Genetic causes of growth disorders

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The severe short stature in two siblings with a heterozygous *IGF1* mutation is not caused by a dominant negative effect of the putative truncated protein

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Abstract

Objective: While in previous studies heterozygosity for an *Insulin-Like Growth Factor 1 (IGF1)* defect only modestly decreased height and head circumference, we recently reported on two siblings with severe short stature with a maternally transmitted heterozygous duplication of 4 nucleotides, resulting in a frame shift and a premature termination codon in the *IGF1* gene. In this paper we describe the structural and functional characteristics of the putative truncated IGF-I protein.

Design: Two children, their mother and maternal grandfather carried the mutation. In addition, two family members who were not affected were included in the study. Mutant (MT) IGF-I was synthesized in oxidized and reduced form using two methods. Neutral gel filtration studies were carried out with wild-type (WT) and synthetic MT IGF-I. Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptors were performed with EBNA-293 cells, stably transfected with the IGF-I receptor, and IM9 cells. L6 cells were used to examine the mitogenic potency and the potential antagonizing effect of synthetic MT IGF-I by [^{3}H]-thymidine incorporation assays.

Results: In the sera of both the carriers and non-carriers the proportion of ^{125}I-IGF-I that was associated with the 150 kDa complex was somewhat less (varying between ~37 and ~52%) than in normal pooled serum (~53– ~ 63%) and, instead, slightly increased amounts of radioactivity were eluted in the 40–50 kDa fraction (consisting of binary IGF–IGFBP complexes) or remained unbound. Synthetic MT IGF-I did not bind to the IGF-I receptor, nor antagonize the growth-promoting effect of IGF-I. It did bind to IGFBPs, but was barely incorporated into 150 kDa complexes. Because in all cases WT IGF-I immunoreactivity was recovered in one peak, corresponding to the MW of WT IGF-I, i.e. ~7.6 kDa, an interaction of circulating truncated mutant peptide with WT IGF- I is very unlikely.

Conclusions: There is no evidence that the severe short stature associated with heterozygosity for this novel *IGF1* mutation in children born from a mother with the same mutation is caused by a dominant negative effect of the truncated protein. We speculate that the growth failure is caused by a combination of partial IGF-I deficiency, placental IGF-I insufficiency, and other genetic factors.

Introduction

IGF-I is a highly conserved, single-chain polypeptide consisting of 70 amino acids, organized into four peptide domains: A, B, C and D. The B-domain and the first A domain helix are mainly responsible for binding to IGFBPs. The A-, B- and C- and, to a lesser extent, the

D-domain contribute substantially to IGF binding to its receptor (IGF1R) (1-8). The A domain also contains residues involved in low affinity binding to the IGF2R (4-9). The IGF-I pro-hormone contains an additional carboxy-terminal E peptide that is cleaved in the Golgi apparatus before secretion (10).

Approximately 80–90% of circulating IGFs form a 150 kDa ternary complex with IGFBP-3 or -5 and the acid-labile subunit (ALS) (11), 10–15% are bound in a 40–50 kDa binary complex to IGFBPs, whereas less than 1% is found in the free form (12). The 150 kDa complexes cannot cross the capillary endothelial barrier, which prolongs the half-life of IGFs, IGFBP-3, and -5 in the circulation (11, 13, 14). This seems to play an important role in the regulation of the bioavailability of IGFs to the tissue compartments. When carried in ternary complexes, the half-life of IGF-I is more than 12 h, much longer than that of binary bound IGF-I (30–90 min) and free IGF-I (~10 min) (15).

In mice IGF-I plays a major role in pre- and postnatal growth (16-17). Recent studies revealed that autocrine and paracrine IGF-I appears crucial for early postnatal and pubertal development of cortical bone, while circulating IGF-I, responsible for the endocrine effect, is mainly important for growth after puberty. In autocrine and paracrine IGF-I deficient mice high levels of serum IGF-I can permit postpubertal "catch-up" growth (18-20).

