Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23

W. Meder[a,1], M. Wendland[b,1], A. Busmann[a], C. Kutzleb[a,*], N. Spodsberg[a], H. John[a], R. Richter[a], D. Schleuder[c], M. Meyer[a], W.G. Forssmann[a]

[a] *IPF PharmaCeuticals GmbH, Feodor-Lynen-Str. 31, D-30625 Hannover, Germany*
[b] *Pharis Biotec GmbH, Feodor-Lynen-Str. 31, D-30625 Hannover, Germany*
[c] *Applied Biosystems, Frankfurter Str. 129 B, D-64293 Darmstadt, Germany*

Received 6 October 2003; revised 4 November 2003; accepted 6 November 2003

First published online 17 November 2003

Edited by Beat Imhof

Abstract The orphan receptor ChemR23 is a G-protein coupled receptor (GPCR) with homology to neuropeptide and chemoattractant receptors. Tazarotene, a synthetic retinoid activating retinoic acid receptor (RAR), up-regulates tazarotene-induced gene-2 (TIG2). The function and molecular target of this protein are now described. By means of reverse pharmacology screening using a peptide library generated from human hemofiltrate, we have isolated and identified TIG2 as the natural ligand of ChemR23 and report the specific molecular form of the bioactive, circulating TIG2, representing the amino-acid residues 21 to 154 of the 163 amino acid-containing prepropeptide. Based on the expression pattern of ChemR23 and TIG2, the physiological role in bone development, immune and inflammatory responses and the maintenance of skin is now being investigated.

Key words: Ligand; Orphan receptor; ChemR23; TIG2; GPCR

1. Introduction

Orphan G-protein coupled receptors (GPCRs) have been successfully used for reverse pharmacology screening in order to establish potential new drug targets. The orphan GPCR ChemR23 exhibits homology to neuropeptide and chemoattractant receptors and is expressed in monocyte-derived dendritic cells, macrophages, antigen presenting cells and, at a lower expression level, in $CD4^+$ T lymphocytes [1]. In vitro, ChemR23 is used as a co-receptor by the immunodeficiency viruses HIV-1 and SIV, although it is not classified with the chemokine receptor family [1]. The mouse ortholog of human ChemR23, sharing 80.3% identity, has highest overall homology with rat GPR1, rat angiotensin II receptor and human C5a anaphylatoxin receptor [2]. Mouse ChemR23 is expressed in adult parathyroid glands, developing osteogenic and cartilaginous tissue and therefore implicated in bone metabolism [2].

Retinoids are analogs of retinoic acid which activate two families of nuclear receptors, retinoic acid receptor (RARα, -β, and -γ) and retinoid X receptor (RXRα, -β, and -γ) [3,4]. RARs and RXRs are heterodimers which change the expression of retinoic acid-responsive genes [3–6]. The retinoid tazarotene (AGN 190168/ethyl 6-[2-(4,4-dimethylthiochroman-6-yl)-ethynyl] nicotinate), is a drug for the treatment of non-malignant (psoriasis, acne), and malignant (squamous cell carcinoma, actinic keratoses, basal cell carcinoma and Kaposi sarcoma) skin diseases, albeit its mechanism of action in dermatological diseases is poorly understood [3,5,7]. Tazarotene up-regulates the expression of TIG1 (tazarotene-induced gene-1), TIG2, and TIG3 [8]. In skin raft cultures, TIG2 expression is increased by RAR-specific retinoids but neither by RXR-specific retinoids nor by 1,23-dihydroxyvitamin D_3. However, TIG2 expression is observed after steroid-induced osteoclastic differentiation of stromal cells [9]. In contrast to TIG1 and TIG3, TIG2 exhibits no anti-proliferation effects, but is implicated in the physiology of skin [10].

Human hemofiltrate is a valuable source for the generation of peptide libraries [11] suitable for receptor screening and for the isolation of circulating forms of peptide hormones in particular, since processed circulating peptide hormones differ considerably from their precursor molecules in sequence and receptor affinity [12–15].

