Insulin-like growth factor-binding protein-3 protease activity in Snell normal and Pit-1 deficient dwarf mice

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Abstract

Partial proteolysis of insulin-like growth factor-binding protein-3 (IGFBP-3) lowers its affinity for IGFs. Presumably, this leads to destabilization of the ternary IGF-IGFBP-3-acid-labile subunit complex in the circulation and an increased bioavailability of IGFs. We investigated the effect of GH on IGFBP-3 proteolysis by comparing serum from normal mice and GH-deficient dwarf mice. While normal mouse serum degraded ^{125}I -IGFBP-3, this activity declined with age. In contrast, serum from dwarf mice displayed strong proteolytic activity at all ages tested (up to 10 weeks). In dwarf mice of 4 weeks and older, this activity could not be inhibited by EDTA and 1,10-phenanthroline, indicating the presence of a divalent

cation-independent protease. Prolonged treatment with GH (4 weeks) did not decrease the overall potency of the serum to degrade IGFBP-3, but partially restored the ability of EDTA to inhibit IGFBP-3 protease activity. GH deficiency therefore appears to induce a new kind of IGFBP-3 protease. Similarly, serum from hypophysectomized rats displayed enhanced IGFBP-3 protease activity compared with control rat serum. These results suggest that a protease induced under conditions of severe GH deficiency may contribute to making IGFs optimally available to the tissues.

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Introduction

The actions of the insulin-like growth factors IGF-I and IGF-II are modulated by the high affinity IGF-binding proteins (IGFBPs), a family of six structurally related proteins (Shimasaki & Ling 1991, Jones & Clemmons 1995). IGFBP-3 is the most abundant carrier protein of the IGFs in the circulation. Together with IGF and an 85 kDa acid-labile subunit (ALS) it forms a 150 kDa ternary complex which protects IGF against rapid degradation and clearance from the circulation (Baxter 1994). In addition, IGFBP-3 sequesters the IGFs away from their receptors, thereby limiting their biological activity.

IGFBP-3, as well as IGF-I and ALS, are regulated by growth hormone (GH). Thus, IGFBP-3 levels are decreased in growth hormone deficiency (GHD) and increased in acromegaly (Baxter & Martin 1986, Blum *et al.* 1990). In GHD dwarf rats and hypophysectomized (hypox) rats, GH induces IGFBP-3 and restores the 150 kDa ternary complex (Gargosky *et al.* 1994, Fielder *et al.* 1996).

Another mechanism by which the inhibitory activity of IGFBP-3 can be regulated is through limited proteolysis. This modulation of IGFBP-3 occurs during pregnancy in humans (Giudice *et al.* 1990, Hossenlopp *et al.* 1990), rats (Davenport *et al.* 1990) and mice (Fielder *et al.* 1990). The generated fragments bind IGFs with lower affinity com-

pared with intact IGFBP-3, resulting in an increased bioavailability of IGF (Blat *et al.* 1994). Increased proteolysis of IGFBP-3 has also been observed in severe illness (Davies *et al.* 1991), during postoperative stress (Cwyfan Hughes *et al.* 1992, Davenport *et al.* 1992a), in malignancies (Coulson *et al.* 1991, Muller *et al.* 1994) and in diabetes mellitus (Bang *et al.* 1994, Bereket *et al.* 1995).

It is not clear if proteolytic activity towards IGFBP-3 is regulated by GH. Binoux *et al.* (1993) reported that GHD patients have an increased proportion of proteolyzed IGFBP-3 in their circulation as compared with normals, while IGFBP-3 proteolysis was decreased in acromegalic subjects. Fielder *et al.* (1990), however, found that hypophysectomy had no effect on IGFBP-3 proteolysis by mouse serum. These results are in conflict with the findings of Rutishauer *et al.* (1993) who used hypox rats to show that GH induces an IGFBP-3 protease in serum.