IGF-I is also needed for normal pre- and postnatal growth in humans, as shown by severe dwarfism in three patients with well documented homozygous *IGF1* defects, caused by the deletion of exons 4 and 5 (21), a missense mutation (22) and a missense mutation with a milder phenotype (23). There may be a fourth case (24), but although the patient's phenotype was very similar, the presumed genetic aberration in the polyadenylation site at the 3'UTR in exon 6 was later found to occur also in healthy controls (25). Patients with a homozygous *IGF1* defect exhibit severely affected intrauterine growth (about –4 SDS), whereas postnatally height SDS varies between –6.9 and –8.5, and head circumference between –4.0 and –8.0 SDS. Heterozygosity for an *IGF1* mutation or deletion may be associated with a mild height loss (in the order of 1 SD), but can present as short stature if occurring in a family with genetic predisposition for decreased height (22).

We recently reported on two siblings with severe short stature carrying a heterozygous duplication of four nucleotides in *IGF1*, resulting in a frame shift at position 35 of the mature IGF-I protein and a premature stop codon (c.243_246dupCAGC, p.Ser83GlnfsX13) (26). In the present paper we describe the laboratory studies that we performed to investigate whether this heterozygous mutation could cause such severe phenotype, either directly or by interfering with the biological action of WT IGF-I, in particular whether the truncated mutant IGF-I may exert a dominant negative or antagonistic effect on the function of wild-type IGF-I.

Subjects and Methods

Subjects

Clinical features, growth curves and plasma levels of IGFs, various IGFBPs and ALS of the 4 carriers of the *IGF1* mutation, as well as the pedigree, have been reported previously (26). In short, the first index case was a 8.2 year old girl (III-1) born small for gestational age (birth weight –2.9 and birth length –3.8 SDS) who presented with severe postnatal short stature (height –4.1 SDS) and microcephaly (head circumference –2.4 SDS). Her 6.2 year old brother (III-2) had a birth weight and length of –1.2 and –1.0 SDS, respectively, a height of –4.6 SDS, and a head circumference of –1.6 SDS. Their mother (II-2) carried the same mutation and had a height SDS of –3.5 and a head circumference of –1.7 SDS, and the affected maternal grandfather (I-1) had a height of –1.4 SDS (corrected for age and secular trend) (27) and a head circumference of –1.8 SDS. The unaffected maternal grandmother and aunt were less short (–1.2 and –2.0 SDS) and had a normal head circumference (0.1 and 0.4 SDS). Serum IGF-I SDS of the 4 carriers was approximately –2 SDS, significantly lower than in non-carriers (P = 0.004). For both carriers and non-carriers of the *IGF1* mutation serum levels of IGFBP-1, -2,-3, -4 and -6, as well as those of ALS, were within the respective normal ranges, i.e. between –2 and +2 SDS (26). Case III-2 showed a 47,XYY karyotype.

Synthesis of mutant IGF-I

The biological properties of mutant (MT) IGF-I were tested with synthesized peptides. Since the B-domain of MT IGF-I contains two unpaired cysteine residues, and it is unknown whether the cysteines remain reduced or become oxidized (in case the peptide is stably expressed *in vivo*), both oxidized and reduced forms were prepared, and used in the various experiments.

One batch of the oxidized mutant peptide was prepared (Dr. L. Schäffer, Novo Nordisk A/S, Maaloev, Denmark) by solid phase peptide synthesis on Tentagel S RAM resin using Fmoc chemistry on a Liberty microwave synthesizer according to the manufacturer's instruction. The formation of disulfide bridges was accomplished by treatment of the peptide with 2% iodine in N-methylpyrrolidone for 1 hour prior to cleavage. The peptide was cleaved from the resin with trifluoroacetic acid/water/triisopropylsilane (95%:2.5%:2.5%) and purified by standard RP-HPLC.

Another batch of oxidized peptide, as well as a batch of reduced peptide, was prepared in the laboratory of Prof. J. D. Wade (Howard Florey Institute, Melbourne, Australia). Region-selectively S-protected A- and B-chains were synthesized separately as their C-terminal amides using the continuous flow Fmoc-SPPS method as previously reported (28), using an automatic PerSeptive Biosystems Pioneer peptide synthesizer (Massachusetts, USA) (29). The identities of the crude peptides were confirmed by MALDI-TOF MS. Subsequently, the

mutant IGF-I peptide was prepared by sequential region-selective disulfide bond formation between the two chains also as previously described (28,29). Following a final RP-HPLC purification step, overall yield of mutant IGF-I was 5.01 mgs (9.5% relative to crude starting B-chain) which was satisfactory given the multiple steps involved in its preparation.

We show the results obtained with the peptides prepared by Prof. J.D. Wade but identical results were obtained with the one prepared by Dr. L. Schäffer.

