about 40–50% at 4 h of incubation, with the maximum effect (~90%) at 16 h (Fig. 1A). A similar (~90%) increase in IGF-IR mRNA level was seen in SH-SY5Y cells using 1 μM bFGF for 16 h (Fig. 1B).

Effect of bFGF on IGF-I Receptor Levels—To assess the relevance of the increase in IGF-IR mRNA levels by bFGF we studied the bFGF effect on IGF-IR protein. Following overnight serum starvation C2C12 cells were incubated in the presence of 1 μM bFGF for 24 h. Cell lysates were assayed for IGF-I receptor and IRS-1 protein levels. bFGF specifically caused a 70% increase in the levels of the IGF-I receptor. Whereas another protein involved in the IGF-I receptor signaling pathway, namely IRS-1, demonstrated no change (Fig. 2).

Effect of bFGF on IGF-IR Promoter Activity—One mechanism that may have been responsible for bFGF-induced increase in IGF-IR mRNA levels was bFGF activation of the IGF-IR gene promoter. To study this possibility C2C12 cells were transiently transfected with a luciferase reporter gene under the control of the proximal promoter region of the IGF-IR gene (476 bp of the 5’-flanking region and 640 bp of the 5’-untranslated region (UTR)) (p–476/+640LUC) together with a β-galactosidase expression vector (pCMV β). bFGF increased IGF-IR promoter activity by ~90%, with the maximum effect at 16 h (Fig. 3A) and at a concentration of 1 μM (Fig. 3B). No effect of bFGF was seen when the promotorless pOLUC or the unrelated promoter pSV2LUC were used (data not shown).

Localization of the bFGF Response Region—To localize the region of the IGF-IR gene promoter responsible for mediating the effect of bFGF on IGF-IR gene expression, C2C12 cells were transiently transfected with different fragments of the IGF-IR promoter cloned upstream of a luciferase reporter gene. After overnight serum starvation, cells were stimulated with 1 μM bFGF for 18 h. As seen in Fig. 4 the stimulatory effect of bFGF was lost when the sequence between nucleotides –476 and –188 of the 5’-flanking region was deleted (compare construct –476/+640 with –188/+640). Removal of the 5’-UTR (compare construct –476/+640 with –455/+30) did not affect bFGF stimulation of transcriptional activity. These result suggest that the major bFGF-responsive element is located between nucleotides –476 and –188 of the 5’-flanking region.

Effect of IGF-I on the Steady-state Levels of IGF-IR mRNA—To characterize the IGF-I effect on the IGF-IR mRNA levels, time course and dose-response experiments were performed in SH-SY5Y cells. After serum starvation, cells were stimulated with the indicated doses of IGF-I for various periods of time (Fig. 5, B and C). IGF-I decreased IGF-IR mRNA levels by ~40–50%, with the maximum effect seen at 8 h and at a concentration of 0.1 μM. IGF-I receptor mRNA down-regulation by IGF-I was similarly seen using C2C12 cells (Fig. 5A).

Effect of IGF-I on IGF-IR Gene Transcription and on mRNA Stability—To determine whether IGF-I treatment affected transcriptional activity, we studied the effect of IGF-I on IGF-IR pre-mRNA levels and on IGF-IR mRNA turnover. An RNA probe complementary to the exon 2:intron 2 boundary was generated as described under “Experimental Procedures.” This probe allows the simultaneous measurement of pre-mRNA and processed IGF-IR mRNA. As shown in Fig. 6, IGF-I down-regulates both IGF-IR pre-mRNA and mature mRNA levels. The decrease relative to controls of pre-mRNA and mature mRNA was similar (~45%). Furthermore, similar results were obtained using an RNA probe complementary to the exon 1:intron 1 boundary (data not shown).

