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A three-step purification strategy for isolation of hamster TIG2 from CHO cells: characterization of two processed endogenous forms

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Abstract

We have recently isolated a bioactive, circulating protein of human tazarotene-induced gene-2 (TIG2) as the natural ligand of the orphan receptor ChemR23. Here we describe a simplified method for the isolation of hamster TIG2 protein from Chinese hamster ovary (CHO) cell supernatant. Using a heparin-affinity column followed by two reversed phase chromatography steps resulted in the isolation of pure biologically active material. Two processed bioactive forms of Chinese hamster TIG2 were identified by Edman sequencing and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) mass fingerprint analysis, representing the amino acid residues T^{20} to F^{156}, and T^{20} to A^{155} of the 163 amino acid propeptide. Comparison with the predicted aa-sequence indicates a mutation or modification within the C-terminal end of the peptide.

Keywords: TIG2; GPCR; Heparin-affinity chromatography; FLIPR; CHO cells

1. Introduction

Originally, the tazarotene-induced gene-2 (TIG2 gene) was discovered in a subtraction hybridization assay which identified upregulated genes of human skin raft cultures by the anti-psoriatic retinoid drug tazarotene [1]. Very recently, using G-protein-coupled-receptor screening, human TIG2 protein was described as a natural ligand for the ChemR23 receptor by Wittamer et al. [2] and also by our group [3]. The ChemR23 receptor exhibits homology to neuropeptide and chemoattractant receptors although it is not classified within the chemokine receptor family and is mainly expressed in immune cells [4]. It conveys TIG2-induced chemotactic activity on macrophages and immature dendritic cells (DC) involving G-proteins of the Gi/o family [2]. Furthermore, the ligand/receptor pair is implicated in bone metabolism [5,6] and in the physiology of the skin [1].

Using human hemofiltrate as a peptide source to discover the ChemR23 ligand Meder et al. have isolated and characterized a circulating bioactive form of TIG2 which is produced from the 163 aa propeptide [18,617 Da] by N- and C-terminal processing and comprises 134 amino acids (E^{21} to F^{154}, 15,566 Da) [3]. Parallel to this, Wittamer et al. described a truncated TIG2 form (137 aa; E^{21} to S^{157}, 15,876 Da) from human ascitic fluid, named chemerin [2]. The N-termini of both human forms discovered in hemofiltrate and ascites start with glutamic acid at position 21 of the propeptide, corresponding to the predicted signal peptide cleavage site [2,3]. In contrast, the C-termini of these forms differ in length. Whereas the biologically active form isolated from hemofiltrate is produced from the propeptide by C-terminal truncation of nine aa [3], the form from ascitic fluid results from truncation of only six aa [2]. Wittamer et al. demonstrated that the removal of a minimum of six amino acids from the C-terminal end is required to develop biological activity measured by intracellular calcium influx [7]. This proteolytic activation of the propeptide presumably

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occurs in the extracellular space. It has been shown that supernatants of Chinese hamster ovary (CHO)-K1 cells are capable of converting exogenous human propeptide into processed bioactive forms, indicating the presence of the responsible protease. However, this enzyme has not yet been identified [2].

In this study, it was intended to develop an improved purification strategy for the isolation of human TIG2 from CHO-K1 cells after stable transfection with human TIG2 cDNA in order to clarify the processing in cell culture. Therefore, the supernatants were chromatographed and isolated peptides were analyzed by Edman sequencing and mass spectrometry. An optimized purification strategy allowed the isolation of only two peptides capable of activating ChemR23 both surprisingly identified as hamster TIG2 forms.

2. Experimental

2.1. Cell lines and cell culture

CHO-K1 cells were grown in F12 nutrient mixture (HAM, Bio Wittaker Inc., Walkersville, MD, USA). Transfected CHO-K1Galpha(16) cells were grown in medium with 200 μg/ml hygromycin and 400 μg/ml G418 (both from Invitrogen GmbH, Karlsruhe, Germany). The cells were cultivated with 100 units penicillin/streptomycin per ml (Invitrogen GmbH, Karlsruhe, Germany) and 5% (v/v) fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) at 37 °C in 5% (v/v) CO_2 atmosphere.

2.2. Cloning and recombinant expression of ChemR23

Stable double transfected CHO-K1 cells carrying the cDNAs of Galpha(16) and hChemR23 were prepared as described previously [3].

