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Endosomal proteolysis of insulin-like growth factor-I at its C-terminal D-domain by cathepsin B

François Authier[a,*], Mostafa Kouach[b], Gilbert Briand[b]

[a] *Institut National de la Santé et de la Recherche Médicale U510, Faculté de Pharmacie, Paris XI, 5 Rue Jean Baptiste Clement, 92296 Châtenay-Malabry, France*

[b] *Laboratoire de Spectrométrie de Masse, Faculté de Médecine, 59000 Lille, France*

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Abstract **IGF-I is degraded within the endosomal apparatus as a consequence of receptor-mediated endocytosis. However, the nature of the responsible protease and the position of the cleavage sites in the IGF-I molecule remain undefined. In vitro proteolysis of IGF-I using an endosomal lysate required an acidic pH and was sensitive to CA074, an inhibitor of the cathepsin B enzyme. By nondenaturing immunoprecipitation, the acidic IGF-I-degrading activity was attributed to the luminal species of endosomal cathepsin B with apparent molecular masses of 32- and 28-kDa. The cathepsin B precursor, procathepsin B, was processed in vitro within isolated endosomes at pH 5 or at 7 in the presence of ATP, the substrate of the vacuolar H^+-ATP-ase. The rate of IGF-I hydrolysis using an endosomal lysate or pure cathepsin B was found to be optimal at pH 5–6 and moderate at pH 4 and 7. Competition studies revealed that EGF and IGF-I share a common binding site on the cathepsin B enzyme, with native IGF-I displaying the lowest affinity for the protease ($IC_{50} \approx 1.5$ μM). Hydrolysates of IGF-I generated at low pH by endosomal IGF-I-degrading activity and analyzed by reverse-phase HPLC and mass spectrometry revealed cleavage sites at Lys^{68}-Ser^{69}, Ala^{67}-Lys^{68}, Pro^{66}-Ala^{67} and Lys^{65}-Pro^{66} within the C-terminal D-domain of IGF-I. Treatment of human HepG2 hepatoma cells with the cathepsin B proinhibitor CA074-Me reduced, in vivo, the intracellular degradation of internalized [^{125}I]IGF-I and, in vitro, the degradation of exogenous [^{125}I]IGF-I incubated with the cell-lysates at pH 5. Inhibitors of cathepsin B and pro-cathepsin B processing, which abolish endosomal proteolysis of IGF-I and alter tumor cell growth and IGF-I receptor signalling, merit investigation as antimetastatic drugs.**

Keywords: Insulin-like growth factor-1; Endocytosis; Protease; Cathepsin; Mass spectrometry

1. Introduction

Insulin-like growth factor I (IGF-I) is a 70-amino acid single polypeptide chain that mediates somatic growth by binding to the IGF-I receptor [1]. It has a high sequence similarity with insulin-like growth factor-II (IGF-II) and the A and B chains of human insulin [2]. IGF-I binding initiates the migration of IGF-I receptors to clathrin-coated pits and the subsequent formation of early endosomes containing internalized but still active receptors [3,4]. Cellular compartmentalization by IGF-I-induced IGF-I receptor internalization is required for cell signalling via the Shc/MAP kinase pathway, but not the IRS-1 pathway [4]. The IGF-I/IGF-I receptor complex ultimately becomes dissociated in the acidic endosomal environment where IGF-I proteolysis takes place [3,5]. Endosomal proteolysis of IGF-I is thought to be required for cellular responses to IGF-I and IGF-I receptor recycling to the cell surface [5].

Endosomal IGF-I degrading activity has been shown to be strongly inhibited by cysteine protease inhibitors and easily extracted from endocytic vesicles of rat liver parenchyma, or carcinoma H-59 and MCF-7 cells [5]. However, the identity of the responsible proteinase remained unknown, and thus far no study has been directed towards identifying the IGF-I degradation products and the proteolytic cleavage sites. Elucidation of these sites in the human IGF-I molecule is essential for a better understanding of the substrate recognition and cleavage site specificity of the endosomal IGF-I degrading enzyme and would facilitate the design of inhibitors and protease-resistant IGF-I analogues.

Recently, we have identified the structural domains of insulin responsible for binding to and degradation by endosomal cathepsin D [6,7]. The aromatic locus Phe^{B24}-Phe^{B25}-Tyr^{B26} at the C-terminal region of the insulin B chain was defined as critical to the high-affinity interaction of insulin with cathepsin D. The insulin B chain is homologous to the amino-terminal 29-residue region of human IGF-I [8]. IGF-I contains an additional connecting C-domain, which joins its B domain to a region corresponding to the A chain of insulin, and an extension at the C-terminus known as the D-domain [2]. In the present study, we show that proteolysis of IGF-I was strongly inhibited, both in vivo and in vitro, in human HepG2 hepatoma cells pretreated with the cathepsin B proinhibitor CA074-Me, and in vitro, using hepatic endosomes incubated with the cathepsin B inhibitor CA074. Using highly purified rat endosomes, the degradation products of native

*Corresponding author. Fax: 33 1 46835844.
E-mail address: francois.authier@cep.u-psud.fr (F. Authier).

Abbreviations: EGF, epidermal growth factor; EN, endosomal fraction; ENs, soluble endosomal extract; HI, human insulin; IDE, insulin-degrading enzyme; IGF-I, insulin-like growth factor I; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reverse-phase high pressure liquid chromatography

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IGF-I were isolated and the amino acid sequence of the peptides determined by mass spectrometry. The data demonstrate that the cysteine protease cathepsin B is responsible for the endosomal processing of IGF-I by inducing proteolytic cleavages that yield C-terminal truncated forms of IGF-I.