Dwarfed Snell mice (genotype *dw/dw*) have a mutation in the gene for the Pit-1 transcription factor, which renders them deficient in GH, prolactin, and thyroid-stimulating hormone (Van Buul-Offers 1983, Li *et al.* 1990). The Snell heterozygous (*+ / dw*) mice are phenotypically normal. We previously used these mice to study the effects of GH and IGFs on the serum levels of IGFBPs (Van Buul-Offers *et al.* 1994a,b). IGFBP-3 levels were almost undetectable in GHD dwarfs, but were

significantly induced by treatment with GH and IGF-I and IGF-II, but not by thyroxine. We now report on the regulation of IGFBP-3 proteolysis in serum of Snell normal and dwarf mice.

Materials and Methods

Materials

BSA, Triton X-100, aprotinin, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, and 2-mercaptoethanol were from Sigma Chemical Co., St Louis, MO, USA.

Human GH was from Kabi-Pharmacia B.V., Woerden, The Netherlands (Genotropin) or from Novo Nordisk A/S, Bagsvaerd, Denmark (Norditropin). Solutions of GH were prepared in PBS, pH 7.4, containing 0.2% BSA.

Recombinant human IGFBP-3 (*E. coli* derived, non-glycosylated, and CHO cell derived, glycosylated) were kindly provided by Drs Christopher A Maack, Jerome A Moore and Andreas Sommer of Celtrix Pharmaceuticals, Inc., Santa Clara, CA, USA (Sommer *et al.* 1991, Tressel *et al.* 1991). The proteins were iodinated with ^{125}I (Amersham International plc, Amersham, Bucks, UK) by the chloramine T method. Unbound radioactivity was removed on a PD-10 (Sephadex G-25) column (Pharmacia Biotech, Uppsala, Sweden). Specific activities for the radioiodinated IGFBP-3 ranged from 40 to 80 $\mu\text{Ci}/\mu\text{g}$ protein.

Serum samples

Pooled serum of 20 healthy adults was used as a normal control. Serum from women in the 3rd trimester of pregnancy was used as a comparison for other serum samples, since it contains strong proteolytic activity for IGFBP-3 (Giudice *et al.* 1990, Hossenlopp *et al.* 1990, Lamson *et al.* 1991). Normal and dwarf Snell mice were bred and kept under standardized laboratory conditions, as described earlier (Van Buul-Offers *et al.* 1986). Dwarf mice between 6 and 8 weeks old were treated with GH (16.6 mU/day) or PBS for 4 weeks. Further details have been described previously (Van Buul-Offers *et al.* 1994b). Two hours after the last injection, the animals were killed by decapitation and blood was collected from the cervical stump and placed on ice. For each experiment, sera from five mice (males and females) were pooled. Sera of untreated mice were pooled from three to seven animals. Sera were frozen at -20°C until use.

Serum from normal and hypox male Wistar rats (5 weeks old) were obtained from Iffa Credo, L'Arbresle, France. Hypophysectomy was performed 3 days before serum collection.

IGFBP-3 protease assay

The presence of proteases in serum which can degrade IGFBP-3 was determined essentially as described by

Lamson *et al.* (1991). Serum (2 μl) was mixed with 40 000 c.p.m. ^{125}I -IGFBP-3 (glycosylated or non-glycosylated as indicated in the text) in a total volume of 30 μl 100 mM Tris-HCl, pH 7.4, containing 0.5 mM CaCl_2 and 1% Triton X-100. Protease inhibitors were added as indicated in the text. The mixture was incubated for 16 h at 37°C and the reaction was stopped by the addition of 50 μl SDS-sample buffer (containing 10% 2-mercaptoethanol) and boiling for 3 min. The samples were electrophoresed on a 12% polyacrylamide gel, followed by staining in Coomassie Brilliant Blue and autoradiography using Fuji RX X-ray film. Quantitation of the gels was performed by phosphorimaging using a GS-363 Molecular Imager System together with Molecular Analyst software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amount of proteolysis was expressed as the intensity in each lane which was associated with the degradation products of IGFBP-3 relative to the total intensity in the lane. The molecular masses of the fragments were calculated using broad-range molecular mass markers (Bio-Rad) as standards.