To study the effect of IGF-I on IGF-IR mRNA stability, cells were stimulated with IGF-I for 6 or 8 h, and DRB (a specific RNA polymerase II inhibitor) was then added to the medium

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**Fig. 2. Regulation of IGF-IR levels by bFGF.** Cells were incubated with 1 nM bFGF for 24 h. Equal amounts of protein (80 μg) were separated by 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filter and blotted either with anti-IRS1 or anti-IGF-IR polyclonal antibodies. IRS-1 and IGF-IR values are presented as the mean ± S.E. of four wells per group.
for various periods of time. As shown in Fig. 7 the decay curves for both control and IGF-I-treated cells were similar, thus suggesting that IGF-I does not significantly affect the turnover rate of the IGF-IR mRNA. Taken together, these data support the hypothesis that IGF-I decreases IGF-IR mRNA levels by affecting IGF-IR gene transcription.

Effect of IGF-I on IGF-IR Promoter Activity—To determine the region of the IGF-IR promoter responsible for mediating this effect of IGF-I, we transiently transfected SH-SYSY with different fragments of the IGF-IR promoter cloned upstream of a luciferase reporter gene. Surprisingly, IGF-I treatment did not decrease luciferase enzyme activity (Fig. 8A). To verify the lack of IGF-I effect on IGF-IR promoter activity, luciferase mRNA levels were measured. Similarly, IGF-I did not significantly affect luciferase mRNA levels (Fig. 8B), while in the same experiments we were able to demonstrate that IGF-I reduced endogenous IGF-IR mRNA levels (Fig. 8C). This discrepancy between the lack of regulation of the activity of transfected promoter fragments and the reduction in endogenous gene expression is not cell type specific, since similar results were obtained using C2C12 and G401 cells (data not shown). These results suggest that the regulatory element responsible for the IGF-I effect is apparently not present in the region extending from nucleotide −2350 in the 5'-flanking region to nucleotide +640 in the 5'-UTR.

**DISCUSSION**

The biological actions of the IGFs are mediated through interaction with their specific cell-surface receptor. The IGF-IR is expressed in most body tissues and the levels of IGF-IR mRNA are modulated in a number of physiological and pathological states, including changes in the levels of various circulating and locally acting growth factors. In this study we characterized the molecular mechanisms by which bFGF and IGF-I regulate IGF-IR mRNA levels. While bFGF increases IGF-IR gene promoter activity by 2-fold, IGF-I decreases transcriptional activity by ~50%.

The stimulation of IGF-IR gene promoter activity by bFGF in transfection experiments correlates with its effect on the expression of the endogenous IGF-IR gene, as measured by mRNA and protein levels; i.e., all three parameters increased approximately 2-fold. By deletional analysis of the IGF-IR promoter region, we determined that the bFGF-responsive region is localized in the proximal promoter, between nucleotides −476 and −188 upstream of the transcription start site.
FIG. 5. Regulation of IGF-IR mRNA levels by IGF-I. C2C12 and SH-SY5Y cells were serum-starved overnight and then stimulated with 13 nM IGF-I for the indicated periods of time (A and B). SH-SY5Y cells following serum starvation, were incubated with varying IGF-I doses for 8 h (C). RNA from two dishes per group was pooled and 10 μg of total RNA were co-hybridized with a 32P-labeled mouse or human IGF-IR and an 18S ribosomal antisense RNA probes. P, undigested probe. Solution hybridization/RNase protection assay was performed as described under “Experimental Procedures” and in the legend to Fig. 1. IGF-IR mRNA values were normalized using the 18S rRNA values (lower panels).
though within the skeletal α-actin gene promoter there is a unique serum response element (SRE1) necessary for induction of promoter activity by bFGF (21), no such consensus sequence was found in the IGF-IR gene proximal promoter. bFGF has also been shown to stimulate proenkephalin gene expression by synergizing with cAMP through a cAMP response element (22). We did not find any cAMP response element in the IGF-IR proximal promoter region; however, there are a number of AP2 sites, which in the absence of a cAMP response element may mediate the stimulatory effect of cAMP (23). Further experiments will be required to address whether these AP2 sites are relevant to the stimulatory effect of bFGF on IGF-IR promoter.