Neutral gel filtration studies with WT and synthetic MT IGF-I

The different molecular-size classes of endogenous IGF-I (i.e. 150 kDa, 40–50 kDa, and unbound, free form) in plasma were determined by neutral gel filtration through a 1.6×60 cm Superdex 200 Hiload column, as described previously (30). Prior to column chromatography, each serum sample (250 µl) was incubated with 100 µl of ~100,000 cpm of either ^{125}I -WT hIGF-I or ^{125}I- MT IGF-I being dissolved in 50 mM sodium phosphate buffer pH 7.4, containing 0.2% BSA, 10 mM EDTA and 0.05% (w/v) Tween-20, for 17 h at 4°C. The various molecular size classes of complexes were eluted from the column at a rate of 1.2 ml/min using 0.05 M NH_4HCO_3 buffer pH 7.4. The ^{125}I content of each 1.2 ml fraction was counted in a gamma counter.

In order to investigate the ability of synthesized MT IGF-I to compete for incorporation into ternary and binary complexes, 250 µl aliquots of pooled normal human serum were incubated with 100 µl of the same amount of ^{125}I radiolabeled WT recombinant IGF-I in the presence of increasing concentrations of either recombinant wild-type IGF-I or synthetic MT IGF-I, as described above. The distribution of ^{125}I-IGF-I among the various molecular-size classes in each aliquot of serum was determined after separation by S200 gel filtration.

The possibility that WT IGF-I and eventually naturally existing MT IGF-I in serum interacted, for example by forming higher MW dimers or multimers, was investigated by Sephadex G-50 exclusion chromatography of Sep-Pak C18 extracts of sera from carriers and non-carriers. The 1.5×90-cm column was calibrated with lactalbumin (14.5 kDa), hIGF-II (7.5 kDa), and insulin-B (3.5 kDa). Acetic acid (0.1 M) was passed in a descending direction at a rate of 4.8 mL/h, and ~3 mL fractions were collected, dried and reconstituted in 50 mM sodium phosphate buffer pH 7.4, containing 0.2% BSA, 10 mM EDTA and 0.05% (w/v) Tween-20. In each column fraction the IGF-I immunoreactivity was determined by RIA (31).

Cell lines

Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptors was performed with EBNA-293 stably expressing the IGF-I receptor (gift from Dr. J. Whittaker, Case Western Reserve University, Cleveland, Ohio) and IM9 human lymphocytes, respectively. The rat myoblast cell line (L6) used for thymidine incorporation was kindly provided by Bo

Falck Hansen, Novo Nordisk, Denmark. These cells express ~175,000 IGF-I receptors per cell and no insulin receptors (Sajeed, W. and De Meyts, P., unpublished data).

EBNA 293 cells transfected with the IGF-1 receptor

The EBNA-293 cells are human embryonic kidney (HEK) cells expressing Epstein-Barr nuclear antigen (EBNA-1) which are stably transfected with the IGF-I receptor (EBNA293t IGF-IR), kindly provided by Dr. J. Whittaker (Case Western Reserve University, Cleveland, Ohio) (32). The cells were cultured in DMEM high glucose with Glutamax-1 supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin (all from Gibco, Invitrogen, USA). 250 µg/ml Hygromycin B (Sigma-Aldrich, USA) and 400 µg/ml Geneticin (Gibco, Invitrogen, USA) were added to the media for selecting the stably transfected cells. The cells were grown at 37°C in 5% CO_2 humidified atmosphere. Cells were sub-cultured three times a week by washing the cells in 10 ml PBS w/o calcium and magnesium (Gibco, Invitrogen, USA), detaching the cells from the culture flask with 2 ml 0.05% Trypsin-EDTA (Gibco, Invitrogen, USA), followed by resuspending in 8 ml fresh media.

IM9 cells

The IM9 human lymphoblast cell line (American Type Culture Collection (ATCC), USA) is derived from a patient with multiple myeloma. The cell line carries the Epstein-Barr virus. The IM9 cells express about 20,000 insulin receptors (isoform A) per cell and have been used for several decades in insulin receptor binding kinetics studies (33). The cells, which grow in suspension, were cultured and used as described previously (33).