In a different approach, pooled normal human serum (1.6 μl) was mixed with the serum to be tested (0.6 μl) in a final volume of 30 μl of the same Tris-HCl buffer as above. After a 16 h incubation at 37°C , the protease-induced disappearance of the intact IGFBP-3 doublet in the reference serum was monitored by Western ligand blotting.

Western ligand blotting

IGFBPs in serum were visualized by non-reducing SDS-PAGE (7–15% gradient gels) and electrotransfer to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) followed by incubation with ^{125}I -IGF-II. The procedure has been described in detail previously (Hossenlopp *et al.* 1986, Van Buul-Offers *et al.* 1994b).

Statistical analysis

Student's *t*-test was used to determine significant differences between two groups.

Results

Proteolysis of ^{125}I -IGFBP-3 by various serum samples is presented in Fig. 1. On reducing SDS-PAGE, non-glycosylated IGFBP-3 migrated as a single band of 35 kDa (Fig. 1A). Human non-pregnancy serum (standard pools) and serum from normal adult mice (FVB strain, 8 weeks old) displayed little proteolytic activity: $32.9 \pm 7.2\%$ (mean $\pm$ s.d., $n=4$) and 39.0% ($n=2$) were degraded by these sera respectively. Human pregnancy serum as well as serum from a normal mouse (FVB strain) at 3 weeks of pregnancy proteolyzed IGFBP-3 and generated a major fragment migrating at 18 kDa. Other bands with varying

Figure 1 Proteolysis of IGFBP-3 by various sera. Non-glycosylated (A) or glycosylated (B) 125 I-IGFBP-3 was incubated with 2 μ l serum as detailed in Materials and Methods. Samples were electrophoresed on SDS gels under reducing conditions. Photographs show composites of autoradiograms of representative experiments. Molecular masses (kDa) of the fragments are indicated. The arrow in (B) indicates the series of fragments which were unique for the degradation of glycosylated IGFBP-3 by serum from dwarf mice. Lanes represent sera from the following: standard, normal pool; pregnancy, human pregnancy (third trimester); FVB, 8-week-old FVB mouse; FVB/preg, FVB mouse at late pregnancy (21 days); normal, $+/\text{dw}$ Snell mouse at 2 and 8 weeks old; dwarf, dw/dw Snell mouse at 2 and 8 weeks old; FVB/dw, FVB intercrossed with dwarf at 8 weeks old.

intensities were observed at 29, 25 and 22 kDa. Also smaller fragments of <10 kDa were prominent near or at the front of the gel. On a 10–20% polyacrylamide gradient gel, these latter bands migrated well behind the dye front, indicating that they did not represent free radioiodine, but indeed were peptide fragments (result not shown). The total amount of proteolysis, both for the human and mouse pregnancy sera, was 88%. Serum from 2-week-old Snell normal mice contained an increased proteolytic activity compared with 8-week-old normal mice (from 36 to 68% in this experiment). By comparison, serum from Snell dwarf mice displayed high protease activity at both ages (76 and 88% at 2 and 8 weeks respectively). Dwarf mice with an FVB genetic background (obtained by intercrossing Snell dw/dw with FVB (Van Buul-Offers *et al.* 1995)) gave results identical to the Snell dwarfs. In contrast to the pregnancy sera and the normal 2-week-old mouse sera, the dwarf sera generated mostly the smaller (<10 kDa) IGFBP-3 fragments.

Serum from Snell mice also proteolyzed glycosylated recombinant IGFBP-3, as is illustrated in Figs. 1B and 4A. The effect (41 and 44% proteolysis for dwarfs at 2 and 8 weeks old) was less prominent than with non-glycosylated

IGFBP-3, suggesting a protective effect by the sugar side chains. Human pregnancy serum generated fragments of 30, 24, and 20 kDa, together with two small bands migrating at or near the dye front of the gel. The same fragments were observed, albeit faintly, with normal mouse serum. They appeared to be degraded further, generating the bands at or near the front. Incubation with sera from dwarf mice of 8 weeks old generated an additional series of bands which migrated just below the 41/44 kDa doublet of the intact IGFBP-3. Pregnancy serum, serum from normal mice or serum from 2-week-old dwarf mice did not induce these bands. We never observed these bands with non-glycosylated IGFBP-3, suggesting that they may be derived from partial deglycosylation of IGFBP-3. Alternatively, glycosylation of the protein makes cleavage sites near the N terminus or the C terminus more susceptible to proteolysis.

