Decreased intracellular degradation of insulin-like growth factor binding protein-3 in cathepsin L-deficient fibroblasts

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Abstract Proteolysis of insulin-like growth factor binding proteins (IGFBPs) is the major mechanism of releasing IGFs from their IGFBP complexes. Analysis of fibroblasts deficient for the lysosomal cysteine protease cathepsin L (CTSL) revealed an accumulation of IGFBP-3 in the medium which was due neither to alterations in IGFBP-3 mRNA expression nor to extracellular IGFBP-3 protease activity. Incubation of CTSL-deficient fibroblasts with radiolabeled IGFBP-3 followed by subcellular fractionation indicates that both intact and fragmented IGFBP-3 accumulate transiently in endosomal and lysosomal fractions of CTSL-deficient cells. This suggests the involvement of CTSL in the intracellular degradation of IGFBP-3 representing a new mechanism to regulate the extracellular concentration of IGFBP-3.

Key words: Insulin-like growth factor binding protein-3; Cathepsin L; Endocytosis

1. Introduction

The binding of insulin-like growth factors (IGF I and II) to IGF and insulin receptors initiates various intracellular processes resulting in an increased rate of cell proliferation, growth and differentiation of cells and tissues [1]. The availability of IGFs is regulated by the presence of six high affinity IGF binding proteins (IGFBP-1–6). The expression pattern and secretion of IGFBPs are cell- and tissue-specific, altered during development, and affected by various cytokines and hormones [2]. IGFBP-3 is secreted by different cell types which may affect IGF actions on cell growth in either an inhibitory or a stimulatory manner. Furthermore, there is also evidence that IGFBP-3 inhibits cell growth and is involved in apoptotic processes in an IGF I-independent manner [3,4].

Limited proteolysis of IGFBPs is believed to be the major mechanism for the release of IGFs from IGFBP/IGF complexes, generating fragments with reduced affinity for IGFs [8]. IGFBP protease activities have been reported to be present in physiological fluids and media of cultured cells differing in their susceptibility against inhibitors, pH optimum, requirements of cations, tissue and IGFBP specificity, and their mechanism of activation and regulation [5]. Recently, the disintegrin metalloprotease ADAM 12-S, the pregnancy-associated plasma protein A, and the complement component C1s have been identified as IGFBP proteases [6–8]. Furthermore, it has been suggested that secreted lysosomal proteases such as cathepsin D may be involved in the regulation of extracellular IGFBP levels [9,10]. In the present study, we report on the decrease in intracellular degradation of endocytosed IGFBP-3 in fibroblasts deficient for the lysosomal cysteine protease cathepsin L (CTSL) resulting in an extracellular accumulation of IGFBP-3.

2. Materials and methods

2.1. Materials

Sodium [^{125}I]iodine, [^{32}P]dCTP, ^{T7}Quickprime kit, Percoll, and prestained protein standard (Rainbow) were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Recombinant human (rh) IGF II from GroPep (Adelaide, Australia) was iodinated by the chloramine T method. Non-glycosylated rhIGFBP-3, a generous gift from Dr. A. Sommer (Celtrix, Santa Clara, CA, USA), and rhIGFBP-4 and -6, kindly provided by Dr. J. Zapf (University Hospital Zurich, Switzerland), were iodinated using Iodo-Gen (Pierce Chemical, Rockford, IL, USA) as described [11]. Pansorbin (10% *Staphylococcus aureus* cell suspension) was from Calbiochem-Novabiochem (Schwalbach, Germany). Human kidney CTSL was from Biogenesis (Poole, UK). Recombinant IGFBP-1 and the rabbit antiserum against human IGFBP-3 were purchased from UBI (Lake Placid, NY, USA).

2.2. cDNA probes

The rat IGFBP-3 cDNA clone pRBP-3-AR, and the rat IGFBP-4 cDNA clone pRBP-4-SH were kindly provided by Dr. Ling (La Jolla, CA, USA [12]) and the *ctsl* cDNA was from Dr. M.M. Gottesman (Bethesda, MD, USA). The 28S rRNA was detected with a complementary oligonucleotide [13].