Rat L6 myoblasts

The L6 WT cells were cultured in 75 cm^2 flasks with DMEM (1000 mg/l glucose, Glutamax-1, Pyruvate) supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin. The cells were grown at 37°C in 5% CO_2 humidified atmosphere. The cells were sub-cultured three times a week, by washing the cells in 10 ml PBS w/o calcium and magnesium, and detaching them by adding 2 ml 0.05% Trypsin-EDTA. Cells were resuspended in 10 ml fresh media, and seeded with a split ratio of 1:6 for two days and 1:12 for three days of maintenance.

Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptor

Binding competition between synthetic mutant peptides and ^{125}I-IGF-I (on EBNA293t IGF-IR cells) and ^{125}I-insulin (on IM9 cells), respectively, as well as accelerated dissociation of labeled ligand by cold ligand (negative cooperativity) were studied according to established protocols (33, 34).

[^{3}H]-Thymidine incorporation assay

[^{3}H]-Thymidine incorporation was used to examine the mitogenic potency of synthetic MT IGF-I using the L6 WT rat myoblast cell line. We used a recently developed optimized assay on cells synchronized in Go/G1 that gives a robust and highly reproducible response to growth factors, as described in detail previously (35).

Results

Mutation analysis

In the two index cases, their mother and maternal grandfather, we found a heterozygous duplication of four nucleotides of the *IGF1* gene, resulting in a frame shift at position 35 of the mature IGF-I protein and a premature stop codon (c.243_246dupCAGC, p.Ser83GlnfsX13) (Fig. 1A). If the peptide would be stably expressed *in vivo*, the mutation should result in a truncated C-domain, followed by a tail of 12 residues with an altered amino acid sequence showing no homology with the wild-type A- and D-domains (Fig. 1B). Therefore, the mutant peptide and wild-type IGF-I protein would have only the B-domain in common. Single-nucleotide polymorphism array analysis showed an interstitial deletion not containing any gene or noncoding RNAs (26) in all carriers and mother's sister (II-1).

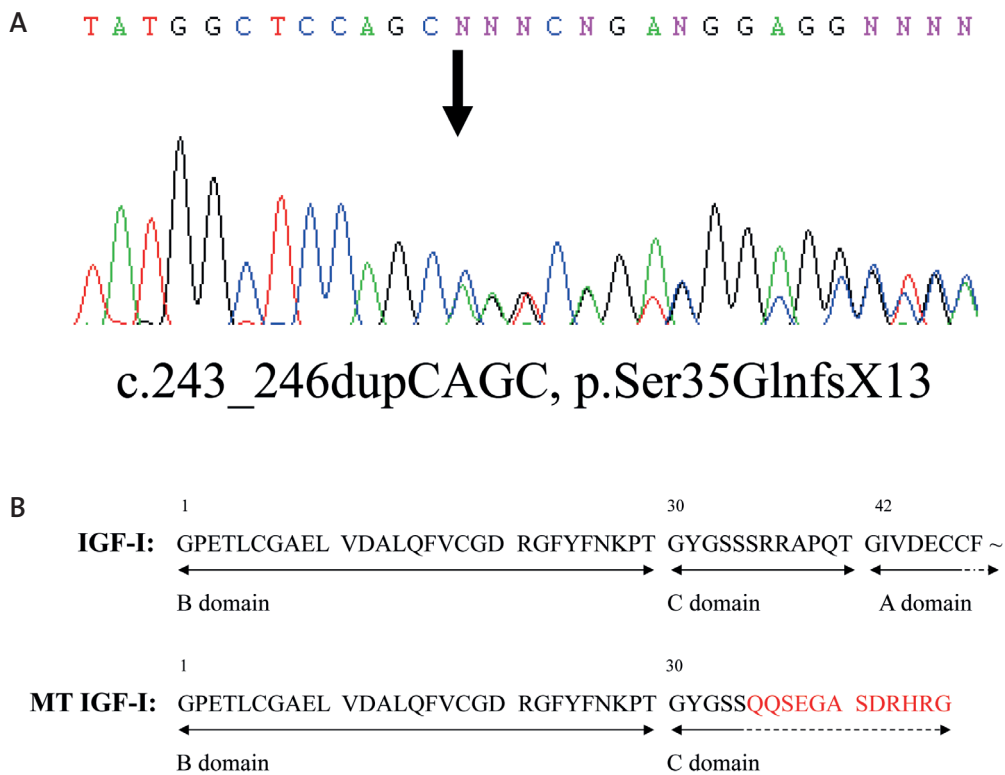


Figure 1

A. DNA sequence chromatogram of the four family members with the heterozygous *IGF1* mutation. The arrow indicates the duplication of the 4 nucleotides.