2.3. Intracellular Ca^{2+} measurement

For the $[Ca^{2+}]_i$ influx assay, CHO cells were seeded into black 96-well plates (Corning B.V., Life Sciences, Schiphol-Rijk, The Netherlands) at 20,000 cells/well and cultured overnight. Cells were incubated at 37 °C in loading medium comprising of HEPES-buffered Hank's balanced salt solution pH 7.4 (HBSS, Invitrogen GmbH, Karlsruhe, Germany), 2.5 mM probenecid (Sigma, Deisenhofen, Germany) and 2 μM Fluo-4 AM (Molecular Probes, Leiden, The Netherlands). After 40 min of incubation at 37 °C, agonist-evoked intracellular Ca^{2+} influx was measured using the fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA, USA). $[Ca^{2+}]_i$ was calculated as peak fluorescence intensity units (FIU) minus basal FIU. These data were presented either as maximal values or transient elevation.

2.4. Isolation of hamster TIG2 from CHO cell supernatant

CHO-K1 cells stably transfected with human TIG2 cDNA were grown to confluence in 175 cm flasks. At confluence the medium was replaced with 20 ml of serum-free medium and the cell supernatants were harvested after 72 h. The cells were repeatedly incubated with fresh serum-free medium for two more periods of 48 h and the supernatants were collected until a total of two liters was obtained. The supernatants were centrifuged (1000 × *g*, 15 min) and filtered (0.2 μm, polyether sulfone membrane, Schleicher & Schuell GmbH, Dassel, Germany). For determination of biological activity a peptide extract from 800 ml CHO-supernatant was centrifuged, filtrated and diluted 1:2 with 0.1% (v/v) TFA (solvent A0). This solution was loaded onto a RPC15 reversed phase HPLC column (250 mm × 20 mm i.d., 15 μm, 300 Å, Amersham Biosciences, Buchinghamshire, England). A three-step gradient of solvent B0 (ACN/0.1% TFA, 80:20 (v/v)) was applied for a 60 min separation at a flow rate of 8 ml/min (0–30% B0 within 4 min, 30% B0 for 16 min, 30–60% B0 within 4 min, 60% B0 for 16 min, 60–100% B0 within 4 min, 100% B0 for 16 min). Three fractions of 20 min each were collected starting at 0 min, lyophilized and resuspended in HBSS buffer to be tested in the $[Ca^{2+}]_i$ flux assay.

The purification of hamster TIG2 was performed in three steps:

- Step 1: Five hundred milliliters cell culture supernatant were diluted with 800 ml of 10 mM Na_2HPO_4, pH 7.0 (solvent A1) and loaded onto a heparin-affinity column (5 ml, HiTrap Heparin HP, Amersham Biosciences, Buchinghamshire, UK). A 60 min gradient from 0 to 60% solvent B1 (10 mM Na_2HPO_4 with 2 M NaCl, pH 7.0), followed by a 9 min gradient from 60 to 100% solvent B1, was applied at a flow rate of 1.5 ml/min. Fractions of 2 min were automatically collected using a fraction collector (Bio Rad Laboratories, Hercules, CA, USA). Aliquots of 10% (v/v) of each fraction (3 ml) were desalted (Empore, high performance extraction disk plates, Varian Inc., Harbor City, CA, USA) and tested for bioactivity using the $[Ca^{2+}]_i$ flux assay. This first isolation step was repeated four times to process 2 l cell culture supernatant.
- Step 2: Fractions of the four chromatographic isolation cycles of step 1 showing bioactivity were pooled, diluted with 100 ml solvent A2 (0.1% TFA) and loaded onto a C18 reversed phase HPLC column (RPC15 source, 250 mm × 4 mm i.d., 15 μm, 300 Å, Amersham Biosciences, Buchinghamshire, England). A gradient from 30 to 55% solvent B2 (ACN/0.1% TFA, 80:20 (v/v)) was applied for separation within 50 min at a flow rate of 0.7 ml/min. Fractions of 700 μl were collected. Aliquots of 5% (v/v) per fraction were used for the $[Ca^{2+}]_i$ flux assay.
- Step 3: Active fractions of step 2 were diluted 1:3 with solvent A3 (0.1% TFA) and loaded onto a C18 reversed

phase HPLC column (RPC18, 200 mm × 2 mm i.d., 5 μm, Phenomenex, Aschaffenburg, Germany). A 60 min gradient from 30 to 50% solvent B3 (ACN/0.1%TFA, 80:20 (v/v)) was used at a flow rate of 0.4 ml/min. Aliquots of 2.5% (v/v) of respective fractions (400 μl) were tested for bioactivity using the $[Ca^{2+}]_i$ flux assay.