2.3. Animals

Target disruption and generation of CTSL-deficient homozygous mice (*ctsl*−/−) on a mixed B6/129/J background is described elsewhere [14]. Age-matched C57/BL76 mice served as controls. The animals were maintained and killed according to institutional guidelines in animal facilities of the University Medical Center, Freiburg, Germany.

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Abbreviations: IGF, insulin-like growth factors; IGFBP, IGF binding protein; CTSL, cathepsin L; DMEM, Dulbecco's modified essential medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

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2.4. Isolation and culture of mouse cells

Fibroblasts were prepared from skin biopsies of adult mice by incubation in 10 mM phosphate-buffered saline (PBS) containing 0.2% collagenase III (Biochrom, Wertheim, Germany) and 2 U/ml dispase I (Roche Molecular Biochemicals, Mannheim, Germany) for 3 h at 37°C. The filtered suspension was centrifuged, and the pellet was resuspended in Dulbecco's modified essential medium (DMEM) containing penicillin/streptomycin supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA)) and plated. For conditioning, the washed cells were preincubated in DMEM containing 0.05% bovine serum albumin (DMEM/BSA) for 8 h and further incubated in fresh DMEM/BSA for 24–72 h.

2.5. Subcellular fractionation

Mouse fibroblasts grown in a 10 cm dish were incubated for 3 h with [^{125}I]IGFBP-3 (200 000 cpm/ml) at 37°C. After removal of the medium, unbound and cell surface-bound [^{125}I]IGFBP-3 were displaced by an acid wash (0.2 M acetic acid containing 0.5 M NaCl) at pH 2.5 for 5 min. A postnuclear supernatant was prepared and fractionated by Percoll (25%) as described [15]. One milliliter fractions were collected and analyzed for radioactivity, density, and β-hexosaminidase activity [16]. Fractions 1–3, 4–6, and 7–9 were pooled (pools I, II, and III, respectively) and after removal of Percoll, the membranes were solubilized in PBS containing 0.2% Triton X-100 and protease inhibitors (buffer B).

2.6. [^{125}I]IGFBP-3 immunoprecipitation

After preabsorption with preimmune serum and Pansorbin, anti-IGFBP-3 antiserum was added to the membrane extracts for 2 h at 4°C followed by the addition of Pansorbin for 1 h. After centrifugation, the immunocomplexes were washed in buffer B and 1 mM PBS. The solubilized samples were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

2.7. Determination of CTSL cleavage sites

Five micrograms of non-glycosylated IGFBP-3 were incubated with 10 μg CTSL in 50 μl 0.1 M Na-acetate buffer, pH 5.6, at 37°C for 20 h and further analyzed as described [10].

2.8. IGFBP protease assay

Fifty microliters of conditioned medium adjusted to the indicated pH were incubated with [^{125}I]IGFBP-3 or -4 (15 000–20 000 cpm) at 37°C for 6–18 h. After solubilization the samples were subjected to SDS–PAGE and visualized by autoradiography [10] or phosphorimaging (Cyclone, Packard, Meriden, CT, USA).

2.9. Other methods

[^{125}I]IGF II ligand blot analysis, isolation of total RNA from rat liver or mouse fibroblasts, and processing for Northern blot analysis have previously been described [17].

3. Results

Inhibitors of cysteine proteases have been reported to prevent the proteolysis of IGFBP-4 in conditioned medium from cathepsin D-deficient mouse liver explants [18]. To determine the IGFBP specificity, recombinant human ^{125}I-labeled IGFBP-1, -3, -4, and -6 were incubated in the presence and in the absence of CTSL for 16 h at various pH values followed by SDS–PAGE and autoradiography. No fragmentation of IGFBP-1 and -6 by CTSL was detected (not shown). When non-glycosylated [^{125}I]IGFBP-3 was incubated with CTSL, two major fragments of 18 and 14 kDa were generated over a broader pH range from 5.0 to 7.0 with a optimum at pH 6.0 (Fig. 1). Microsequencing of a 17 kDa fragment (separated under reducing conditions instead of non-reducing conditions as shown in Fig. 1) formed by large scale proteolysis of IGFBP-3 by CTSL at pH 5.6 resulted in the sequence IIIIKKGH, indicating isoleucine 172 as cleavage site. When [^{125}I]IGFBP-4 was used as a substrate for CTSL, optimal activity was observed at pH 5.0 resulting in the formation of 17 and 10 kDa IGFBP-4 fragments (Fig. 1).