B. Sequence alignment of wild-type human IGF-I and the mutant IGF-I (MT IGF-I).
The MT *IGF1* has a heterozygous duplication of 4 nucleotides at position 247. The duplication leads to an IGF-I molecule truncated in its C-domain, which therefore only contains 5 of the 12 residues (Gly^{30}–Ser^{34}) originally seen in the C-domain in a wild-type IGF-I molecule. The truncation is followed by a tail of 12 residues with an altered amino acid sequence (red colour), that show no homology with the following A- or D-domain observed in wild-type IGF-I. Consequently, the MT IGF-I has no A- and D-domain though it still has an intact B-domain. The arrows mark the location of the different domains. The dotted arrow marks the altered C-domain, whereas the red colour marks the twelve altered residues in the truncated C domain.

Neutral gel filtration studies with WT and synthetic MT IGF-I

After equilibration with ^{125}I-IGF-I, in aliquots (n = 4) of pooled normal control serum a substantial part of the radioactivity (~53 – ~63%) was incorporated into the 150 kDa complex (Fig. 2A). In the sera of both the carriers and non-carriers the proportion of ^{125}I-IGF-I that was associated with the 150 kDa complex was somewhat less (varying between ~37 and ~52%) and, instead, slightly increased amounts of radioactivity were eluted in the 40–50 kDa fraction (consisting of binary IGF-IGFBP complexes) or remained unbound (data not shown).

Superdex 200 gel filtration column chromatography of normal serum after incubation with either ^{125}I-labeled WT hIGF-I or MT IGF-I (reduced form) revealed that the radiolabeled mutant peptide is able to bind to IGFBPs in serum but, in contrast to WT IGF-I, is hardly incorporated into 150 kDa complexes (Fig. 2A). Similar results were obtained for the oxidized form of MT IGF-I (data not shown).

In addition, competition gel filtration experiments were performed in which ^{125}I-WT hIGF-I was equilibrated with different aliquots of pooled normal serum in the presence of increasing amounts of either unlabeled WT-hIGF-I or MT IGF-I. As shown in figure 2B, in contrast to WT hIGF-I, MT IGF-I (reduced form) did not compete significantly with radiolabeled ligand for 150 kDa complex formation and only modestly for incorporation into 40–50 kDa complexes. The same results were encountered for the oxidized form of MT IGF-I (data not shown).

The results of acid G50 column chromatography of Sep-Pak C18 extracted sera from carriers of the *IGF1* mutation did not support the possibility that the putative circulating truncated mutant peptide forms heteromultimers with WT IGF- I, because in all cases WT IGF-I immunoreactivity was recovered in one peak corresponding to the MW of WT IGF-I, i.e. ~7.6 kDa (data not shown).

MT IGF-I (both reduced and oxidized) did not show any cross-reactivity in the semi-automated chemiluminescence IGF-I assay employed (30) nor in an in house RIA (31).

Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptor

We tested the ability of the reduced and two oxidized preparations of MT IGF-I to compete for ^{125}I-WT IGF-I binding to EBNA293t IGF-IR cells, as well as for ^{125}I-insulin binding to IM9 cells (oxidized peptide only). No competition was observed in either system at concentrations as high as 10 µM, indicating that the peptides do not bind to IGF-I receptors (Fig. 3) or insulin receptors (data not shown). Likewise, the peptides did not accelerate the dissociation of ^{125}I-IGF-I (negative cooperativity) and did not antagonize the negative cooperativity of cold IGF-I (data not shown).