We report here on the isolation and characterization of TIG2 as the natural ligand of the orphan receptor ChemR23 showing the circulating bioactive molecular form of this ligand.

2. Materials and methods

2.1. Cell lines and cell culture

Transfected Chinese hamster ovary (CHO)-Gα(16) cells (Molecular Devices, Sunnyvale, CA, USA) are grown in nutrient mixture F12 (HAM), 200 μg/ml hygromycin, 400 μg/ml G418. The cells are cultivated with 100 units/ml penicillin/streptomycin, 5% fetal calf serum at 37°C in 5% CO_2 atmosphere.

2.2. Cloning and recombinant expression of ChemR23 and TIG2

A 1186 bp DNA fragment containing the complete open reading frame of ChemR23 was cloned by polymerase chain reaction (PCR) from genomic DNA using a primer set: 5′-TGG TCC CTG TCT TCT CTT GC-3′ (sense) and 5′-TGT CCC TGG GTT GAG AGA

*Corresponding author: Fax: (49)-511-5466-132.
E-mail address: c.kutzleb@ipf-pharmaceuticals.de (C. Kutzleb).

[1] Dr. W. Meder and Dr. M. Wendland contributed equally to this work.

Abbreviations: GPCR, G-protein coupled receptor; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; PCR, polymerase chain reaction; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CHO, Chinese hamster ovary

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01312-7

GT-3′ (antisense). The amplified fragment was subcloned into the expression vector pCl and the sequence was verified by DNA sequencing as described previously [16]. This ChemR23 expression vector was transfected into a CHO-Gα(16) expressing cell line (Molecular Devices, Sunnyvale, CA, USA) and cell clones were selected and tested for stable expression of ChemR23 as described [17].

A 537 bp DNA fragment containing the complete open reading frame of human TIG2 was cloned by reverse transcription-PCR from human liver RNA using primer set: 5′-GCC AGG GTG ACA CGG AAG-3′ (sense) and 5′-GAG GCA CCA CGC AGC TC-3′ (antisense) and subcloned into vector pGEM-T. The cDNA encoding the mature form of TIG2 (corresponding to AA 21–154) was amplified from this template and subcloned in frame into a genetically modified YEpFLAG-1 yeast expression vector carrying the α factor secretion signal. The transformation was performed by electroporation of the linearized TIG2 expression cassette into BJ3505. ADH2+ transformants were selected and analyzed for stable integration of TIG2 cDNA into the yeast genome by PCR analysis of genomic DNA from obtained yeast clones. Cultivation and induction of recombinant gene expression was performed according to the protocol by the manufacturer. Daily aliquots of induced ADH2+ transformants were subjected for SDS–PAGE to analyze the protein bands by Coomassie staining. This material was assayed for ChemR23 receptor activation.

2.3. Preparation of human hemofiltrate peptide libraries

8000 l human hemofiltrate were ultrafiltered over a 50 kDa membrane with a filtration buffer containing 0.1 M citrate (pH 3.0) and then prepared as described previously [12]. For bioassay testing, lyophilized aliquots were dissolved in HBSS buffered with 20 mM HEPES (pH 7.4) and transferred into 96-well plates.

2.4. Intracellular Ca^{2+} measurement

Cells were seeded in 96-well black well plates (Costar Corp., Cambridge, USA) at 20 000 cells/well and cultured overnight. After 40 min incubation at 37°C in loading medium (HEPES-buffered HBSS, pH 7.4, containing 2.5 mM probenecid and 2 μM Fluo-4 AM; Molecular Probes, Leiden, The Netherlands); agonist evoked intracellular Ca^{2+} measurements $[Ca^{2+}]_i$ were performed on a Fluorometric Imaging Plate Reader (FLIPR) (see [17]) and calculated as peak fluorescence intensity units (FIU) minus basal FIU. These data were either presented as maximal values or transient elevation.

2.5. Isolation of the ChemR23 ligand from human hemofiltrate

700 mg of lyophilized fractions, corresponding to 7100 l hemofiltrate equivalent, were pooled and purified in six steps.