2.5. Capillary zone electrophoresis

Purity of bioactive fractions of the third isolation step was confirmed by capillary zone electrophoresis (CZE, P/ACE MDQ, Beckman, München, Germany) at 214 nm using a fused silica uncoated capillary (50 cm × 75 μm i.d.) and 0.1 M H_3PO_4 (0.02% (w/v) hydroxypropyl-methylcellulose, pH 2.5). Separation was carried out with a constant current of 80 μA at 25 °C.

2.6. Edman sequencing

The N-terminal sequence (first 23 amino acids) was determined by Edman degradation using a Procise 494 amino acid sequencer (Applied Biosystems, Darmstadt, Germany).

2.7. Mass spectrometric analysis

Mass spectrometric analysis was carried out on a matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-Pro, Applied Biosystems, Darmstadt, Germany) in different modes: linear mode for wide range mass detection, reflector mode for improved mass determination with internal standard and PSD mode for generating sequence tags for identification. The settings for all modes used were optimized to detect TIG2 fragments.

2.7.1. Peptide mass fingerprint

The two isolated peptides were cleaved into fragments by endoproteinase Lys-C treatment and analyzed by peptide mass fingerprint (PMF) analyses to confirm the aa-sequence. Lyophilized samples (peptide content of 0.5–1 μg) were reconstituted in 100 μl 25 mM NH_4HCO_3 (pH 7.8) and 2 μl dithiothreitol solution (DTT, 10 mg/ml H_2O) were added prior to incubation for 45 min at 50 °C. Two microliters iodoacetamide solution (10 mg/ml H_2O) were added and the reaction continued for 45 min at room temperature in the dark. Subsequently, 5 μl of an endoproteinase Lys-C stock solution (100 μg/ml H_2O, endoproteinase Lys-C, sequencing grade, Roche Diagnostic GmbH, Penzberg, Germany) were added and incubated at 37 °C for 18 h for enzymatic cleavage of the isolated peptides. In parallel, both isolated peptides were dissolved in 100 μl 25 mM Tris/HCl buffer (1mM EDTA, pH 8.5) to perform the enzymatic cleavage with Lys-C without reduction and carboxyamidomethylation. The incubation mixtures were desalted using 50 μl POROS 50 R2 material (PerSeptive Biosystems, Framingham, MA, USA) and composition of the reaction solutions was determined off-line by MALDI-MS in the linear positive mode. Alpha-cyano-4-hydroxycinnamic acid (Sigma, Deisenhofen, Germany) was used as the matrix. The resulting mass spectrometric signals were assigned database entries using PeptideCutter (http://us.expasy.org/tools).

2.7.2. Analysis of cleaved peptides after HPLC separation

Enzymatically cleaved proteins were loaded onto a C18 reversed phase HPLC column (Reprosil, 250 mm × 1 mm i.d., 3 μm, Dr. Maisch GmbH, Ammerbach-Entringer, Germany) to separate the internal peptidic products. A 110 min gradient of solvent B4 (ACN/0.06%TFA, 80:20 (v/v)) from 5 to 60% (v/v) was applied at a flow of 20 μl/min (solvent A4: 0.06% TFA (v/v)). Fractions of 1 min (20 μl) were collected and analyzed by MALDI-TOF-MS.

3. Results and discussion

3.1. CHO culture supernatant evokes $[Ca^{2+}]_i$ increase in ChemR23-expressing cells

The aim of this study was the isolation and structural characterization of a bioactive human TIG2 peptide from CHO cell supernatant. As shown in our previous studies, human TIG2 is a high affinity ligand for the ChemR23 receptor [3]. In this study we observed Ca^{2+} mobilization in receptor-overexpressing cell lines by subjecting fractionated CHO cell culture supernatants to the FLIPR assay. A dose-dependent activity on ChemR23-overexpressing cells was detected (Fig. 1A) whereas no $[Ca^{2+}]_i$ influx was observed on CHO-K1 wildtype or Galpha(16) transfected cells, indicating the agonistic specificity. The amount of cell supernatant required for a significant $[Ca^{2+}]_i$ increase was relatively high (10 ml equivalent) indicating only low concentrations of the active peptides.