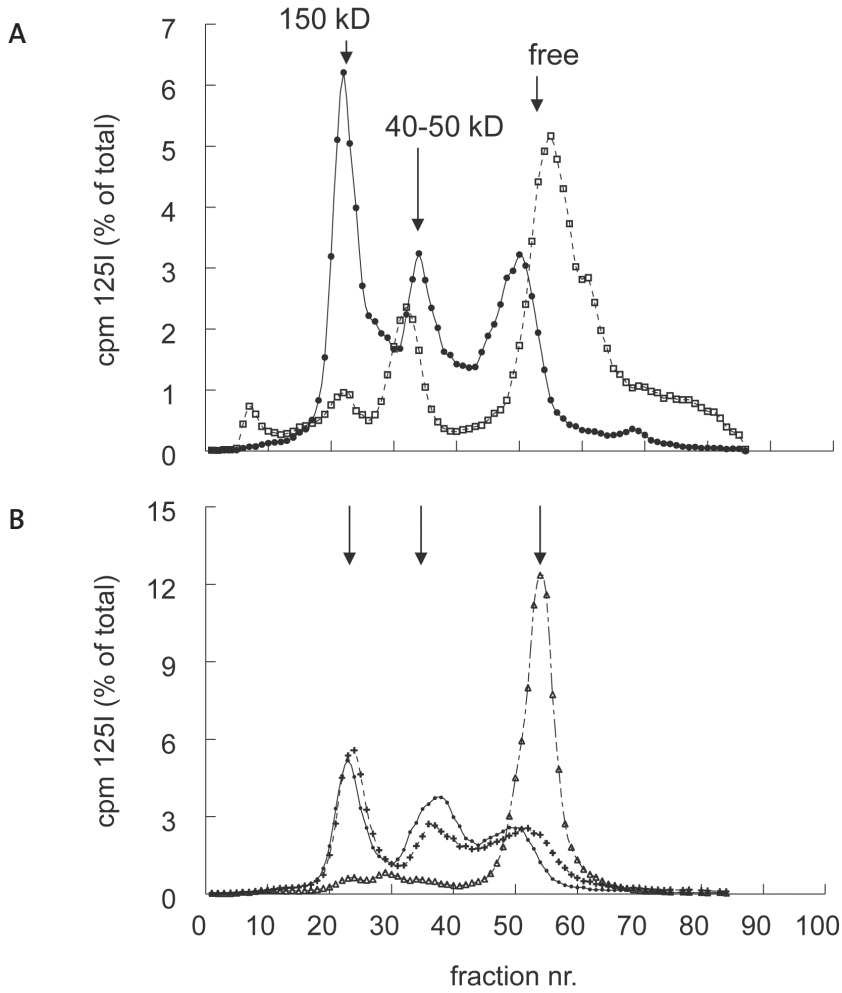
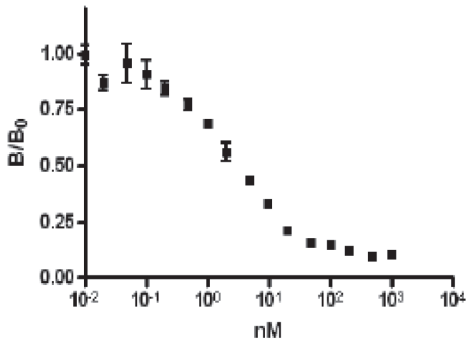


Figure 2

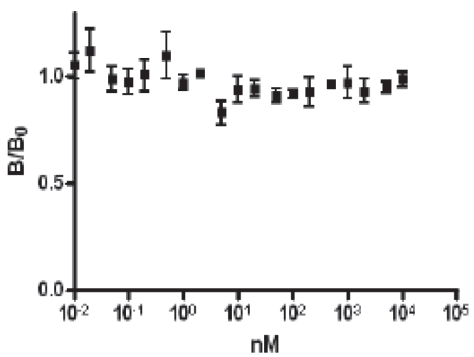
A. Aliquots of pooled normal serum were equilibrated with either ^{125}I radiolabeled WT hIGF-I (• —— •) or synthetic MT IGF-I (reduced form)(□- - -□) , followed by Superdex 200 gel filtration column chromatography.

B. Distribution of radioactivity among the various molecular weight classes, as determined by Superdex 200 gel filtration in aliquots of pooled normal serum that had been equilibrated with ^{125}I radiolabeled WT in the absence (• —— •) or presence (Δ- - - -Δ) of increasing amounts of unlabeled WT hIGF-I or reduced synthetic MT IGF-I (+ - - - +). Only the highest amounts of unlabeled WT hIGF-I and MT IGF-I employed are included in the figure, i.e. 26 pmol and 64 pmol, respectively.