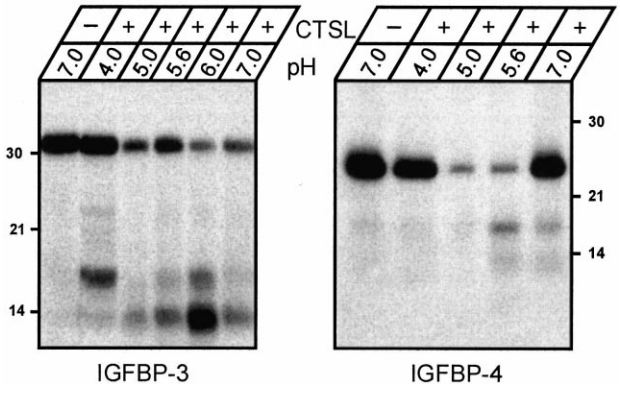

Fig. 1. Proteolysis of IGFBP-3 and -4 by CTSL. [^{125}I]IGFBP-3 and -4 were incubated in the presence or absence of human CTSL (1 μg for 16 h or 0.5 μg for 4 h, respectively) at the indicated pH at 37°C. The reaction products were separated by SDS–PAGE (12.5% acrylamide) and visualized by autoradiography. The position of the molecular mass marker proteins (in kDa) are indicated.

To assess the role of CTSL in IGFBP degradation in vivo, IGFBP levels were studied in conditioned medium from fibroblasts of CTSL-deficient mice by ligand blotting and compared with the IGFBP pattern of mouse serum. The amount of the 42/45 kDa IGFBP-3 doublet was found to be strongly increased (2.7-fold, mean of four experiments estimated by densitometry) compared with medium from control mouse fibroblasts (Fig. 2). The abundance of the 26 kDa IGFBP (presumably IGFBP-4) was about 1.8-fold higher in CTSL-deficient cells whereas the activity of secreted β-hexosaminidase was comparable with control cells (2.9 vs. 4.0 mU/ml). The identity of the IGFBPs could not be determined due to the failure of tested antibodies to cross-react specifically with mouse IGFBPs. Northern blot analysis showed similar or even higher intensities of the 2.6 kb specific IGFBP-3 and -4 transcripts, respectively, in CTSL-deficient and control fibroblasts (Fig. 3) excluding higher IGFBP transcriptional rates in CTSL-deficient cells. Reprobing of the IGFBP-4 blot with

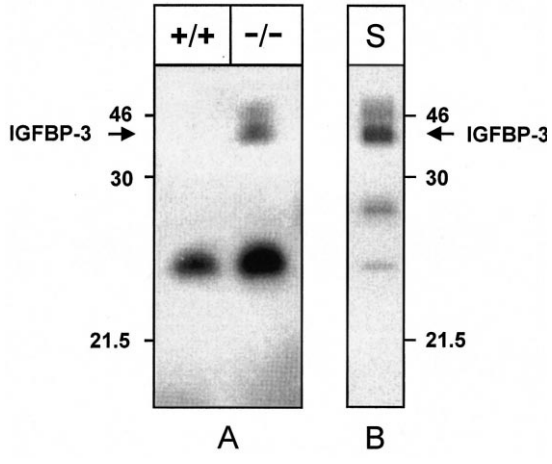

Fig. 2. Ligand blot analysis. Control (+/+) and CTSL-deficient (−/−) fibroblasts were incubated in serum-free medium for 72 h. Aliquots of (A) the medium (0.3 ml) or (B) mouse serum (S) were analyzed by [^{125}I]IGF II ligand blotting. This autoradiograph is representative of four independent experiments.