Step 1 (Fig. 1C): Pooled fractions were dissolved in 1000 ml of 10 mM HCl (solution A), filtrated, loaded onto C18 reverse-phase high performance liquid chromatography (HPLC) column (Bakerbond, J.T. Baker, Philippsburg, USA, 47×300 mm, 15–30 μm). A gradient from 15 to 55% of solvent B (80% acetonitrile/10 mM HCl) at a flow rate of 40 ml/min was applied. Aliquots of ∼0.5% of each fraction (50 ml) were tested for bioactivity.

Step 2 (Fig. 1D): Fractions with bioactivity were pooled and loaded onto a C4 reverse phase HPLC column (Biotek, Oestringen, Germany, 20×250 mm, 5 μm). A gradient of 15–70% solvent B (100% MeOH/10 mM HCl) was applied for 65 min and 6 ml fractions were collected. Aliquots of ∼1% per fraction were tested for activity.

Step 3: The active fractions were pooled, diluted with solvent A (0.1% trifluoroacetic acid (TFA)) and loaded onto a C18 reverse phase HPLC column (Vydac, Hesperia, USA, 20×250 mm, 5 μm) followed by a 34–56% gradient application (solvent B: 0.1% TFA/80% acetonitrile) for 55 min at a flow rate of 7 ml/min. Aliquots of ∼2.5% of respective fractions (3.5 ml) were tested for bioactivity.

Step 4 (Fig. 1E): The active fractions were pooled and loaded onto a C5 reverse phase HPLC (Phenomenex, Torrance, USA, 10×250 mm, 5 μm). A 40–55% gradient (solvent B: 0.1% TFA/80% acetonitrile) was applied for 45 min at a flow rate of 2 ml/min and aliquots of ∼1% per fraction (1 ml) were used for the $[Ca^{2+}]_i$ assay.

Step 5 (Fig. 1F): The active fractions were pooled, diluted 4-fold with solvent A (85% acetonitrile/10 mM NH_4Ac, pH 3.0) and loaded onto a normal phase HPLC (Poly-hydroxyethyl HILIC, Western Analytical Products, Murietta, USA, 4.6×250 mm, 5 μm). A 20–50% gradient (solvent B: 10% aceto-nitrile/10 mM NH_4Ac, pH 3.0) was applied for 60 min at a flow rate of 1 ml/min and aliquots of ∼3% per fraction (1 ml) were used for bioactivity testing.

Step 6: In order to remove salt, the active fractions were pooled, diluted 3-fold with solvent A (0.1% TFA) and loaded onto a C5 reverse phase HPLC (Phenomenex, 4.6×250 mm, 5 μm). A 40–55% gradient (solvent B: 0.1% TFA/80% acetonitrile) was applied for 45 min at a flow rate of 1 ml/min and 500 μl fractions were collected. Approximately 4% of the respective fractions were used for the $[Ca^{2+}]_i$ assay.

2.6. Peptide analysis

Purity of bioactive fractions of the sixth isolation step was confirmed by capillary zone electrophoresis (P/ACE 2000, Beckman, München, Germany) at 220 nm. Molecular weight determination was carried out on a Sciex API III quadrupole mass spectrometer (Perkin-Elmer, Überlingen, Germany). Sequencing was performed on a 473 A gas-phase sequencer (Applied Biosystems, Weiterstadt, Germany). For mass fingerprint analysis, lyophilized activating fractions (peptide content of 0.5–1 mg) were reconstituted in 100 μl of 100 mM Tris/HCl buffer (pH 8.5), and 1 μl of a 1 mg/ml trypsin stock solution (in 1 mM HCl) was added for enzymatic cleavage. The N-terminus (first 33 amino acids) was determined by Edman degradation; whole sequence was confirmed by trypsin digestion after carboxyamidomethylation, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis and subsequent alignment of resulting mass peaks with database entries (NCBI; National Center for Biotechnology Information). In addition, trypsinated samples were loaded onto a C18 reverse phase HPLC column (Reprosil, Dr. Maisch GmbH, Ammerbach-Entringer, Germany, 1×250 mm, 3 μm), a 5–60% gradient (solvent B: 0.06% TFA/80% acetonitrile) was applied and single fractions were analyzed by Edman degradation. Alternatively, trypsinated fractions were loaded onto a nano liquid chromatography column (C18, reverse phase 75 μm diameter), a standard gradient was applied (solvent A: 5% acetonitrile, 0.2% formic acid, 0.005% TFA; solvent B: 90% acetonitrile, 0.2% formic acid, 0.005% TFA) and electrospray ionization (ESI) results were subsequently acquired. Liquid chromatography-mass spectrometry data (LC-MS/MS) were automatically aligned with database entries (SwissProt, NRBD).