A



B



C

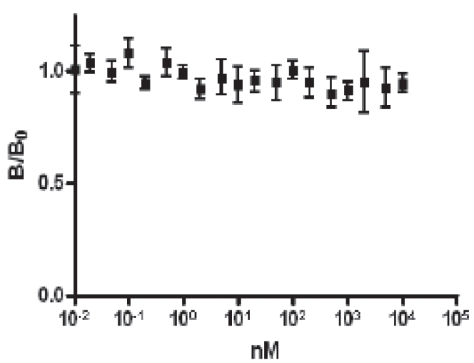


Figure 3

Competition of unlabeled IGF-I (A) and reduced (B) and oxidized (C) MT IGF-I for ^{125}I -IGF-I binding to EBNA-293 cells stably transfected with the IGF-I receptor. The data are plotted as bound tracer (as a fraction of tracer bound without unlabeled ligand), as a function of the concentration of competing ligand. IGF-I competes for tracer binding with a high affinity Kd of 1.3 nM, while both forms of the MT IGF-I are unable to compete at concentrations as high as 10 μM.

[^{3}H]-Thymidine incorporation assay

The MT IGF-I peptides were tested for their ability to stimulate thymidine incorporation in L6 myoblasts. The cells showed a robust response to WT IGF-I, while the MT peptides showed no effect in the physiological molar range of WT IGF-I (0.13–13 nmol/L) (36). Only a very small increase in thymidine incorporation was observed at very high non-physiological concentrations of MT IGF-I, which is probably an artificial effect since at these concentrations there was no binding to the IGF-I receptor. Figure 4 shows data for the reduced mutant peptide; similar data were obtained with the oxidized peptides. The mutant oxidized and reduced peptides did not antagonize the response to WT IGF-I (data not shown).

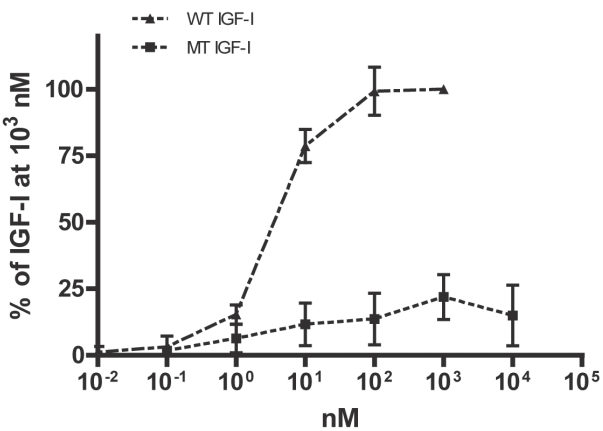


Figure 4

[^{3}H]-Thymidine incorporation assay. Dilution series of MT IGF-I (0–10,000 nM) were used to stimulate L6 cell proliferation. The data were plotted as MT IGF-I response compared to WT IGF-I response at 10^3 nM in percent, as a function of the MT IGF-I concentration. WT IGF-I was included in each assay as a control and data were plotted as percent of WT IGF-I response at 10^3 nM. Data presented display three independent experiments performed in triplicates ± SD. MT IGF-I is not able to stimulate mitogenesis in normal physiological concentrations.

Discussion

In a previous study on a patient with a homozygous missense mutation in the *IGF1* gene, presenting with severe intrauterine and postnatal growth retardation, microcephaly, and sensorineural deafness, we also investigated family members who were heterozygous for this *IGF1* mutation. These subjects exhibited SDS values for height, head circumference, and birth weight that were lower than those for non-carriers from the same family, but still within the normal range (22). Hence, it was surprising that the severity of the short stature of the two siblings with the heterozygous *IGF1* mutation described in the present study was, in fact, of a similar order of magnitude as observed in one of the three subjects with a homozygous *IGF1* mutation (23), though less severe than the patients with a complete loss of function of IGF-I (21,22). This finding prompted us to investigate the possible functional effects of the putative truncated IGF-I peptide as encoded by the mutated *IGF1* gene.

Although we could not establish whether any MT IGF-I was present in the circulation, we first hypothesized that in case it would, this truncated peptide may exert a dominant negative effect on the biological action of the WT IGF-I. Insulin and IGF-I bind to their respective dimeric receptors with a complex mechanism that involves cross-linking of two partial binding sites (sites 1 and 2) on each receptor half. Two distinct sites of the ligand are involved, leading to high affinity binding (37). We reasoned that while the truncated IGF-I clearly lacks the whole set of determinants needed to cross-link the two receptor halves, it may still have sufficient affinity to bind to one of the two receptor binding sites, which would thus enable it to prevent the cross-linking by native IGF-I, resulting in a dominant negative or antagonistic effect.