2. Materials and methods

2.1. Peptides, ligand radioiodination, antibodies, protein determination and materials

Recombinant human IGF-I and insulin were purchased from Sigma and Preprotech Inc. Mouse EGF was purchased from Collaborative Research. Bovine cathepsin B (EC 3.4.22.1; 10 U/mg) and bovine cathepsin D (EC 3.4.23.5; 5 U/mg) were purchased from Sigma. IGF-I was radioiodinated by the lactoperoxidase method to specific activities of 350–500 Ci/mmol and purified by gel filtration on Sephadex G-50 [5]. Rabbit anti-mouse cathepsin D R291, rabbit anti-rat cathepsin B 7183, rabbit anti-rat cathepsin L R958 and rabbit anti-rat cathepsin B propeptide 1149 were obtained from Dr. J.S. Mort (Shriners Hospital for Crippled Children, Montreal, Quebec) and used as described previously [7,9,10]. Sheep anti-human cathepsin B PC049 was purchased from The Binding Site. Mouse monoclonal antibody 9B12 directed to the human insulin-degrading enzyme [11] was obtained from Dr. R.A. Roth (Stanford University, Stanford, CA). HRP-conjugated goat anti-rabbit IgG was from Bio-Rad. The protein content of isolated fractions was determined by the method of Lowry et al. [12]. Nitrocellulose membranes and Enhanced ChemiLuminescence (ECL) detection kit were from Amersham. Protein G-Sepharose was from Pharmacia. Pepstatin-A, E-64, PMSF and bafilomycin A1 were from Sigma. CA074 and its methyl ester form, CA074-Me, were purchased from Peptides International. HPLC grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Baker Chemical Co. All other chemicals were obtained from commercial sources and were of reagent grade.

2.2. Cell culture

Human HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin in an atmosphere of 95% air/5% CO_2 as described previously [13]. CA074-Me, a membrane permeable analog of CA074 [14], was dissolved in DMSO (stock concentration of 1 mM) resulting in a final DMSO concentration of 0.1% (v/v) in the medium. Control cells received 0.1% DMSO only. HepG2 cells were incubated in the presence of 1 μM CA074-Me 60 min before addition of radiolabeled IGF-I or preparation of cell lysates.

2.3. IGF-I internalization and degradation in hepatoma cells

A modification of the procedure of Zapf et al. [15] was followed. HepG2 cells were grown to subconfluency on 55 mm culture dishes, rinsed three times in HBS buffer (50 mM HEPES, pH 7.5, 150 mM NaCl) and incubated for various times at 37 °C with 1 ml of HBS containing 17000 cpm [^{125}I]IGF-I. At the indicated times, cells were placed on ice and washed three times with ice-cold HBS. To remove the surface-bound ligand, cells were acid washed three times for 5 min at 4 °C in the presence of 1 ml 50 mM citrate–phosphate, pH 3.5, 150 mM NaCl and the acid-washes were counted in a Packard γ-counter. The integrity of radiolabeled IGF-I remaining cell-associated was assayed by adding 2 ml of ice-cold 6% trichloroacetic acid (TCA). The samples were then centrifuged at $10000 \times g$ for 20 min at 4 °C, and the supernatants and pellets were evaluated for their radioactive content.

Proteolysis of [^{125}I]IGF-I was also measured using cell lysates derived from HepG2 cells which had been pretreated for 60 min with or without 1 μM CA074-Me as described previously [5]. Cells were washed three times with HBS, scraped and lysed in HBS buffer containing 1% Triton X-100 and 0.5% deoxycholate. The lysates were clarified by centrifugation for 60 min at $60000 \times g$. The clarified lysates were then incubated for various times at 37 °C with 10000 cpm [^{125}I]IGF-I in 200 μl of 300 mM citrate–phosphate, pH 5, and the integrity of [^{125}I]IGF-I was assessed by reducing Tris–Tricine gels.

2.4. Animals and liver subcellular fractionation

Male Sprague-Dawley rats, body weight 180–200 g, were obtained from Charles River France (St. Aubin Les Elbeufs, France) and were fasted for 18 h prior to sacrifice. All in vivo procedures were approved by the institutional committee for use and care of experimental animals.

Subcellular fractionation was performed using established procedures [7,9–11]. The endosomal (EN) fraction was isolated by discontinuous sucrose gradient centrifugation and collected at the 0.25–1.0 M sucrose interface [7,9–11]. The soluble endosomal extract (ENs) was isolated from the EN fraction by freeze/thawing in 5 mM Na-phosphate pH 7.4, and disrupted in the same hypotonic medium using a small Dounce homogenizer (15 strokes with Type A pestle) followed by centrifugation at $150000 \times g$ for 60 min as previously described [7,9–11].

For some experiments, freshly isolated endosomes were suspended at 0.5 mg/ml in 0.15 M KCl, 5 mM $MgCl_2$ and 25 mM citrate–phosphate, pH 5 or 7, in the presence or absence of 3 mM ATP and 0.01 μM bafilomycin A1. Fractions were then incubated at 37 °C for 2–60 min, after which reducing Laemmli sample buffer was added. Samples were heated at 65 °C for 15 min and then subjected to SDS–PAGE followed by Western-blotting to determine the content of the precursor and mature species of cathepsin B.

2.5. Immunoblot analysis

Electrophoresed samples were transferred onto nitrocellulose membranes for 60 min at 380 mA in transfer buffer containing 25 mM Tris base and 192 mM glycine. The membranes were blocked by a 3 h incubation with 5% skim milk in 10 mM Tris–HCl pH 7.5, 300 mM NaCl and 0.05% Tween-20. The membranes were then incubated with the primary antibody [sheep IgG against human cathepsin B PC049 (diluted 1:100), rabbit polyclonal antisera against rat procathepsin B 7183 (diluted 1:250) or rat cathepsin B propeptide 1149 (diluted 1:250)] in the above buffer for 16 h at 4 °C. The blots were then washed 3 times with 0.5% skim milk in 10 mM Tris–HCl pH 7.5, 300 mM NaCl and 0.05% Tween-20 over a period of 1 h at room temperature. The bound immunoglobulin was detected using HRP-conjugated goat anti-rabbit IgG.