3.2. Isolation of hamster TIG2 from CHO cell supernatant

In our previous work, we isolated human TIG2 following a purification scheme comprising six separation steps. Only minimal amounts of pure human TIG2 were obtained. To find a more efficient method for purification, we tested the binding properties of human TIG2 to various matrices and observed a complete adsorption to heparin. Elution of human TIG2 was achieved with high sodium chloride concentration after most contaminating proteins have been washed off the matrix. Therefore, in this study heparin-affinity chromatography was used as the first isolation step to remove about 90% of the total protein amount of the cell supernatant, which did not bind to the column. A further separation of contaminating material is achieved by elution with a sodium chloride gradient (0–2 M). More than 80% of the gradient-eluted material was characterized as inactive in the $[Ca^{2+}]_i$ assay. Compounds of fractions 22–25 elicited an increase of $[Ca^{2+}]_i$ in ChemR23-expressing

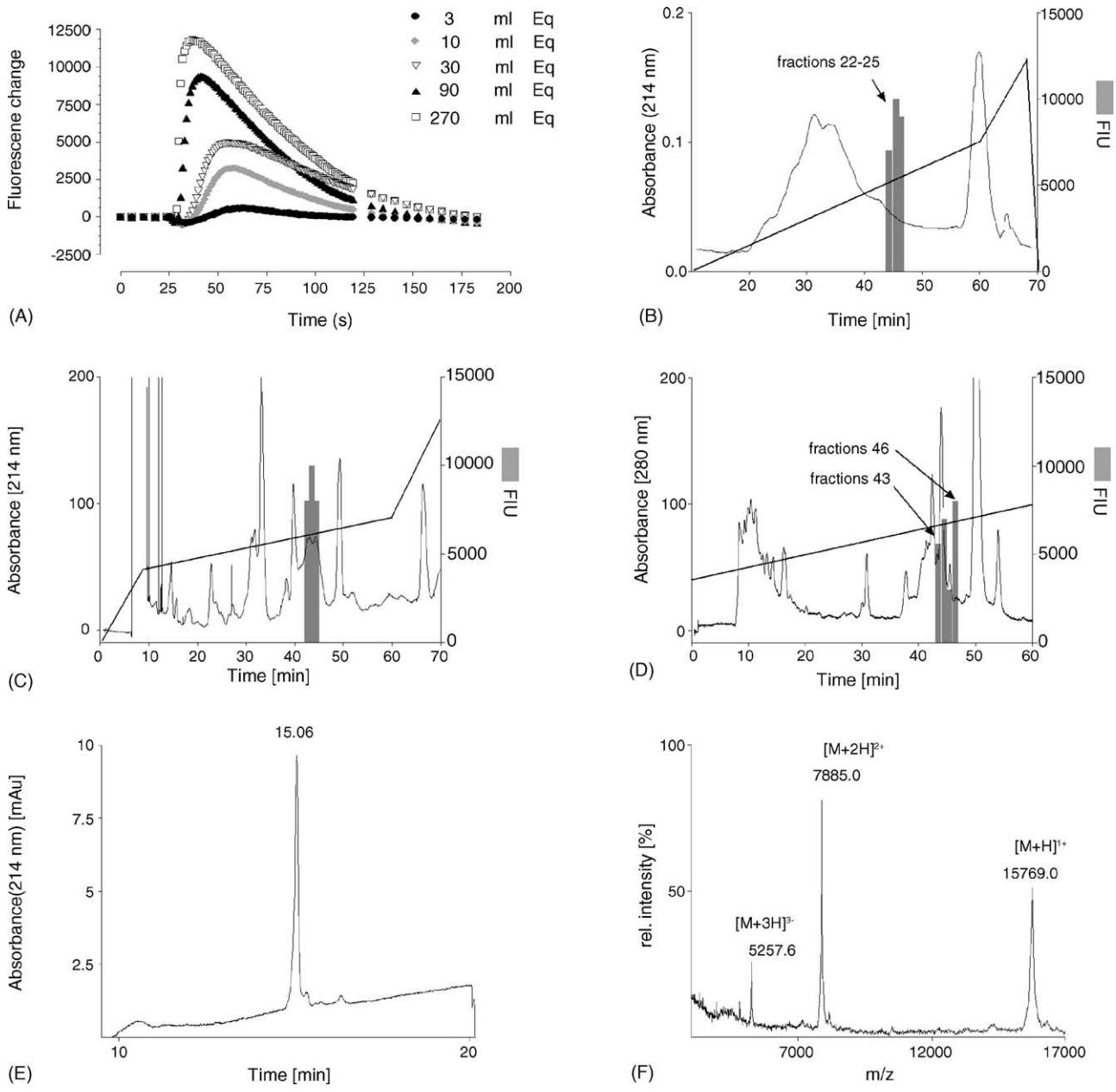

Fig. 1. Isolation of hamster TIG2 from CHO cell supernatant. (A) Concentration-dependent activity of CHO-K1 peptide extract (fraction 2) in the range of 3–270 ml supernatant equivalents. Transient elevation of $[Ca^{2+}]_i$ are presented as fluorescence change. One typical experiment out of three replicates is shown. (B–D) Chromatographic purification of hamster TIG2; gradient profiles are indicated as solid lines and activating fractions are marked as shaded bars. (B) Step 1: Heparin-affinity chromatography (Hitrap) of 500 ml cell supernatant diluted 1:3 with solvent A1 (10 mM Na_2HPO_4, pH 7.0). A 60 min gradient from 0 to 60% solvent B1 (10 mM Na_2HPO_4 with 2 M NaCl, pH 7.0) followed by a 9 min gradient from 60 to 100% solvent B1 were applied; flow rate: 1.5 ml/min; fraction size: 2 min. (C) Step 2: Bioactive fractions of step 1 were pooled, diluted with solvent A2 (0.1% TFA), loaded onto a Source™ reversed phase column (RPC15; 250 mm × 4 mm i.d., 15 μm, 300 Å) and eluted with a gradient from 30 to 55% solvent B2 (ACN/0.1%TFA, 80:20 (v/v)) within 