The amount of intact IGFBP-3 in normal mouse serum (as measured with Western ligand blotting) increases with age (Van Buul-Offers *et al.* 1994a). Therefore we were interested in determining the IGFBP-3 protease activity during postnatal development in more detail. Figure 2 shows that in normal mice the protease activity declined

Figure 2 Age-dependent protease activity in sera from normal and dwarf mice. Serum pools from Snell normal (●, ○) and dwarf mice (▲, △) of different ages were tested for their ability to degrade non-glycosylated IGFBP-3. The amount of proteolysis was determined and expressed as described in Materials and Methods. The closed symbols represent values in the absence of EDTA, the open symbols in the presence of 10 mM EDTA. For the points at 2 weeks old, three different serum pools were tested (each measured once or twice); for the points at 8 weeks old, two different pools were tested (each measured twice); the points at the other ages are single measurements.

with increasing age, while in dwarf mice this activity remained at the same high level throughout the 8-week period tested. However, the ability of EDTA to inhibit the activity in dwarf mice disappeared between 2 and 4 weeks old, indicating that at this age proteases appear which differ from the pregnancy-associated proteases and which are not dependent on divalent cations.

In order to compare further the proteolytic activities in dwarf mice, young normal mice and human pregnancy,

we tested various protease inhibitors for their ability to inhibit the proteolytic activity of the respective sera (Fig. 3). EDTA and the Zn^{2+} -chelator phenanthroline as well as the serine protease inhibitors PMSF and aprotinin inhibited the proteolytic activity in human pregnancy serum, consistent with the notion of a divalent cation-dependent serine protease being the enzyme responsible for IGFBP-3 proteolysis in pregnancy (Hossenlopp *et al.* 1990, Lamson *et al.* 1991). The inhibition pattern in Snell normal mice was similar, although PMSF seemed less potent than in pregnancy serum, because it could only prevent the formation of the smallest fragments (<10 kDa), while in its presence the intensity of the 18 kDa band was increased. Two-week-old Snell dwarf mice were similar to normal mice with respect to EDTA, PMSF and aprotinin, but the proteolytic activity in this serum was totally unsusceptible to phenanthroline. As in human pregnancy serum, the protease in serum from pregnant mice was inhibited by all four inhibitors tested, including phenanthroline (not shown). This indicates that the inability of phenanthroline to inhibit the protease in dwarf mice is limited to the GH deficiency state, and is not peculiar to the mouse species. Also at 4 and 8 weeks old, the protease in serum from dwarf mice was unsusceptible to phenanthroline, while in normal mice of these ages, phenanthroline remained inhibitory (not shown).

GH treatment can partially restore the deficiency in IGFBP-3 in dwarf mice (Van Buul-Offers *et al.* 1994a,b). We therefore investigated the effect of GH treatment on the protease activity. Figure 4A shows a representative example of the degradation of non-glycosylated and glycosylated IGFBP-3 by sera of PBS- and GH-treated dwarf mice. The results are presented in a quantitative fashion in Fig. 4B. After treatment for 4 weeks, the proteolytic degradation of non-glycosylated IGFBP-3 decreased from

Figure 3 Effect of protease inhibitors. ^{125}I -IGFBP-3 (non-glycosylated) was incubated with human pregnancy serum and serum from normal and dwarf Snell mice (2 weeks old) in the absence and presence of protease inhibitors. (A) Autoradiograms of a representative experiment. (B) Proteolysis of IGFBP-3, expressed as percentage of IGFBP-3 that appeared as fragments on the gel. Values for pregnancy serum are from one representative experiment. Values for the mouse sera are the means of two separate serum pools, which were each measured once or twice. c, control without inhibitor; E, 10 mM EDTA; ph, 2 mM 1,10-phenanthroline; P, 10 mM PMSF; ap, 2 mg/ml aprotinin.