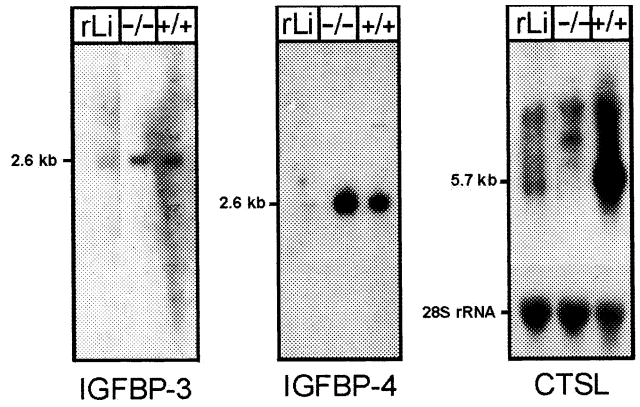

Fig. 3. IGFBP mRNA expression in control and CTSL-deficient fibroblasts. Total RNA (12.5 μg RNA/lane) from primary control (+/+), CTSL-deficient mouse fibroblasts (−/−), and rat liver (rLi) was analyzed for IGFBP-3 and -4 expression using radiolabeled cDNA probes. The sizes of the hybridization bands (in kb) are indicated. Ethidium bromide-stained 28S and 18S rRNAs of the same preparation were tested for equal RNA loading (not shown). As representative, the IGFBP-4 blot was stripped and rehybridized with labeled CTSL cDNA and the complementary 28S rRNA oligonucleotide.

CTSL cDNA and the 28S rRNA complementary oligonucleotide confirmed the *ctsl* gene targeting and equal loading of RNA, respectively.

To determine whether proteases affect the IGFBP-3 level in the medium of CTSL-deficient fibroblasts, IGFBP-3 protease activity was assessed at pH 4.0, 5.6, and 7.0 in vitro. During cell-free incubation at each pH distinct IGFBP-3 cleavage pattern were observed but without differences between media from control and CTSL-deficient cells (not shown). In contrast, when primary fibroblasts from control and CTSL-deficient mice were incubated with [^{125}I]IGFBP-3 for 1–4 h at 37°C, a continuous disappearance of the intact [^{125}I]IGFBP-3 in the medium was observed (Fig. 4). In parallel, the amount of trichloroacetic acid-soluble radioactivity in the medium increased with the incubation time (not shown) demonstrating the continuous degradation of [^{125}I]IGFBP-3 in the presence

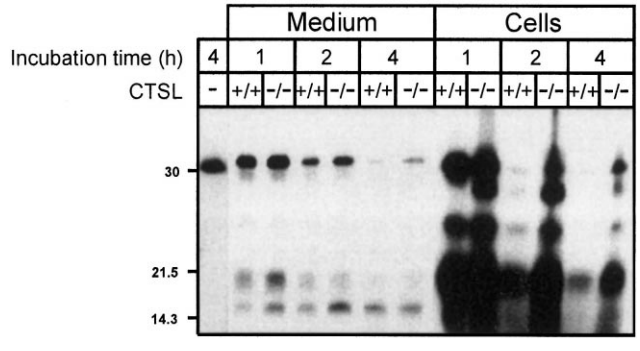

Fig. 4. Cell-dependent degradation of IGFBP-3. Control (+/+) and CTSL-deficient fibroblasts (−/−) were incubated for 1, 2, and 4 h at 37°C with [^{125}I]IGFBP-3 (130 000 cpm per plate) in serum-free medium. After removal of the medium, the washed cells were scraped and collected by centrifugation. Aliquots of the medium (13% of total) and the cells were solubilized and analyzed by SDS–PAGE and autoradiography. An aliquot of [^{125}I]IGFBP-3-containing medium incubated in the absence of cells was used as control. The experiment was repeated with cells from three different preparations resulting in moderate alterations in the kinetics of [^{125}I]IGFBP-3 degradation but with similar differences in cellular accumulated [^{125}I]IGFBP-3.