50 min; flow rate: 0.7 ml/min. Fractions of 700 μl were collected. (D) Step 3: RP-HPLC of the marked fractions in panel C; RP C18 Phenomenex, 200 mm × 2 mm i.d.; solvent A3: 0.1% TFA, solvent B3: ACN/0.1%TFA, 80:20 (v/v), gradient of B3 from 30 to 50% within 60 min, flow rate: 0.4 ml/min. Aliquots of 2.5% (v/v) of respective fractions were tested for bioactivity using the FLIPR system. (E) Capillary zone electrophoresis of fraction 46 of step 3 (D). Fused silica uncoated capillary, 50 cm × 75 μm i.d., 0.1 M Na_3PO_4 with 0.02% (w/v) hydroxypropyl-methylcellulose pH 2.5, T: 25 °C, I: 80 μA. (F) MALDI-TOF-MS positive ion mode spectrum of fraction 46 from step 3 (D).

cells (Fig. 1B) and were pooled for the second isolation step. Resolved peaks were obtained using a Source™ RPC15 reversed phase column and ACN/TFA as eluent (Fig. 1C). In the last purification step (step 3) using an analytical C18 column, two bioactive pools (fractions 43/44 and 45/46, Fig. 1D) were isolated. CZE analysis of fractions 43 and 46 showed one predominant major peak each, indicating one main component of high purity (Fig. 1E). Mass spectrometric analysis revealed a molecular weight of 15,768 Da (Fig. 1F) for the compound in fraction 46 and 15,621 Da for the other in

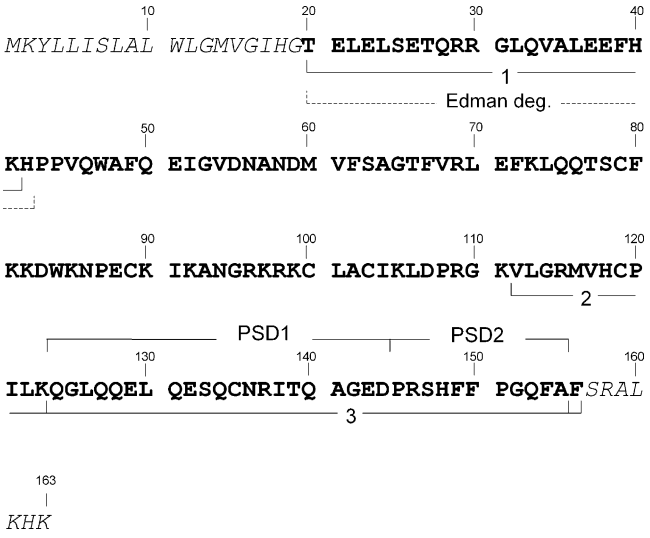

Fig. 2. Amino acid sequence of hamster TIG2 as deduced from the cDNA. The two processed forms acting as ligands to the ChemR23 receptor are displayed in bold letters: V1 (T^{20}–F^{156}) and V2 (T^{20}–A^{155}). Sequence written in italics represents the signal peptide. The numbered sequence tags (1–3) represent the peptidic fragments shown in the PMF spectra of hamster TIG2 (Fig. 3). Both fragments identified by MALDI-MS/MS analysis are labeled with PSD1 and PSD2. The dotted line depicts the N-terminus identified by Edman degradation.

fraction 43 (not shown). For easier discussion, in the following sections the 15,768 Da compound is referred to as variant 1 (V1) and the 15,621 Da as variant 2 (V2). Using Edman degradation and MALDI-TOF-MS analysis, two processed forms of hamster TIG2 were detected (Fig. 2, bold letters).