Figure 4 Effect of GH treatment on IGFBP-3-proteolytic activity. Dwarf mice were treated with GH or PBS vehicle as described in Materials and Methods. Proteolytic activities in serum pools (from five animals) were tested with ^{125}I -IGFBP-3 (both non-glycosylated and glycosylated) in the absence or presence of 10 mM EDTA. (A) Autoradiogram of a representative gel. (B) Quantitation of proteolytic activities of serum pools from four independent experiments. Proteolysis is expressed as the percentage (mean $\pm$ S.D.) of IGFBP-3 that appeared as fragments on the gel. Each serum pool was measured between one and three times. Statistical significance between groups is indicated by their *P* value. The molecular masses (kDa) of marker proteins are indicated on the left.

85.5 to 69.9%, a non-significant decrease. Similarly, the assay using glycosylated IGFBP-3 showed proteolysis of 47.0 and 33.4% without and with treatment respectively. This decrease was also not significant. As mentioned above (Fig. 2), the inhibitory effect of EDTA on the protease activity of these (older) dwarf mice was minor, but in the GH-treated animals EDTA could significantly inhibit the protease activity (Fig. 4B). In addition, in the presence of EDTA, GH treatment significantly decreased the proteolysis of glycosylated IGFBP-3 (from 37.2 to 20.6%). This suggests that during GHD a cation-independent protease is induced, which is largely suppressed by GH treatment.

The pronounced proteolysis in GHD mice is in contrast with the results of Rutishauser *et al.* (1993) who described a GH-dependent IGFBP-3 protease in rats which was lacking from hypox rats. In order to directly compare hypox rats with dwarf mice, we performed an experiment in which the degradation of both ^{125}I -IGFBP-3 tracers was compared for normal and GHD samples of both species. As shown in Fig. 5, hypox rats displayed about twice as much protease activity as normal rats when non-glycosylated IGFBP-3 was used. In contrast with the dwarf mice, the activity in the hypox rats could be well inhibited by EDTA. The activity towards glycosylated IGFBP-3 was negligible (compare with Fig. 1B). This result supports the conclusion reached with the dwarf mice

that the protease is induced under conditions of GH deficiency, and not by GH as was suggested earlier (Rutishauser *et al.* 1993).

In order to supplement some of the data obtained with radiolabeled IGFBP-3 preparations, we performed mixing experiments with normal human serum as substrate for proteolytic activity. Disappearance of intact IGFBP-3 as visualized by Western immunoblotting (Fig. 6A) was seen for human pregnancy serum and serum from 2-week-old dwarf mice. In both these sera, EDTA inhibited the protease. The amount of proteolysis by dwarf serum (46% of the endogenous IGFBP-3 in the reference serum) in this system was comparable to proteolysis of glycosylated ^{125}I -IGFBP-3 (Fig. 1B), which was 41%. Incubation of reference serum with hypox rat serum resulted in a 49% decrease in the intensity of the IGFBP-3 doublet as compared with a control incubation with normal rat serum (not shown). Figure 6B shows that also in this system, an age-dependent decrease of protease activity was observed in normal mice (compare with Fig. 2).

Discussion

In this study we have characterized proteolytic activity for IGFBP-3 in normal mouse serum and in dwarf mouse

Figure 5 Proteolysis by sera from normal and hypox rats, compared with normal and dwarf mice. ¹²⁵I-IGFBP-3 (non-glycosylated and glycosylated) was incubated with sera from normal rats (contr), hypox rats (hypox), Snell normal mice (+/dw) and Snell dwarf mice (dw/dw) in the absence or presence of 10 mM EDTA. The mice were 10 weeks old, the rats 5 weeks. Autoradiograms of the gels are shown, together with the percentage of proteolysis. The experiment was repeated once with similar results.

serum. While young normal mice (2 weeks old) showed considerable activity, this declined with age (Figs 1, 2 and 6B). This correlates with a rise in IGFBP-3 levels as measured by Western ligand blotting (Van Buul-Offers *et al.* 1994a). In contrast, dwarf mice displayed a strong activity well into adulthood. The results obtained with a protease assay using ¹²⁵I-IGFBP-3 (Lamson *et al.* 1991) were confirmed when normal serum was used as a source of IGFBP-3 substrate, which was detected by Western ligand blotting.