In addition, there are a number of SP1 sites. However, the significance of these sites to bFGF action has not been previously described.

bFGF and PDGF are competence factors that stimulate entry of cells into the cell cycle (11, 12). Both growth factors increase IGF-IR gene expression. It has been shown that PDGF stimulates IGF-IR promoter transcriptional activity (15). Likewise, we show in the present study that bFGF stimulates IGF-IR gene expression by activating promoter activity. This stimulatory effect on IGF-IR promoter activity correlates with the IGF-IR transcriptional activation by bFGF recently shown in aortic smooth muscle cells (24).

Both bFGF and PDGF synergize with IGF-I in their biological actions (25, 26). The results of the present study suggest that one mechanism may be the increased expression of the IGF-I receptor by these growth factors. Other investigators have demonstrated that increased expression of the IGF-I receptor enhances cellular responses (27), whereas decreased levels by as little as 50% using antisense technologies decreases responsiveness to IGF-I (28).

bFGF also regulates the expression and secretion of various IGF binding proteins (IGFBPs). For instance, in the newborn rat olfactory bulb, bFGF enhanced IGFBP-2 and IGFBP-4 mRNA levels, whereas IGFBP-5 mRNA was not affected (29). In addition, IGFBP secretion by rat neuronal and glial cells in culture was differentially affected by the presence of bFGF in the culture medium (30). Since IGFBPs modulate both positively and negatively the actions of IGF-I (31–33), the effect of bFGF on the expression of the IGFBPs represent another level of regulation of IGF-I action.

IGF-I is a progression factor that is required by cells to progress through the cell cycle. In this study we have shown that IGF-I down-regulates endogenous IGF-IR mRNA levels in a neuroblastoma cell line and a muscle cell line. Similar results were described previously in a separate muscle cell line (10). The direct demonstration that IGF-I down-regulates IGF-IR gene expression supports the hypothesis that both circulating and locally produced IGF-I are responsible for the regulation of IGF-IR mRNA levels under several physiological and pathological situations in animal models as well as in humans. Further support of the hypothesis that IGF-I represses expression of
the IGF-IR gene “in vivo,” comes from developmental studies in which we showed that increasing postnatal levels of IGF-I are correlated with decreased levels of IGF-IR mRNA in developing rats (20).

Results of experiments in which we measured IGF-IR pre-mRNA levels and mRNA stability suggest that down-regulation of IGF-IR mRNA levels by IGF-I is due to a decrease in transcription activity rate with no change in mRNA stability. Thus, IGF-I decreases pre-mRNA and mature mRNA levels to a similar extent as measured with two different exon:intron boundary probes, whereas, on the other hand IGF-I does not affect the IGF-IR mRNA turnover.

We were unable to demonstrate regulation of the IGF-IR promoter by IGF-I using fragments of the promoter extending from nucleotide −2350 in the 5′-flanking region to nucleotide +640 in the 5′-UTR. The promoter activity of this region has been previously characterized and several cis-acting regulatory elements have been described, including EGR/WT1 binding sites (34), Sp1 binding sites (35), a PDGF response region (15) and a potential FGF response region (this study). In addition, the IGF-IR promoter contains a sequence between nucleotides −583 and −555, which is similar to a putative IGF-I response element described in the elastin gene that mediates activation of the elastin promoter by IGF-I (36). However, our present data would suggest that the putative IGF-I response element probably lies entirely outside this region (−2350/+640) or, alternatively, that it is within this region but requires other cis- or trans-activating elements for this effect. A similar discrepancy between transcriptional and promoter activity regulation has been seen in the transcriptional activation of the IxBo gene by glucocorticoids (37, 38). Thus, while increasing transcription from the IxB0 gene as measured by run-on transcription assay, dexamethasone had no effect on IxBo gene promoter activity. Based on our results we cannot totally exclude the possibility that the luciferase reporter vector used in this study was the inappropriate vector, albeit similar results were obtained with two different luciferase reporter vectors (pOULUC and pGL2) (data not shown).

In summary we have characterized the regulation of IGF-IR gene expression by two growth factors that exhibit opposite effects on the transcriptional activity of the IGF-IR gene. bFGF increases IGF-IR promoter activity by acting in the proximal promoter region whereas IGF-I down-regulates transcriptional activity through a different mechanism that may involve other as yet uncharacterized, regions of the IGF-IR gene.

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