3. Results and discussion

The aim of this study was to identify the natural ligand of the ChemR23 receptor. For this purpose, overexpressing cells were screened with a hemofiltrate peptide library. Fractions which specifically activated Gα(16) ChemR23 cells but not CHO wild-type cells, Gα(16) control cells or Gα(16) cells transfected with another receptor were detected in pool 7 (fractions 22–25; Fig. 1A). A clear dose–response relation for the activity is detected (Fig. 1B).

The ChemR23-stimulating activity was purified in six chromatography steps, including five reverse-phase and one normal phase separation (Fig. 1C–F). By means of Edman degradation, MALDI-MS and ESI-MS analysis (Fig. 2), a processed form of TIG2 was identified, previously known only from the corresponding cDNA. In comparison to the human precursor protein, which consists of 163 amino acids, the circulating TIG2 is truncated at the N- and the C-terminus (Fig. 2B). Circulating TIG2 comprises 134 amino acids (AA 21–154, compared with precursor) and has six cysteine residues most likely linked by three disulfide bonds. The molecular weight is 15 566 Da. The isoelectric point is 8.59 and the charge at pH 7.0 is 3.17 according to ProtParam analysis (http://us.expasy.org/tools/prot-param.html). To confirm that TIG2 is the ligand for ChemR23 receptor we tested the recombinant TIG2 expressed in yeast cells transfected with a DNA-construct leading to the secretion of TIG2 (AA 21–154). This expression of the processed TIG2 resulted in ma-