2.6. In vitro proteolysis of IGF-I by hepatic endosomes and cathepsin B

ENs (≈1 μg) was incubated for varying lengths of time at 37 °C with 10–30 μg native IGF-I in 150 μl of 30 mM citrate–phosphate buffer (pH 4–7) in the presence or absence of protease inhibitors. To determine the integrity of IGF-I, the ENs samples were acidified with acetic acid (15%) and immediately loaded onto a RP-HPLC column.

For some experiments, native IGF-I was digested in vitro with bovine cathepsin B. IGF-I (10 μg) was incubated with cathepsin B (0.2 U) in 30 μl of 33 mM citrate–phosphate buffer, pH 4–7, for 2–15 min at 37 °C. The proteolytic reaction was stopped by addition of reducing Tricine sample buffer and the samples were resolved on 16.5% Tris–Tricine gels according to the method of Schaffer & von Jagow [16]. Bands were revealed by Coomassie Brilliant Blue staining of the gels.

For the in vitro degradation of [^{125}I]IGF-I by ENs, radiolabeled IGF-I (13000 cpm) was incubated with ENs (≈1 μg) and various concentrations of native IGF-I, human insulin or EGF in 50 mM citrate–phosphate buffer, pH 5, for 30 min at 37 °C. The amount of degraded [^{125}I]IGF-I was assayed by precipitation with 2 ml of ice-cold TCA (10%) for 2 h at 4 °C. The samples were then centrifuged at $10000 \times g$ for 20 min at 4 °C, and the supernatants and pellets evaluated for their radioactive content using a Packard γ-counter.

2.7. Immunodepletion studies

ENs was immunodepleted of active cathepsin B, cathepsin D, cathepsin L or insulin-degrading enzyme prior to the digestion step by incubating ENs (0.25 mg/ml) with antibodies precoated onto protein G-Sepharose beads for 16 h at 4 °C in 600 μl of 20 mM sodium phosphate buffer (pH 7). The fractions were then centrifuged for 5 min at $10000 \times g$, and the resultant immunodepleted supernatants were used in the peptide degradation assay. The reaction was terminated by the addition of 15% acetic acid and immediately analyzed by RP-HPLC.

2.8. HPLC separation of IGF-I peptides

RP-HPLC was performed using a Beckman Coulter System Gold model 127 liquid chromatograph equipped with a Rheodyne sample injector fitted with a 0.5 ml loop and a μBondapak C18 column (Waters, 0.39 × 30 cm; 10 μm particle size). Samples were chromatographed using a mixture of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) with a flow rate of 1 ml/min. Elution was carried out using three sequential linear gradients of 0–5% solvent B (10 min), 5–15% solvent B (5 min) and 15–39% solvent B (32 min), followed by an isocratic elution of 39% solvent B (20 min). Eluates were monitored on-line for absorbance at 214 nm with a LC-166 spectrophotometer (Beckman Coulter).

2.9. Mass spectrometry

Samples were prepared and analyzed using ion spray mass spectrometry and HPLC-electrospray ionization mass spectrometry coupling as previously described [6,7].

3. Results

3.1. Catalytic properties of endosomal IGF-I-degrading activity

IGF-I proteolysis has previously been demonstrated in isolated liver parenchymal endosomal fractions following addition of exogenous IGF-I [5]. Therefore, rat hepatic endosomes were used in the present study to characterize the proteolytic activity responsible for the endosomal degradation of internalized IGF-I. A soluble endosomal fraction (ENs) was first assessed for its ability to proteolyze native IGF-I in vitro (Fig. 1A–C). Fig. 1A shows the RP-HPLC elution profile of native IGF-I peptide alone (HPLC profile IGF-I), and a mixture of IGF-I and ENs incubated for 15 min at pH 5 (HPLC profile IGF-I+ENs). Major intermediate peptide peaks were observed in addition to the