The N-termini of both isolated fragments are identical and correspond to T^{20}-H^{42} of hamster TIG2 precursor, thereby confirming the signal peptide cleavage site predicted by the SignalP-2.0 software [8]. We conclude that the difference in molecular weight of the two fragments, determined by MALDI-TOF MS ($\Delta m/z$: 147 Th), represents a C-terminal truncation of the phenylalanine at position 156 of variant 1. The calculated average masses for the unmodified aa-sequences considering disulfide linkages are 15,781.2 Da for V1 (T^{20}–F^{156}) and 15,634.0 Da for V2 (T^{20}–A^{155}). Compared to the measured molecular weights, a difference of −13 Da appeared. To analyze this discrepancy, the C-terminus was experimentally characterized further by peptide mass fingerprint.

Although using CHO cells intended for recombinant expression of human TIG2, we isolated the endogenous hamster ortholog from CHO cells (*Cricetulus griseus*) by an improved purification strategy using heparin-affinity chromatography. The missing evidence of human TIG2 peptide might be due to the lack of those regulatory proteins or enzymes, which enable the processing of human TIG2 protein precursors during translation or to those, that are needed for the secretion into the cell supernatant. Nevertheless, these explanations seem less probable since Wittamer et al. showed synthesis and secretion of human TIG2 in CHO cells after transfection of the human cDNA. For recombinant production, we used an expression cassette containing the human TIG2 cDNA together with an antibiotic resistance gene to obtain stable expressing clones by antibiotic selection, which is a broadly accepted method for this purpose. A clone with high expression level of human TIG2 mRNA was identified by RT-PCR and propagated for continued cultivation to produce human TIG2 peptide. In general, a genetic drift within 15–25 passages of cultivation does not affect transgene expression but this is not guaranteed when synthesis of the transgene causes slow cell growth and/or recombination events uncouple the transgene from antibiotic resistance. This might have contributed to lose human TIG2 expression in the selected clone which initially was identified in the screening. However, this is the first time that hamster TIG2 has been isolated and characterized on the protein level. The sequence has been known so far only from the corresponding cDNA. As previously described for human TIG2, the hamster precursor protein, which consists of 163 amino acids, is processed at the N- and the C-terminus (Fig. 3).

3.3. Mass fingerprint analysis

To analyze the amino acid residues of the C-terminus and clarify the discrepancy between calculated and experimentally obtained masses, peptide mass fingerprint was performed. First, both variants were cleaved with Lys-C without prior reduction of disulfides and subsequently analyzed by MALDI-TOF-MS. In contrast to common PMF analyses using trypsin, we chose Lys-C to generate C-terminal peptide fragments of sufficient molecular weights reliably detectable by MALDI-TOF-MS without signal suppression due to MALDI matrix interferences. The mass spectra of the peptides showed only one fragment each at *m/z* 2614.9 (data not shown). This signal is assigned to the N-terminal cleavage product T^{20}–K^{41} (2613.9 Da, Fig. 2). The lack of additional signals may be due to steric hindrance of enzymatic cleavage presumably caused by three expected cysteine bridges.

To improve the accessibility of the enzyme, both peptides were reduced and carboxyamidomethylated prior to Lys-C cleavage and MALDI analysis. Mass spectra of digested V1 (15,768 Da) and V2 (15,621 Da) showed very similar signal patterns (Fig. 3). The three signals at *m/z* 1926.5, *m/z* 3091.4, and *m/z* 4947.4 labeled with 4–6 (Fig. 3A) were assigned to fragments of autoproteolyzed Lys-C and therefore emerged as unrelated to hamster TIG2. In addition, both spectra show two singly charged ions at *m/z* 2614.9 labeled with 1 and *m/z* 1423.8 labeled with 2 (Fig. 3). These fragments are assigned to the sequences T^{20}–K^{41} (2613.9 Da) and to V^{112}–K^{123} (1422.8 Da) of hamster TIG2. The latter cleavage product is carboxyamidomethylated at the cysteine residue in position 119. The most important differences between the spectra of V1 and V2 are the two signals labeled with 3 at *m/z* 3838.0 (V1, Fig. 3A) and at *m/z* 3691.0 (V2, Fig. 3B). These two ions show a difference of $\Delta m/z$ = 147.0 Th which is in accordance with the molecular mass of phenylalanine,