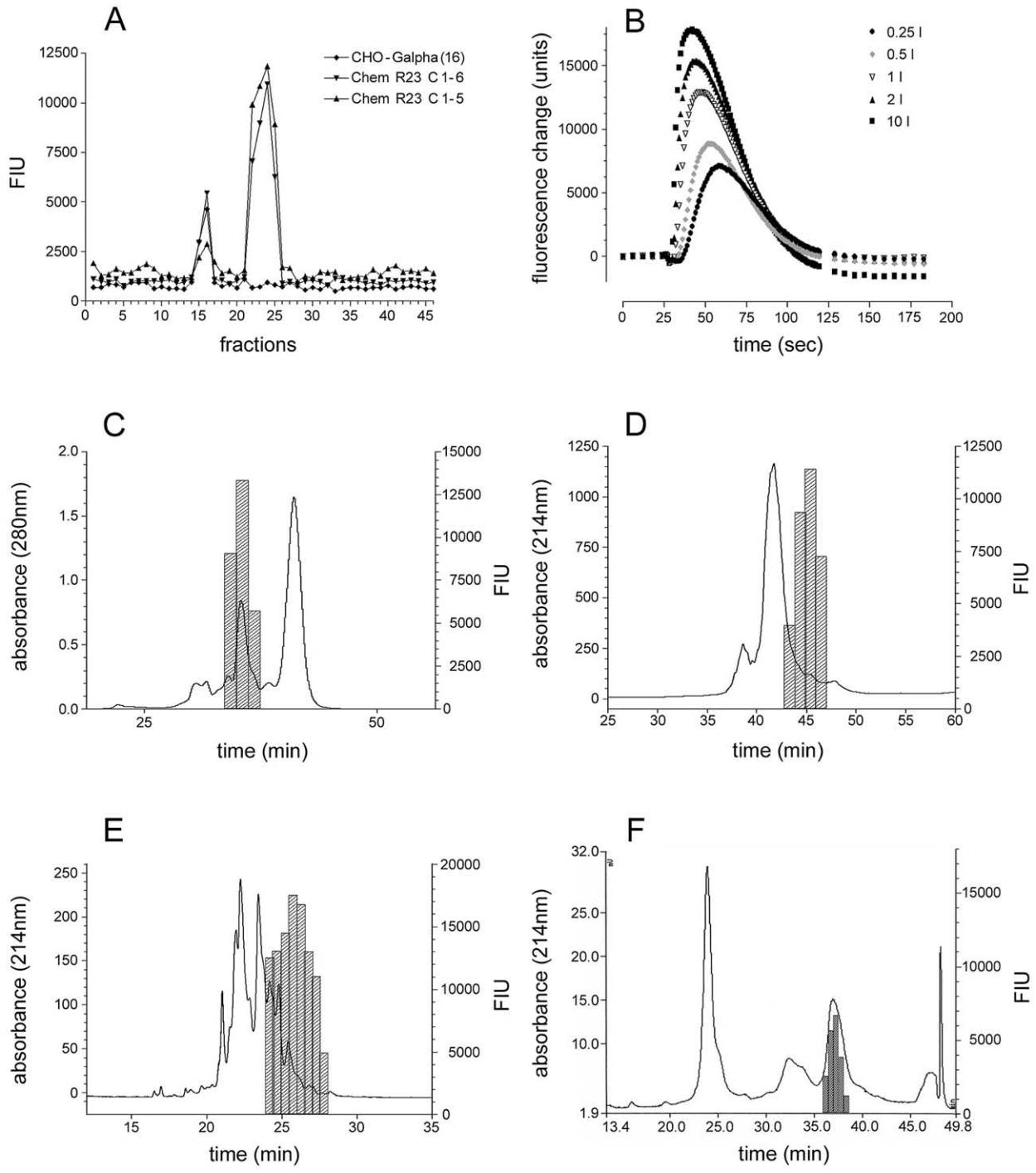

Fig. 1. Isolation of TIG2 from human hemofiltrate. A: Activity profile of hemofiltrate pH pool 7 in CHO-Gα(16) cells overexpressing the ChemR23 receptor presented as maximal FIU of $[Ca^{2+}]_i$ changes. Fractions 22–25 specifically activate ChemR23-cells (clone 1-5 and 1-6), but not CHO-Gα(16) control cells. Non-specific activity can be observed in fractions 15 and 16. B: Concentration-dependent activity in hemofiltrate fraction 24 in the range of 0.25–10 l hemofiltrate equivalents. Transient elevation of $[Ca^{2+}]_i$ are presented as fluorescence change. One typical experiment out of 4 replicates is shown. C–F: Chromatographic purification of TIG2. Elution profile and activating fractions as maximal FIU of $[Ca^{2+}]_i$ changes are included in the respective chromatograms; (C) step 1; C18 reverse-phase HPLC (Bakerbond, 47×300 mm), (D) step 2; C4 reverse-phase HPLC (Biotek, 20×250 mm), (E) step 4; C5 reverse-phase HPLC (Phenomenex, 10×250 mm) and (F) step 5; normal phase HPLC (Poly-Hydroxyethyl HILIC, 4.6×250 mm).

terial showing full bioactivity compared to the isolated truncated TIG2 of hemofiltrate (Fig. 3).