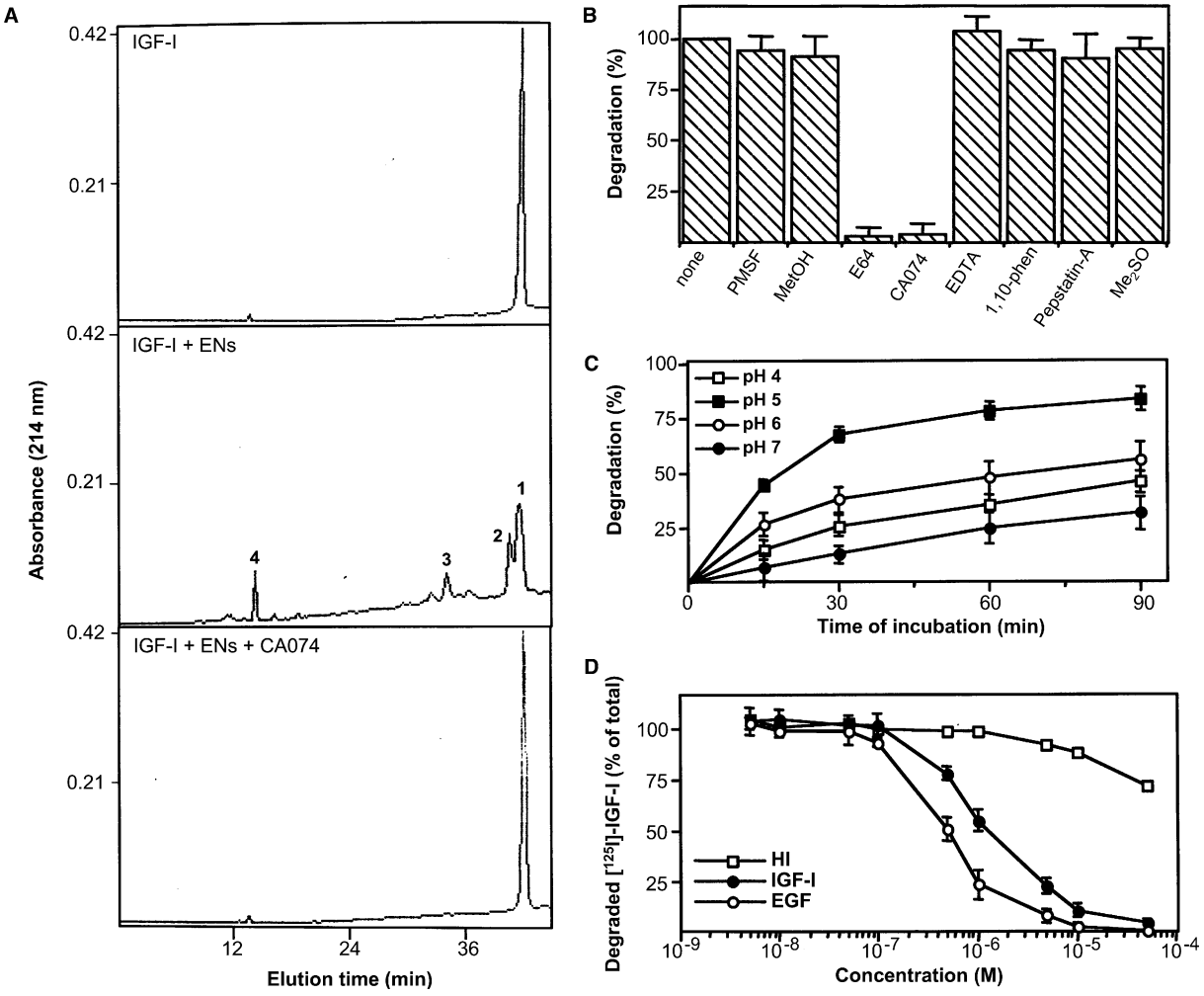

Fig. 1. Assessment of IGF-I proteolysis by hepatic endosomes. Panels A–C, Soluble endosomal fraction (ENs, ≈1 μg) was incubated with 10 μg native IGF-I at 37 °C for 15 min (panels A and B) or 15–90 min (panel C) in 30 mM citrate–phosphate buffer pH 5 (panels A and B) or 4–7 (panel C) in the absence or presence of 5 μg/ml pepstatin-A (PA), 1% Me_2SO (DMSO), 1 mM PMSF, 1% MetOH, 1 μM E64, 0.1 μM CA074, 1 mM EDTA or 0.1 mM 1,10-phenanthroline. At the end of the incubation, the proteolytic reaction was stopped with acetic acid (15%), and the incubation mixtures were analyzed by RP-HPLC. Panel A, representative absorbance profiles at 214 nm obtained following the incubation of native IGF-I with ENs in the absence or presence of 0.1 μM CA074. The major degradation products, pools 1–4, were collected from the elution profile IGF-I + ENs as indicated and subjected to mass spectrometry analyses (see Table 1). Intact IGF-I had an elution time of 42 min. Panels B and C, The rate of hydrolysis of IGF-I was determined by following the disappearance of the peak area corresponding to the parent peptide. The results are expressed as the amount of peptide degraded (% of control) (panel C) and normalized to that seen in the absence of added compound (panel B). Panel D, Competition of native IGF-I, human insulin (HI) and EGF for the degradation of [^{125}I]IGF-I by ENs. ENs (≈1 μg) was incubated with [^{125}I]IGF-I at 37 °C and pH 5 with the indicated concentrations of unlabeled peptides. The amount of degraded radiolabeled IGF-I was determined by precipitation with TCA. The percentage of [^{125}I]IGF-I degraded in the absence of unlabeled peptide was 32% ± 5.2. Results are the means ± S.D. of three separate experiments and are expressed as a percentage of degradation observed in the absence of added unlabeled peptides.

undegraded IGF-I peptide (peak 1), which had decreased in peak height. The effect of various protease inhibitors on the acidic IGF-I-degrading activity was next examined (Fig. 1A and B). The proteolytic activity observed at pH 5 was inhibited by the cysteine-protease inhibitor E64 and a rapid inactivator of cathepsin B, CA074 (Fig. 1A (HPLC profile IGF-I + ENs + CA074); Fig. 1B) [14]. No significant effect was observed using serine, metallo- or aspartic acid protease inhibitors. ENs was also examined for its ability to degrade native IGF-I at various pH (pH 4–7) (Fig. 1C). Degradation of IGF-I was pH dependent with maximal degradation obtained at pH 5. A lower but significant rate of hydrolysis of IGF-I was observed at pH 4, 6 and 7.

Substrates of the same protease would be expected to compete with each other for the enzyme binding site. We therefore used a competition assay to evaluate the ability of native IGF-I, HI and EGF to inhibit degradation of the radiolabeled substrate [^{125}I]IGF-I at pH 5 by ENs (Fig. 1D). The cathepsin B substrate EGF [10] was found to inhibit [^{125}I]IGF-I proteolysis by ENs in a dose-dependent manner with a IC_{50} of 0.5 μM. Native IGF-I was ≈3 times less effective than EGF at competing for proteolysis of radiolabeled IGF-I (IC_{50} of 1.5 μM). HI, a well-defined cathepsin D substrate [7], was found to be less potent than EGF and IGF-I.