The proteolytic activity in dwarf mice, especially at later ages, differs in nature from the activity found in young normal mice and in human pregnancy. First, incubation of non-glycosylated IGFBP-3 with serum from dwarf mice resulted in more extensive degradation than with the other sera; only fragments of <10 kDa remained. Secondly, a characteristic set of degradation bands migrating just below the doublet of glycosylated IGFBP-3 was seen with serum from dwarfs of 4 weeks and older (Fig. 1B shows 8 weeks, but we also observed this pattern at 4 weeks). These bands did not appear when glycosylated IGFBP-3 was degraded by serum from normal mice or pregnant women. Also hypox rat serum generated these bands, although they are only faintly visible on the right panel of Fig. 5. Thirdly, from 4 weeks old onward the proteolytic activity in dwarf serum could not be inhibited by EDTA. While phenanthroline usually inhibits the activity in human pregnancy serum even more strongly than EDTA does, we consistently found no effect of this zinc chelator on the activity in dwarfs (at all ages tested). This is in contrast with most IGFBP-3 proteases described so far, which have been characterized as divalent cation-dependent serine proteases or metalloproteinases (Fielder *et al.* 1990, Hossenlopp *et al.*

1990, Cohen *et al.* 1992, Davenport *et al.* 1992b, Fowlkes *et al.* 1994). The only IGFBP-3 proteases which have been described that are not inhibitable by EDTA or phenanthroline are plasmin (Campbell *et al.* 1992), cathepsin D (Conover & De Leon 1994) and nerve growth factor (Rajah *et al.* 1996). The activity in dwarf mice appears to be caused by a mixture of at least two different enzymes since treatment with GH partially restored the inhibitory effect of EDTA. Apparently, GH suppresses the divalent cation-independent enzyme, whereas the total proteolytic activity in the circulation is only minimally altered. There is a striking difference between the inhibitory activity of aprotinin, which largely prevented proteolysis in mouse sera, and PMSF, which prevented the generation of the small (<10 kDa) fragments, while an 18 kDa product accumulated (Fig. 3A). Although both are serine protease inhibitors, their specificities and mechanisms of action differ. In addition, PMSF is labile in aqueous solutions, so proteases which may be activated during the 16 h incubation period can no longer be inhibited.

GHD rats showed high protease activities already in early pregnancy, while in GH-normal rats protease activity did not occur until late pregnancy (Gargosky *et al.* 1993). Also the cleavage pattern was slightly different from that produced in late pregnancy. This underscores our finding that in GHD a distinct IGFBP-3 protease is induced. A more detailed study with more specific enzyme inhibitors and/or antibodies will be needed to further characterize the responsible enzyme(s).

The role of GH in the regulation of IGFBP-3 proteases has received limited attention so far. Rutishauer *et al.* (1993) found proteolytic activity in rat serum, which disappeared upon hypophysectomy. GH treatment restored

Figure 6 Western ligand blot from mixing experiments. Reference serum (pool of normal human donors) was incubated with human pregnancy serum or mouse serum in the absence or presence of 10 mM EDTA as described in Materials and Methods. (A) Autoradiogram of the Western ligand blots. The mouse serum was from dwarfs of 2 weeks old. (B) Quantitation of the density of the IGFBP-3 doublet (arbitrary units). The left and middle panel correspond to the two panels in (A). The right panel of (B) shows the decline of the proteolytic activity in normal mice with increasing age. ref, ref+BSA, reference serum (1.6 µl) incubated with BSA; pregn, pregnancy serum and dwarf, dwarf serum (0.6 µl) without incubation; E, EDTA (10 mM). The results shown are from a representative experiment. The experiment was repeated with similar results.