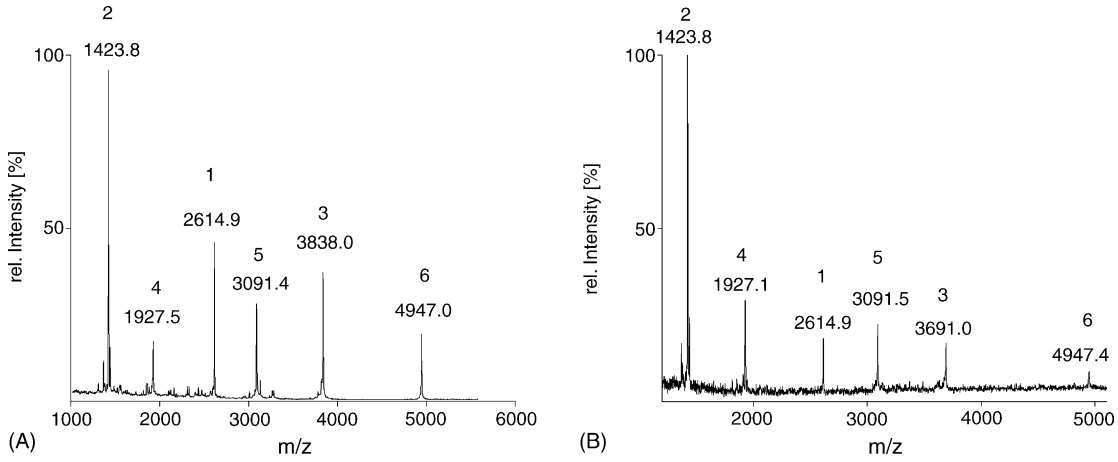

Fig. 3. Identification of hamster TIG2 by peptide mass fingerprint (PMF) of V1 (A) and V2 (B) after reduction, carboxyamidomethylation and Lys-C cleavage. The incubation mixture was desalted and measured by MALDI-TOF-MS. Protonated peptides: *m/z* 2614.9 (1), *m/z* 1423.8 (2) and *m/z* 3691.0 (3) are assigned to peptidic cleavage products of hamster TIG2 as shown in Fig. 2. Signals labeled with 4–6 are assigned to autoproteolyzed Lys-C (4: Q^{31}–K^{48}, 5: G^{1}–K^{30} and 6: R^{156}–K^{203}).

the expected C-terminal amino acid of the processed hamster TIG2 (V1 Fig. 2). The same difference in molecular weight was observed for the intact TIG2 peptides V1 and V2. Therefore, we conclude that both ions represent the C-terminal fragments of the two isolated bioactive forms of hamster TIG2, which differ in the truncation of the last phenylalanine residue.

The expected molecular weight for the carboxyamidomethylated C-terminal cleavage product of V1 is 3853.0 Da (Q^{124}–F^{156}) and of V2 it is 3706.0 Da (Q^{124}–A^{155}). The measured ions differ by −15 Da, when compared to the theoretical aa-sequences deduced from the cDNA. Within the MALDI method accuracy, this difference could be also observed between the calculated and measured processed full-length hamster peptides V1 and V2. Based on these consistent mass determinations for the intact peptides and their fragments, we assume that this difference is due to at least one aa mutation or modification within the last C-terminal residues (from Q^{124} to A^{155}). To narrow down this aa mutation or modification, we performed an improved mass determination of the V2 fragment by MALDI-TOF-MS with internal standards in the reflector mode. Using this technique, we detected a monoisotopic molecular mass $[M+H]^+$ of 3688.7 Da (data not shown). Compared with the theoretical monoisotopic mass $[M+H]^+$ of 3703.7 Da (V2, Q^{124}–F^{155}), a difference of 15.0 Da was detected. This mass difference fits to a replacement of glutamine (Q, 128.1 Da) by leucine or isoleucine (L/I, 113.2 Da), resulting in a loss of 15.0 Da, within the given mass accuracy.

3.4. MALDI-MS/MS analysis

To clarify the position of this potential aa exchange within the C-terminal fragment, an MS/MS experiment (MALDI-PSD) was performed for the signal at *m/z* 3688.7. The PSD (post source decay) spectrum showed only two major peaks at *m/z* 1293.6 and at *m/z* 2403.6 and no sequence ladder tag (data not shown). These two peaks are due to an N-terminal fragmentation at proline (P^{145}) typical for MS fragmentations. Nevertheless, these two signals are a further hint towards narrowing down the region containing the assumed aa exchange (Q → I/L). In accordance with the expected theoretical sequence, the signal at *m/z* 1293.6 is assigned to the C-terminal part (P^{145}–A^{155}) of the peptide. The other signal at *m/z* 2403.6 results from the N-terminal region (Q^{124}–D^{144}), when considering the 15.0 Da difference. Therefore, we conclude that the assumed aa exchange (Q → I/L) occurs in the region between Q^{124} and D^{144}. This region contains six glutamine residues. However, all of them appear to be strongly conserved between hamster, rat, mouse and humans (see Fig. 4). Considering the glutamine coding triplets and assuming a single base exchange, a conversion is only possible to leucine coding triplets.