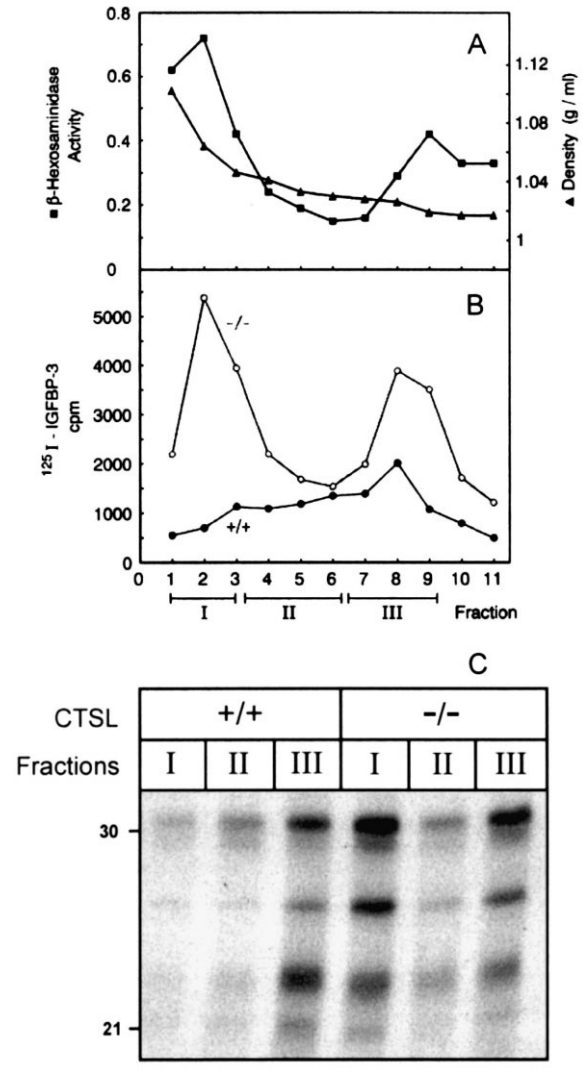

Fig. 5. Subcellular localization of endocytosed [^{125}I]IGFBP-3. Control (+/+) and CTSL-deficient fibroblasts (−/−) were incubated for 3 h at 37°C with [^{125}I]IGFBP-3. The postnuclear supernatant was subjected to Percoll density gradient centrifugation. The collected fractions were tested for density (▲) and β-hexosaminidase activity in mU/ml (■) (A). The distribution of [^{125}I]IGFBP-3 radioactivity from both gradients are shown (B). The fractions 1–3, 4–6, and 7–9 were afterwards combined to prepare the membrane fractions I, II, and III, respectively, which were used for immunoprecipitation of [^{125}I]IGFBP-3 (C).

of cells. Analysis of the solubilized fibroblasts by SDS–PAGE and autoradiography showed that similar amounts of intact [^{125}I]IGFBP-3 as well as various fragments of 25, 18, and 14 kDa were associated with control and CTSL-deficient cells after an incubation period of 1 h. An additional 28 kDa IGFBP-3 fragment was detected only in CTSL-deficient cells. After 2 and 4 h of incubation weak signals of [^{125}I]IGFBP-3 fragments were found in control cell extracts whereas intact [^{125}I]IGFBP-3 and its fragments were still detectable in CTSL-deficient cells (Fig. 4). To define the intracellular site of IGFBP-3 cleavage, postnuclear supernatants prepared from control and CTSL-deficient fibroblasts after an incubation for 3 h at 37°C with [^{125}I]IGFBP-3 were fractionated by Percoll density gradient centrifugation. The density profile and the distribution of β-hexosaminidase, a lysosomal marker, are