However, we demonstrated that synthetic MT IGF-I could not bind to the IGF1R or the insulin receptor at all and lacked significant mitogenic potency. Moreover, MT IGF-I did not show any antagonistic effects on either the binding of WT IGF-I to the IGF1R and the insulin receptor or its mitogenic capacity. This result could be explained by the fact that MT IGF-I lacks critical A- and C-domain residues involved in receptor binding. Furthermore, it likely has a disordered structure with the potential for an abnormal disulfide bond as in the oxidized peptide. Thus, the short stature of the carriers of the heterozygous *IGF1* mutation cannot be attributed to a blockade of WT IGF-I action at the receptor level.

On the other hand, MT IGF-I still does contain various domains important for binding to IGFBPs. Therefore, we investigated the possibility that MT IGF-I might tightly bind to IGFBPs (including 150 kDa complexes with ALS) in such a way that it would not be readily exchangeable with WT IGF-I. In theory, this would lead to a decrease in the half-life of circulating WT IGF-I (as observed in ALS deficient patients), and, as a consequence, deregulation of its bioavailability. However, the results of our gel filtration experiments

with both synthesized forms of MT IGF-I did not provide any evidence for this possibility, probably due to a disordered structure as discussed above. In addition, there was no indication that MT IGF-I directly interacted with WT IGF-I.

The observation that the incorporation of WT ^{125}I-IGF-I into 150 kDa complexes in sera from both carriers and non-carriers of the *IGF1* mutation appeared to be somewhat less than in normal sera is difficult to explain. Subtle individual differences between the serum concentrations of the various IGFBPs or ALS may account for this finding, although for all the subjects investigated the levels of these proteins were within their normal ranges (26). In all members of this family, the serum levels of IGF-II appeared to be either in the upper normal range or even elevated (26). A relatively higher endogenous serum IGF-II pool may have competed with the WT ^{125}I-IGF-I for 150 kDa complex formation.

Circulating endogenous levels of WT IGF-I (MT IGF-I could not be detected by the IGF-I assays employed) in the four heterozygous carriers of the *IGF1* mutation were found to be significantly reduced, i.e. varying between – 1.8 and – 2.6 SDS (26). In each case, this appears to represent about half of that of the corresponding normal mean serum IGF-I level when adjusted for age and gender.

We therefore conclude that the severe short stature of the two index cases is not caused by a dominant negative or antagonistic effect on the function of WT IGF-I. We speculate that the severe short stature might be caused by a cumulative effect of three factors: 1) WT *IGF1* haploinsufficiency; 2) placental dysfunction due to maternal WT *IGF1* haploinsufficiency; and 3) other genetic factors associated with (mild) short stature (26). Our studies showed that MT IGF-I is not able to bind to the IGF1R, therefore we speculate that reduced height can be caused by WT *IGF1* haploinsuffiency; it seems that the WT copy of *IGF1* does not produce enough IGF-I to reach normal height. Another factor that may contribute to the short stature observed is placental dysfunction due to maternal WT *IGF1* haploinsufficiency, as discussed in our earlier paper (26). There is a strong correlation between the rate of maternal IGF-I increase during pregnancy and placental weight (38). Various studies have been performed to investigate IGF-I in intrauterine growth retardation pregnancies. Contradicting results have been observed in studies concerning placenta IGF-I and IGF1R mRNA and protein expression. Some studies reported increased expression of IGF-I and IGF1R in placentas from small for gestational age (SGA) newborns compared with appropriate for gestational age (AGA) newborns (39, 41), while other studies showed decreased or equal expression. Inconsistencies could be due to differences in the selection criteria for IUGR, length of gestation and the methodology used (40, 42). Based on the observation that birth length and childhood height of case III-1 is 1–2 SD lower than of her mother and our previous observations in a family with an *IGF1R* mutation (38), in which the proband's length was much more affected than in her mother, who did not inherit the

mutation maternally, we hypothesize that IGF-I and IGF1R play an important role in normal placental function and consequently in intrauterine growth.

Interestingly, we observed that GH therapy was efficacious in increasing height in the first two years of treatment, without adverse events, which may be explained by the normalisation of IGF-I availability.

Acknowledgments
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