3.5. Amino acid sequence alignment of hamster, human, mouse and rat TIG2

The amino acid sequence alignment of TIG2 from four mammalian species represents a highly conserved molecule interrupted by a few short regions displaying low homology. Seven amino acids of the C-terminus are 100% identical in all four species (P^{151}GQFAFS^{157}), as depicted in Fig. 4. This C-terminal end was shown to be crucial for the biological activity. A synthetic peptidic fragment identical to the C-terminus of human TIG2 (Y^{149}-S^{157}, chemerin-9) exerts full functional activity [7]. Furthermore, elongation or truncation by single amino acids significantly decreases the functional activity. Interestingly, the residues G^{152}, F^{154}, and F^{156}, which are crucial for full bioactivity, as shown by alanine-scanning mutagenesis [7], are strictly conserved in the different species (Fig. 4). In addition, it was shown that the replacement of Y^{149} by phenylalanine was well tolerated [7]. This is consistent with our results, showing that hamster TIG2, with the

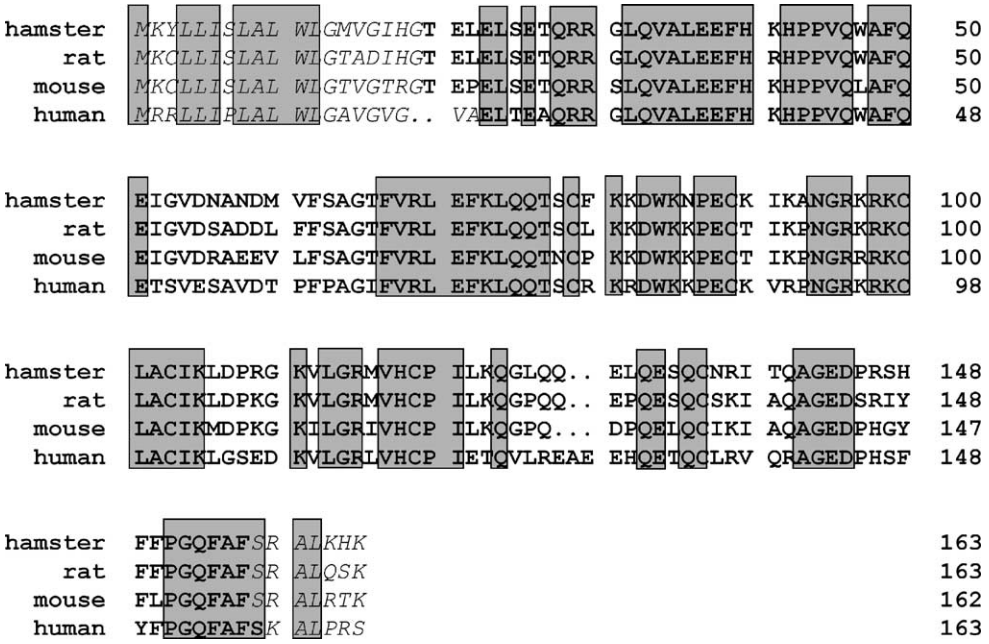

Fig. 4. Amino acid sequence alignment of TIG2 from four different species: hamster, rat, mouse, and human. Amino acids identical to hamster TIG2 sequence are boxed. The biologically active forms predicted in analogy for mouse and rat TIG2 are displayed in bold letters. Sequences were taken from data base entries with the accession numbers BAC45229.1, NP_002880.1, BAB21997.1 and XP_216142.1.

predicted phenylalanine at position 149, activates the human ChemR23 receptor.

4. Conclusions

An optimized isolation strategy for the purification of hamster TIG2 was developed by introducing heparin-affinity chromatography, which reduced the number of chromatographic steps required from six to three. Using this modified procedure, two biologically active hamster TIG2 forms from hamster (CHO cells) were identified, previously only described as corresponding cDNA. These forms differ by the presence or absence of the last C-terminal phenylalanine residue. Despite this truncation, both forms show activating potency for the ChemR23 receptor. From our mass spectrometric data, we conclude that the described variants of hamster TIG2 bear at least one aa mutation or a modification when compared to the theoretically deduced sequence from cDNA. Comparing the C-terminus of the two earlier described human TIG2 forms [2,3] and our newly discovered hamster forms reveals some heterogeneity in the processing reaction. Our results give new insights into the molecular structure and the processing of endogenous hamster TIG2 in CHO cell culture and provide an improved purification regime.

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