3.2. Sites of cleavage of IGF-I intermediates

Each of the major HPLC peaks (see Fig. 1A; HPLC profile IGF-I+ENs) was analyzed using mass spectrometry to determine the molecular mass of the peptide products. Table 1 lists the HPLC peaks, their retention times, theoretical and experimental molecular masses, and structures. After a 15 min incubation at neutral pH, only HPLC pool 1 can be detected. It contained intact IGF-I (7648 Da). After a 15 min incubation at pH 5, three additional peaks were observed (HPLC pools 2–4). HPLC pool 1 contained both intact IGF-I and truncated IGF-I with the C-terminal dipeptide Ser^{69}-Ala^{70} clipped off. HPLC peaks 2, 3 and 4 correlated with the sequential removal of the N-terminal Lys^{68}, Ala^{67} and Pro^{66}. The Gly^{1}-Pro^{66} and Gly^{1}-Lys^{65} were not detected after a 15 min incubation at pH 4.

3.3. Identification of endosomal IGF-I-degrading enzyme as cathepsin B

The inhibition of IGF-I-degrading activity by E64 and CA074, its affinity binding to EGF, its low pH optimum and its presence in the endosomal lumen as a soluble form suggested cathepsin B as a likely candidate for this activity. We therefore used well characterized polyclonal antibodies to mature cathepsin B and its proform [9] to deplete cathepsin B from ENs (Fig. 2A and B). Quantitative immunoprecipitation of cathepsin B using antibody directed against the rat enzyme (7183) removed greater than 75% of the endosomal proteolytic activity directed towards IGF-I as assessed by RP-HPLC. Immunodepletion of ENs with antibodies to IDE [11], cathepsin D [7] or cathepsin L [9] failed to remove the proteolytic activity (Fig. 2A and B).

To strengthen the physiological relevance of our observations obtained using cell-free endosomes, we compared the rate of hydrolysis of IGF-I using pure cathepsin B at various pH, followed by reducing Tris–Tricine gel and Coomassie Blue staining (Fig. 2C). Optimal proteolytic conversion of native IGF-I into IGF-I-intermediates (experimental molecular masses ranging from 6- to 3.5-kDa) was observed at pH 5 and 6 with a cathepsin B concentration of 6.7 U/ml. IGF-1 degradation products were less abundant at pH 4 and 7.

To determine whether endosome acidity alters a processing state of both the latent and mature forms of the cathepsin B enzyme within endocytic vesicles, we developed a method for assaying procathepsin B processing in intact endosomes (Fig. 2D). Freshly prepared endosomes were incubated at 37 °C for various times at pH 5 or 7 in an isotonic buffer which mimicked the intracellular milieu. At zero time, immunoblot analyses using antibodies directed against mature cathepsin B and its proform (antibody 7183) or rat cathepsin B propeptide (antibody 1149) showed intense immunoreactivity for the 45-kDa cathepsin B proenzyme (antibodies 7183 and 1149) and low but detectable levels of the 32- and 28-kDa forms of active cathepsin B (antibody 7183). Rapid conversion of the proenzyme to both active forms was evident by 2 min of incubation of cell-free endosomes at pH 5 or 7 in the presence of ATP, the substrate of the endosomal ATPase proton pump. No digestion of the proenzyme occurred when hepatic endosomes

Table 1
Masses and assigned structures of the cleavage products generated from native IGF-I by endosomal acidic IGF-I-degrading activity

HPLC pools	Retention time (min)	Theoretical calculation (Da)	Mass analysis (Da)	Assigned structure
IGF-I + ENs/pH7				
1	42	7648.7	7648	Gly^{1}-Ala^{70}
IGF-I + ENs/pH5				
1	42	7648.7	7648	Gly^{1}-Ala^{70}
1	42	7490.5	7490	Gly^{1}-Lys^{68}
2	40	7362.3	7363	Gly^{1}-Ala^{67}
3	34	7291.3	7291	Gly^{1}-Pro^{66}
4	14	7194.2	7194	Gly^{1}-Lys^{65}
IGF-I + ENs/pH4				
1	42	7648.7	7648	Gly^{1}-Ala^{70}
1	42	7490.5	7490	Gly^{1}-Lys^{68}
2	40	7362.3	7364	Gly^{1}-Ala^{67}

Selected HPLC pools (see Fig. 1A, HPLC profile IGF-I + ENs) generated at pH 4, 5 and 7 by endosomal acidic IGF-I-degrading activity were analyzed by RP-HPLC coupled with electrospray ionization-mass spectrometry. Average molecular masses were used for the calculations. HPLC pool 1 generated at pH 4 and 5 is heterogeneous. HPLC pool 1 contains intact IGF-I. Recovery of Gly^{1}-Lys^{65} peptide (HPLC pool 4) was low.