The discovery of this receptor–ligand pair allows some conclusions concerning the function of ChemR23 activation by TIG2. TIG2 is not only expressed in skin, but also in pancreas, liver, spleen, prostate, ovary, small intestine, and colon [9]. In situ hybridization experiments show a high expression of TIG2 in the non-lesional epidermis of psoriatic patients and a lower expression in psoriatic lesional skin. In contrast, TIG2 is strongly induced in psoriatic lesions after treatment with tazarotene [9]. Taken together, the high expression of TIG2 message in uninvolved relative to lesional skin shows

its role in maintaining the normal physiology of skin. The TIG2 protein constitutes two motifs for casein kinase II phosphorylation (AA 50–53, 54–57), for myristylation (AA 16–21, 29–34), and a potential site for protein kinase C phosphorylation (AA 76–78) according to PROSITE database analysis. However, our mass data indicate that such processing is not required for bioactivity. ChemR23 is expressed in antigen-presenting cells, macrophages and dendritic cells [1]. Due to its expression pattern and due to the observation that ChemR23 is used as a co-receptor by several SIV strains and a primary HIV-1 strain [1], this receptor is suggested to have functional meaning in inflammation and immunodeficiency virus infection. The mouse counterpart of the receptor is expressed in developing osseous and cartilaginous tissue and in adult parathyroid glands and may therefore be involved in bone metabolism [2].

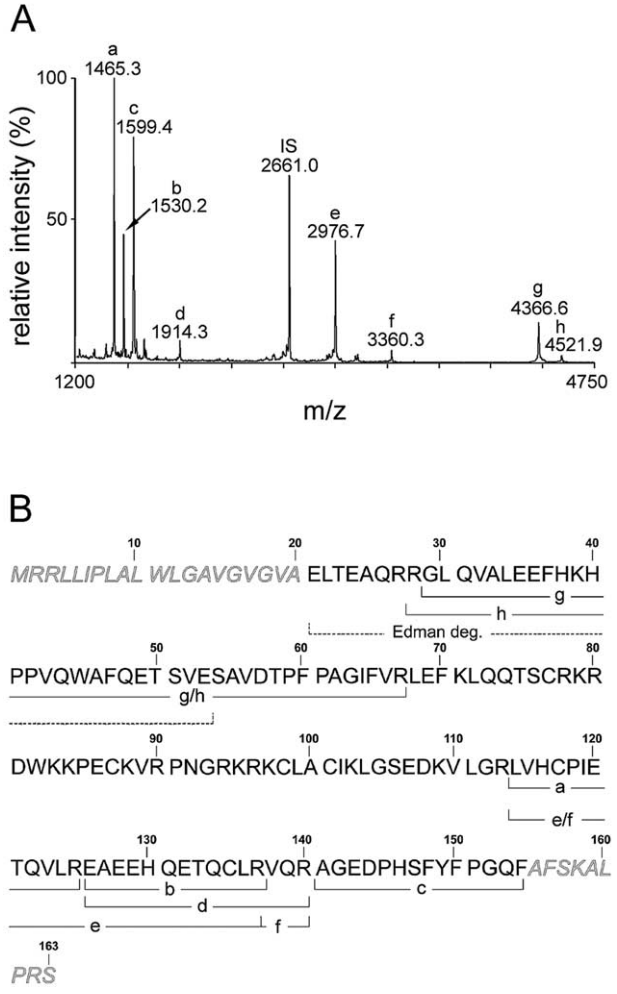

Fig. 2. Identification of TIG2 by peptide mass fingerprint (PMF) and Edman degradation. A: PMF after tryptic cleavage. The tryptic incubation mixture was desalted and measured by MALDI-TOF-MS using an internal standard (IS) for improved mass accuracy. Labeled signals are assigned to peptidic cleavage products after carboxyamidomethylation as shown in panel B. B: Amino acid sequence of human TIG2. The active circulating metabolite acting as ligand of ChemR23 is displayed in bold letters. It constitutes 134 amino acids and is truncated both at the N- and C-terminus (AA 21–154) compared with the precursor. The labeled lines (a–h) represent the peptidic fragments as shown in the PMF of TIG2 after tryptic cleavage (A). The dotted line depicts the N-terminus identified by Edman degradation.