shown in Fig. 5A. The highest radioactivity values were estimated in the light membrane fractions (fractions 7–9) of control cells comprising the endoplasmic reticulum, Golgi, plasma membranes and endosomes [15]. In contrast, the total radioactivity in all subcellular fractions from CTSL-deficient fibroblasts was about 2.5-fold higher (mean of two experiments) than in fractions of control cells, showing two peaks in the dense lysosomal (fractions 1–3) and in the light membrane fraction (Fig. 5B). To explore the distribution of intact and IGFBP-3 fragments, [^{125}I]IGFBP-3 was immunoprecipitated from solubilized membrane pools I, II, and III. The majority of immunoreactive intact and [^{125}I]IGFBP-3 fragments were precipitated from the light membrane fraction (III) of control cells (Fig. 5C). Both intact and [^{125}I]IGFBP-3 fragments were detected in a similar ratio in the fractions I, II, and III from CTSL-deficient fibroblasts.

4. Discussion

In this study we demonstrated that the cysteine protease CTSL cleaves IGFBP-3 and -4 in vitro. The pH optimum between 5.6 and 6.0 for the proteolysis of IGFBPs is similar to that described for artificial substrates [19]. CTSL as well as the aspartyl protease cathepsin D are highly abundant, ubiquitously expressed lysosomal enzymes which are secreted in low amounts as inactive precursor forms in normal cells. However, the physiological significance of secreted cathepsins in the regulation of extracellular IGFBP proteolysis appears to be questionable. In this study we have demonstrated that CTSL plays an important role in the intracellular degradation of IGFBP-3. Fibroblasts from CTSL-deficient mice are characterized by (i) an extracellular accumulation of IGFBP-3, and (ii) a transient accumulation of intact and proteolytic fragments of endocytosed IGFBP-3 which is rapidly and completely degraded in normal mouse fibroblasts. It appears that the intracellular accumulation of IGFBP-3 affects the uptake of IGFBP-3 in CTSL-deficient cells suggesting rather limitations in the transport capacity of a putative IGFBP-3 receptor than pinocytotic uptake. Several membrane-associated proteins and the type V transforming growth factor-β receptor have been described as putative IGFBP-3 receptors [20–22]. Endocytosis of IGFBP-3 has also been reported in rat megakaryocytes [23] and cocultures of rat hepatocytes and Kupffer cells [13]. The identity of the IGFBP-3 receptor in these three cell systems remains to be determined.

Upon subcellular fractionation of mouse fibroblasts, we showed that the endocytosed IGFBP-3 is transported via the endosomal compartment to lysosomes for final degradation. In rat megakaryocytes the endocytosed intact IGFBP-3 accumulated in α-granules [23]. Interestingly, the present data showed that endocytosed IGFBP-3 is completely degraded with different kinetics in control and CTSL-deficient cells, whereas purified CTSL or cathepsin D [10] generated in vitro IGFBP-3 fragments of defined sizes. This suggests that several acidic proteases are involved in cellular IGFBP-3 degradation which can compensate either the total deficiency of a single protease such as CTSL or cathepsin D [18], or the partial deficiency of several lysosomal enzymes [24]. Thus, the generation and transient accumulation of IGFBP-3 fragments may be explained by the inefficient initial cleavage(s) of IGFBP-3 required for subsequent proteolytic steps which can be overcome by other (cysteine) proteases with lower efficiency. This is supported by the identification of one new CTSL cleavage site in IGFBP-3, isoleucine 172, which was not found in any of the previous studies [10,25,26].

In summary, our findings demonstrate that mouse fibroblasts are capable of endocytosing IGFBP-3 from extracellular fluid. This study also shows that CTSL is required but not essential for an efficient and rapid degradation of IGFBP-3 along the endocytic pathway. Whereas CTSL is supposed to function in non-specific terminal degradation of proteins in lysosomes, recent studies investigating CTSL-deficient mice have reported more specific functions of CTSL in the processing of the invariant chain of MHC II in cortical thymic epithelial cells [27] and in the control of proliferation of hair follicle epithelial cells and basal keratinocytes [14]. Since growth factors including IGF I play an essential role in the balance between proliferation and differentiation of epithelial cells [28], the sequestration of IGFs by accumulating IGFBP-3 derived from CTSL-deficient dermal fibroblasts may shift the balance toward proliferation causing epidermal and hair follicle hyperplasia.

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