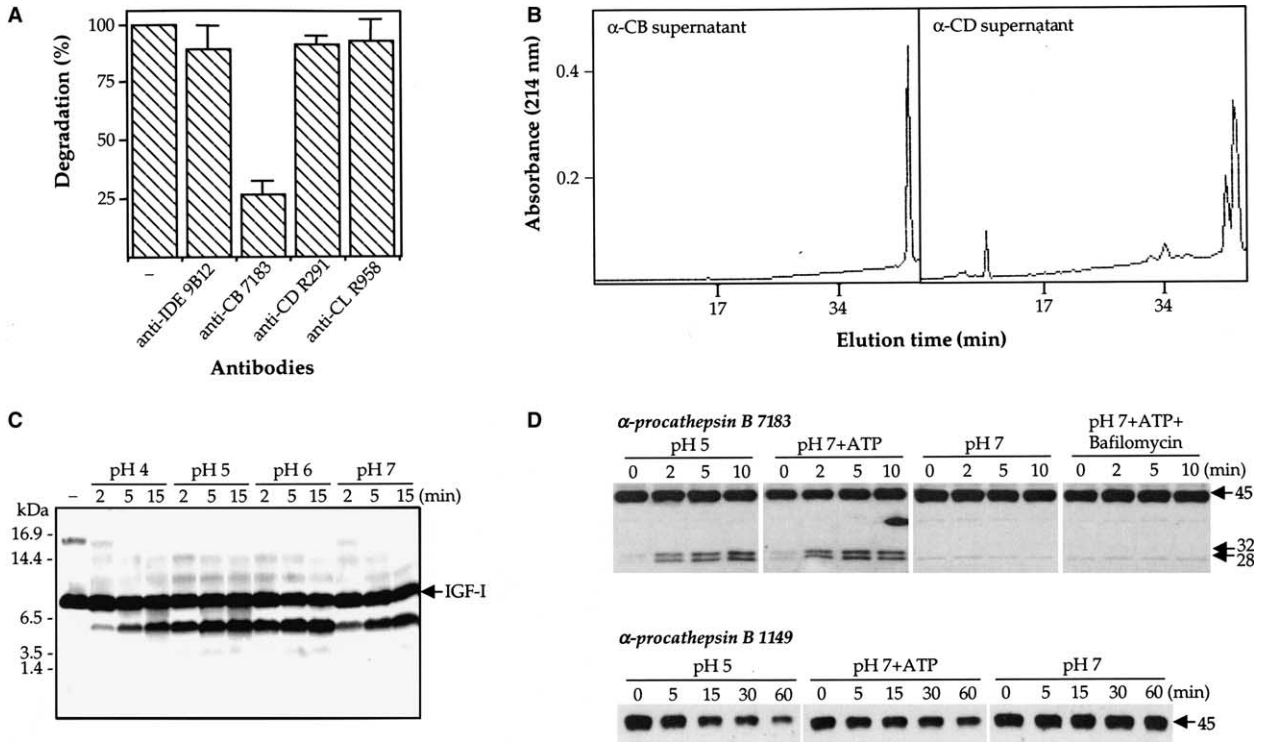

Fig. 2. Identification of endosomal IGF-I-degrading enzyme as cathepsin B. Panel A, ENs fractions were immunodepleted of active insulin-degrading enzyme (IDE), cathepsin B (CB), cathepsin D (CD) or cathepsin L (CL) using their respective antibodies which had been precoated onto protein G-Sepharose beads. Following centrifugation, the resultant supernatants were incubated with 10 μg native IGF-I in citrate–phosphate buffer pH 5 at 37 °C, and then analyzed by RP-HPLC. The rate of IGF-I proteolysis was determined by following the disappearance of the peak area corresponding to the parent peptides. Panel B, Representative absorbance profiles at 214 nm obtained following the incubation of native IGF-I with ENs fractions immunodepleted of active cathepsin B (α-CB supernatant) or CD (α-cathepsin D supernatant). Panel C, IGF-I (10 μg) was incubated with cathepsin B (0.2 U) at 37 °C in citrate–phosphate buffer pH 4–7 for the indicated times. The incubation mixtures were then analyzed by reducing Tris–Tricine gel followed by Coomassie Brilliant Blue Staining. Molecular mass markers are indicated to the left of the panel. Arrow on the right indicates the mobility of intact IGF-I (≈7.6-kDa). Panel D, EN fractions were isolated from control rats and incubated in isotonic buffer at 37 °C and pH 5 or 7 in the presence or absence of 3 mM ATP or 3 mM ATP + 0.01 μM bafilomycin A1. After various incubation times, the samples were evaluated for their content of procathepsin B and mature cathepsin B by immunoblotting with the indicated polyclonal antisera. Bands on the blot were visualized by ECL. Arrows indicate the mobility of procathepsin B (≈45-kDa) and mature cathepsin B (≈32- and 28-kDa).

were incubated at pH 7 alone and 7 in the presence of ATP and bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase.

3.4. IGF-I internalization and degradation in human hepatoma cells

To strengthen the physiological relevance of our observations obtained with cell-free endosomes, we studied the metabolic fate of [^{125}I]IGF-I internalized in intact HepG2 cells (Fig. 3). This human hepatoma cell line expresses detectable levels of IGF-I and IGF-I/insulin hybrid receptors [17,18]. Based on the acid-wash procedure, time-course studies of IGF-I internalization yielded a $t_{1/2}$ value of ≈10 min (Fig. 3A). Steady state was reached by ≈45 min (Fig. 3A) and represented 34% of the total cell-associated [^{125}I]IGF-I. The proportion of internalized [^{125}I]IGF-I that was TCA-soluble increased with time, representing ≈24% at 60 min. Treatment of HepG2 cells with the cathepsin B proinhibitor CA074-Me caused a 4–6-fold decrease in the intracellular proteolysis of IGF-I (Fig. 3A). These data suggest that cathepsin B, whose presence in HepG2 cell-lysates was confirmed by Western-blotting (see Fig. 3B), is responsible for most receptor-mediated [^{125}I]IGF-I degradation in vivo.

IGF-I proteolysis was also investigated using lysates from control and CA074Me-treated HepG2 cells which were incubated with exogenous [^{125}I]IGF-I at pH 5 (Fig. 3C). Tris–Tricine gel analysis revealed that [^{125}I]IGF-I proteolysis was rapid using lysates isolated from control cells (>90% degradation after a 20 min incubation), whereas the proteolysis of radiolabeled IGF-I was clearly reduced using lysates from cells treated with CA074-Me (Fig. 3C).