the protease activity in hypox rats. These results are in sharp contrast with our findings. Also in experiments in which we used glycosylated ^{125}I -IGFBP-3, as Rutishauser *et al.* (1993) did, or when we compared normal rat serum with hypox rat serum, we consistently found that IGFBP-3 proteolysis is favored under GH-deficient conditions. In analogy to the results with hypox rats (Rutishauser *et al.* 1993), we found that GH-deficient dwarf mice have very low IGFBP-3 levels as measured by Western ligand blotting, which are increased by GH administration (Van Buul-Offers *et al.* 1994a,b). An increase in protease activity, as reported by Rutishauser *et al.* (1993) would instead be expected to lead to a decrease of IGFBP-3 as measured by this method, because partially proteolyzed IGFBP-3 cannot be detected by this method (Gargosky *et al.* 1992, Liu *et al.* 1992). At this time, we cannot reconcile the discrepancies between our results and those of Rutishauser *et al.* (1993). It is evident, however, that differences exist between the hypox rat and

dwarf mouse models. GH treatment can restore IGFBP-3 to normal levels in hypox rats (Rutishauser *et al.* 1993, Fielder *et al.* 1996), while it only partially restores IGFBP-3 in dwarf mice (Van Buul-Offers *et al.* 1994a,b). Furthermore, our results (Fig. 5) indicate that the IGFBP-3 protease activity in the hypox rat serum is lower than in the dwarf mouse serum and can be better inhibited by EDTA.

Our results with dwarf mice appear to correlate better with a study in which GH-deficient patients were compared with normal controls (Binoux *et al.* 1993). IGFBP-3 in the patient serum was proteolyzed to a greater extent (53%) than in the controls (37%).

It remains an open question why GH treatment only has a limited effect on the protease activity in dwarf mice. As mentioned earlier, also IGF-I and IGFBP-3 levels are only minimally elevated (Van Buul-Offers *et al.* 1994a,b). Also the 150 kDa complex of IGFBP-3 with IGF-I and ALS is not restored (Van Buul-Offers *et al.* 1994a). The effects of

GH treatment on growth pattern (Van Buul-Offers & Van den Brande 1978, Van Buul-Offers *et al.* 1994a,b) and growth plate morphology (Smeets & Van Buul-Offers 1983) are much more pronounced, however, suggesting that paracrine or autocrine actions of locally induced IGF-I prevail. Others have also reported GH-induced stimulation of growth in hypox rats (Orlowski & Chernausk 1988) and Snell dwarf mice (Pell & Bates 1992) while circulating IGF-I levels were not or only modestly increased. It appears that administration of GH by continuous infusion restores circulating concentrations of IGF-I and IGFBP-3 much better than administration by twice-daily injections does (Gargosky *et al.* 1994). It would therefore be of interest to determine if also the IGFBP-3 protease activity decreases more after continuous administration. In addition, the proposed local actions of GH warrant study of the regulation of IGFBP-3 proteases at the cellular level.

Taken together, our results suggest that in GH deficiency a complex protease system is present that results in cleavage of IGFBP-3. Presumably, this leads to release of IGF-I and/or IGF-II from the 150 kDa ternary complex, making these growth factors available for binding to the type I IGF receptor. This mechanism resembles the enhanced availability of IGF-I through IGFBP-3 degradation in other catabolic states such as pregnancy (Blat *et al.* 1994), chronic renal failure (Lee *et al.* 1993), insulin-dependent (Bereket *et al.* 1995) and non-insulin-dependent diabetes mellitus (Bang *et al.* 1994), and severe illness (Davies *et al.* 1991). In the same context, prostate specific antigen is an IGFBP-3 protease which potentiates IGF action (Cohen *et al.* 1994). Similarly, IGFBP-3 proteases have been suggested to play a role in tumor progression by allowing IGF-I to exert its mitogenic actions on tumor cells (Muller *et al.* 1994). Clearly, proteolysis of IGFBP-3 and in particular IGFBP-3 is an important defense mechanism and therefore further investigations into its nature and regulation are certainly warranted.

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