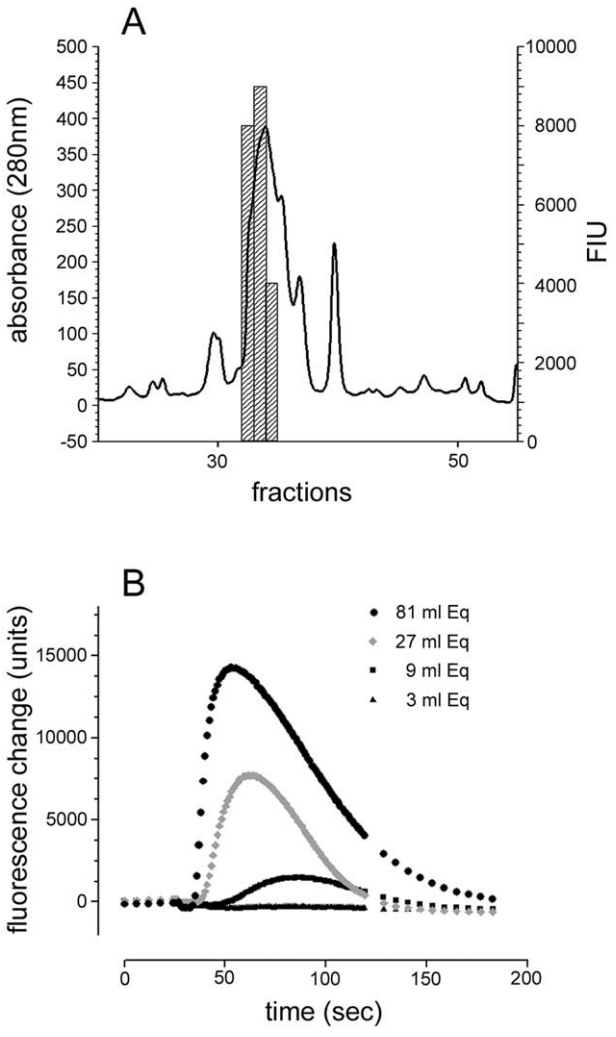

Fig. 3. Specificity of the recombinant TIG2 shown by ChemR23 activation in the FLIPR assay. A: Activity profile of fractionated supernatants from a TIG2 overexpressing yeast clone, presented as maximal FIU of $[Ca^{2+}]_i$ changes. Fractions 32 to 34 specifically activate ChemR23, but not CHO-Gα(16) control cells. B: Concentration-dependent activation of ChemR23 in the range of 9–81 ml of yeast culture supernatant. Transient elevations of $[Ca^{2+}]_i$ are presented as fluorescence change. One typical experiment out of four replicates is shown.

Human hemofiltrate is a rich source for circulating peptides [11] which are processed from precursor molecules [12–15]. After initial isolation of human CC chemokine (HCC)-1 comprising 74 amino acids [18], an HCC-1 (9–74) variant, lacking the first eight amino acids, was later found in hemofiltrate. The N-terminal truncated variant was shown to be a potent agonist of the chemokine receptors CCR1, CCR3 and CCR5 [14]. Thus, HCC-1 represents a precursor that can be rapidly transferred into the active chemokine by proteolytic processing.

In conclusion, we have identified TIG2 as a natural ligand of the orphan G protein-coupled receptor, ChemR23. The human circulating molecular form consists of 134 amino acids and is truncated at the N- and C-terminus compared with the precursor. Signal transduction due to activation of ChemR23 by TIG2 indicates a role in bone metabolism, in the maintenance of the normal physiology of the skin and/or in inflammatory processes.

After submission of our manuscript, the isolation of TIG2 as ligand for the ChemR23 receptor was also described by another group [19]. However, the molecular form purified from ovarial carcinoma fluid differs in the C-terminal cleavage.

Acknowledgements: We thank Ute Block, Stefanie Schulz, Ina Trotz, and Ilka Uhrlandt for their excellent technical assistance.