4. Discussion

In this study, the soluble cysteine protease cathepsin B has been identified as the IGF-I-degrading activity responsible for the primary cleavages of internalized IGF-I within endosomes. Using similar approaches, we have previously reported that hepatic endosomes contain active cathepsin B that processes internalized glucagon [9] and EGF [10]. Recently, we have also shown that cysteine proteinase inhibitors prevented the endosomal processing of internalized IGF-I in MCF-7 and H-59 tumor cells [5]. Using highly purified hepatic endosomes, we have demonstrated in this present work that the endosomal IGF-I-degrading enzyme and cathepsin B are identical, as indicated by the following observations: (a) the optimum pH and inhibitor profile of the endosomal proteolytic activity were similar to those of cathepsin B; (b)

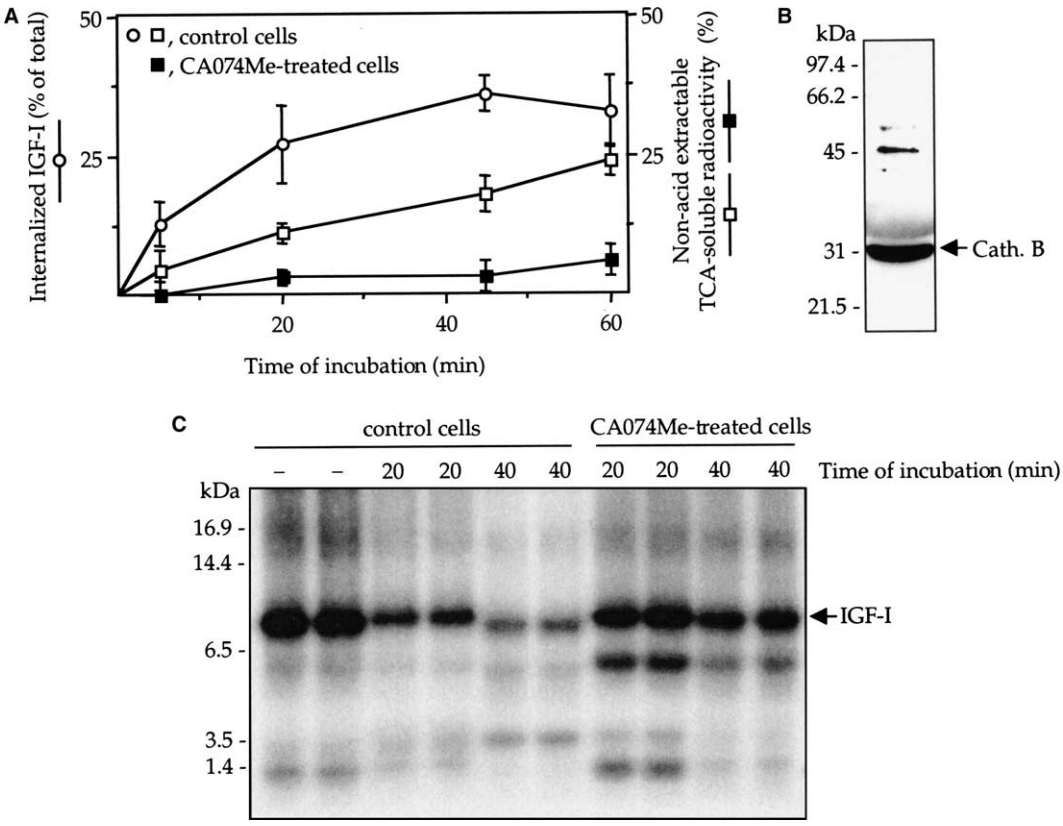

Fig. 3. IGF-I internalization and degradation in human HepG2 hepatoma cells. Panel A, HepG2 cells were treated with or without 1 μM CA074-Me for 1 h prior to the addition of [^{125}I]IGF-I. At the indicated times, cells were placed on ice, acid washed and lysed in 6% TCA. The acid washes, non-acid extractable TCA-soluble and -precipitable radioactivity were counted in a γ-counter. Results are the means ± S.D. of two experiments performed in duplicate wells. Panel B, HepG2 cell lysate was subjected to reducing SDS–PAGE, transferred to nitrocellulose and immunoblotted with sheep anti-human cathepsin B PC049. Panel C, HepG2 cells were treated for 1 h with or without 1 μM CA074-Me. The cells were lysed, and the clarified cell lysates were incubated at 37 °C and pH 5 with [^{125}I]IGF-I for the indicated times. The integrity of [^{125}I]IGF-I was then analyzed on a reducing Tris–Tricine gel followed by autoradiography. Results represent two different experiments. Molecular mass markers are indicated to the left of the panel. Arrow on the right indicates the mobility of intact IGF-I (≈7.6-kDa).

immunodepletion of cathepsin B from an endosomal lysate led to a loss of IGF-I-degrading activity; (c) endosomal IGF-I-degrading activity and pure cathepsin B produced cleavage patterns for IGF-I that were in accordance with the carboxypeptidase activity of cathepsin B; (d) IGF-I and the high-affinity cathepsin B substrate EGF displayed comparable IC_{50} values; and (e) Western-blot experiments demonstrated the presence of the 32- and 28-kDa active forms of cathepsin B in the endosomal lumen, as well as its 45-kDa inactive precursor which was processed to the active forms under ATP-dependent endosomal acidification conditions.

In addition, using human HepG2 hepatoma cells, we provide evidence that internalized [^{125}I]IGF-I is a physiological substrate for the intracellular cathepsin B protease. A rapid internalization of [^{125}I]IGF-I has previously been demonstrated in CHO cells stably transfected with the human IGF-I receptor [4], in Rat-1 fibroblasts [15] and in human osteosarcoma MG-63 cells [3]. In these studies [3,15], the internalization of [^{125}I]IGF-I reached a maximum at ≈1 h and slowly declined thereafter. Moreover, a progressive degradation of internalized [^{125}I]IGF-I was demonstrated up to 2 h of incubation and was inhibited by the addition of lysosomotropic agents or leupeptin [3]. Our present work extends these observations to human HepG2 cells which express IGF-I and IGF-I/insulin hybrid receptors [17,18], and indicates that cathepsin B is mainly responsible for the intracellular degradation of internalized [^{125}I]IGF-I. However, the cathepsin B proinhibitor CA074-Me did not completely inhibit [^{125}I]IGF-I proteolysis either in vivo in HepG2 cells or in vitro using HepG2 cell lysates, suggesting the potential minor contribution of another endosomal or lysosomal protease to the processing of radioactive IGF-I.

Originally, it was believed that the active forms of cathepsin B resided solely in lysosomes, leading to the proposal that activation occurred in the same compartment [19]. Recent reports demonstrated that both precursor and mature cathepsin B were also present in the endosomal compartment [9,10] suggesting that proteolytic activation may occur in the endosomal apparatus, as already demonstrated for the maturation of the precursor of the aspartic proteinase cathepsin D [20]. Procathepsin B contains a highly accessible region, close to the NH_2 terminus of the mature enzyme, which is very susceptible to proteolytic attack by several endosomal proteinases [19]. Our studies with intact cell-free endosomes support this hypothesis and show that ATP-dependent endosomal acidification is required for proenzyme activation. It still remains to be determined whether maturation of endosomal procathepsin B occurs by an unimolecular autoprocessing event [21] or intermolecular processing by endosomal cathepsin B or other endosomal cathepsins [22,23].

Our present work is in agreement with other studies suggesting that 20–40% of newly synthesized lysosomal hydrolases can be detected in early endosomes [24]. In hepatic endosomes, cathepsin B processes internalized glucagon, EGF and EGF receptor at acidic pH [9,10]. In macrophage endosomes, cathepsin B is responsible for proteolytic cleavage of ricin A chain at both neutral and acidic pH [25]. In pancreatic β-cells, a pool of endosomal procathepsin B was identified [26] and, in thyroid epithelial cells, procathepsin B is first transported to compartments of the late endocytic pathway where it matures before being secreted [27]. Finally, in Vero cells, cathepsin B is responsible for the endosomal proteolysis of the Ebola virus glycoprotein-1 [28].

According to the sequences of the peptide products, the main proteolytic activity participating in the endosomal degradation of IGF-I at acidic pH can be attributed to a carboxypeptidase activity that generates four major IGF-I metabolites: Gly^{1}-Lys^{68}, Gly^{1}-Ala^{67}, Gly^{1}-Pro^{66} and Gly^{1}-Lys^{65}. Thus, the pattern of IGF-I peptides produced within endosomes is in accordance with the dipeptidyl carboxypeptidase activity of cathepsin B observed at acidic pH [22]. The resistance of Gly^{1}-Lys^{65} to digestion by cathepsin B and/or other endosomal proteases, even after a long digestion time, may result from inaccessibility of the enzyme(s) to the cleavage sites. Using a similar approach, we found C-terminally truncated forms of EGF [10] and glucagon [9] produced by endosomal cathepsin B.

The cathepsin B cleavage sites within IGF-I are located at the carboxyl terminus of the D-domain of the peptide which contains eight amino acids [2]. Studies on the structural requirements for IGF-I/IGF-I receptor binding have shown that the removal of the D-region of hIGF-I has little effect on binding to the type-1 IGF receptor [29]. Moreover, [1-62]hIGF-I displays a 2-fold higher affinity for the insulin receptor and a 4-fold higher affinity for IGF serum-binding proteins. Thus, as previously demonstrated for PTH-(1-84) [30], glucagon [31] and endocytosed protein antigens for major histocompatibility class II presentation [32], changes in the biological activity of the IGF-I peptide were observed following processing in endosomes.

We have shown that binding and cleavage of IGF-I (this study; [6]) and EGF [6,10] were not due to the endosomal insulinase cathepsin D. Moreover, we have previously demonstrated that the aromatic locus Phe^{B24}-Phe^{B25}-Tyr^{B26} of the insulin B chain and the corresponding Phe^{24}-Phe^{25}-Tyr^{26} bonds in the insulin moiety of proinsulin dictate insulin and proinsulin binding to and degradation by endosomal cathepsin D [7,33]. The differences in primary sequence between positions Phe-Phe-Tyr of the insulin and proinsulin peptides and the corresponding positions Phe-Tyr-Phe of IGF-I might explain the lack of binding and cleavage of the IGF-I peptide by cathepsin D which displays a high selectivity for the Phe-Phe bond [34]. Another contributing factor may be the steric interference caused by the presence of D-chain residues in IGF-I but not in insulin and proinsulin [29]. Thus, since the eight amino acid D-chain region of IGF-I may interfere with binding to the 23–25 region [2], any protein that interacts with the B^{24}-B^{26} region of insulin will exhibit lower affinity for IGF-I.

A connecting peptide links the carboxyl terminus of the B-region to the amino terminus of the A-region in the proinsulin (35 amino acid C-peptide) and IGF-I (12 amino acid C-domain) polypeptides [2]. Whereas proinsulin was cleaved at eight bonds within the C-peptide by endosomal cathepsin D [33], we did not observe any cleavage in the connecting C-domain of IGF-I. Our inability to show cleavage of IGF-I in the Gly^{30}-Thr^{41} peptide despite the presence of cathepsin D in our endosomal fractions [7] may be explained by the major differences observed in the length and amino acid composition of the proinsulin and IGF-I connecting peptides.

A previous report has shown that cathepsin D can efficiently proteolyze IGF-I and -II as well as IGFBP-1 to -5 in vitro at pH 4 in a time and concentration-dependent manner [35]. This contrasting report may have reflected the very low pH (pH 4) and/or the extremely high enzyme/substrate ratio (≈1 μg cathepsin D per assay) and time of incubation (6–20 h) used in their digestion assay. Moreover, in these studies, the nature of the alterations of IGF-I was based on comparison of the electrophoretic mobility of intact radioactive IGF-I to that of cathepsin D-digested radiolabeled peptide. Thus, radioactive IGF-I might not display all the structural elements of the natural peptide substrate and the radioactive iodine on the molecule might alter the enzyme-substrate recognition. Whatever the physiological relevance of these studies, our work identifies an IGF-I-degrading enzyme in endosomes which is distinct from cathepsin D and functional at the endosomal pH (pH 5.5–7).

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