References

[1] Samson, M., Edinger, A.L., Stordeur, P., Rucker, J., Verhasselt, V., Sharron, M., Govaerts, C., Mollereau, C., Vassart, G., Doms, R.W. and Parmentier, M. (1998) Eur. J. Immunol. 28, 1689–1700.
[2] Methner, A., Hermey, G., Schinke, B. and Hermans-Borgmeyer, I. (1997) Biochem. Biophys. Res. Comm. 233, 336–342.
[3] Nagpal, S. and Chandraratna, R.A. (1996) Curr. Pharm. Des. 2, 295–316.
[4] Chambon, P. (1994) Semin. Cell. Biol. 5, 115–125.
[5] Boehm, M.F., Heyman, R.A., Patel, S., Stein, R.B. and Nagpal, S. (1995) Exp. Opin. Invest. Drugs 4, 593–612.
[6] Nagpal, S., Friant, S., Nakshatri, H. and Chambon, P. (1993) EMBO J. 12, 2349–2360.
[7] Peck, G.J. and DiGiovanna, J.J. (1994) in: The Retinoids: Biology, Chemistry, and Medicine (Sporn, M.B., Roberts, A.B. and Goodman, D.S., Eds.), pp. 631–638, Raven Press, New York.
[8] Duvic, M., Nagpal, S., Asano, A.T. and Chandraratna, R.A. (1997) J. Am. Acad. Dermatol. 37, 18–24.
[9] Adams, A.E., Abu-Amer, Y., Chappel, J., Stueckle, S., Ross, F.P., Teitelbaum, S.L. and Suva, L.J. (1999) J. Cell Biochem. 15, 587–595.
[10] Nagpal, S., Patel, S., Jacobe, H., DiSepio, D., Ghosn, C., Malhotra, M., Teng, M., Duvic, M. and Chandraratna, R.A. (1997) J. Invest. Dermatol. 109, 91–95.
[11] Forssmann, W.G., Schulz-Knappe, P., Meyer, M., Adermann, K., Forssmann, K., Hock, D. and Aoki, A. (1992) In: Characterization of Natural Posttranslationally Processed Peptides from Human Blood: A New Tool in the Systematic Investigation of Native Peptides (Yanaihara, N., Ed.), pp. 553–557, Escom, Leiden.
[12] Schulz-Knappe, P., Schrader, M., Ständker, L., Richter, R., Hess, R., Jürgens, M. and Forssmann, W.G. (1997) J. Chromatogr. A 776, 125–132.
[13] Richter, R., Schulz-Knappe, P., Schrader, M., Ständker, L., Jürgens, M., Tammen, H. and Forssmann, W.G. (1999) J. Chromatogr. B 726, 25–35.
[14] Detheux, M., Ständker, L., Vakili, J., Münch, J., Forssmann, U., Adermann, K., Pöhlmann, S., Vassart, G., Kirchhoff, F., Parmentier, M. and Forssmann, W.G. (2000) J. Exp. Med. 192, 1501–1508.
[15] Hock, D., Magerlein, M., Heine, G., Ochlich, P.P. and Forssmann, W.G. (1997) FEBS Lett. 400, 221–225.
[16] Mägert, H.J., Reinecke, M., David, I., Raab, H.R., Adermann, K., Zucht, H.D., Hill, O., Hess, R. and Forssmann, W.G. (1998) Regul. Pept. 73, 165–176.
[17] Lammerich, H.P., Busmann, A., Kutzleb, C., Wendland, M., Seiler, P., Berger, C., Eickelmann, P., Meyer, M., Forssmann, W.G. and Maronde, E. (2003) Br. J. Pharmacol. 138, 1431–1440.
[18] Schulz-Knappe, P., Mägert, H.J., Dewald, B., Meyer, M., Cetin, Y., Kubbies, M., Tomeczkowski, J., Kirchhoff, K., Raida, M., Adermann, K. and Forssmann, W.G. (1996) J. Exp. Med. 183, 295–299.
[19] Wittamer, V., Franssen, J.D., Vulcano, M., Mirjolet, J.F., Le Poul, E., Migeotte, I., Brezillon, S., Tyldesley, R., Blanpain, C., Detheux, M., Mantovani, A., Sozzani, S., Vassart, G., Parmentier, M. and Communi, D. (2003) J. Exp. Med. 198, 977–985.