ELSEVIER

FEBS Letters 342 (1994) 214–216

FEBS 13871

FEBS LETTERS

An isoform of the human calcitonin receptor is expressed in TT cells and in medullary carcinoma of the thyroid

J.L. Frendo, F. Pichaud, R. DeLage Mourroux, Z. Bouizar, N. Segond, M.S. Moukhtar, A. Jullienne*

INSERM U349, Centre Viggo Petersen, 6 rue Guy Patin, 75010 Paris, France

Received 14 February 1994

Abstract

We amplified, using the polymerase chain reaction and calcitonin receptor (CTR) specific primers, RNA extracted from medullary thyroid carcinoma (MTC) and the derived TT cell line. Both secrete large amounts of calcitonin. Electrophoresis of amplification products revealed, in both cases, an ethidium bromide-stained band that hybridized to a CTR probe. Sequencing the band amplified from TT cells revealed an open reading frame identical to the sequence of H-CTR but lacking 16 amino acids in the first intracellular loop. This demonstrates the existence of an mRNA coding for a subtype of H-CTR which is expressed in TT cells and MTC.

Key words: RNA; Expression; Truncated; Autocrine; Paracrine; Polymerase chain reaction

1. Introduction

Calcitonin, a hypocalcemic hypophosphatemic hormone [1,2], acts through specific receptors in kidney and bone [3]. Calcitonin receptors, characterized by specific binding of the hormone or activation of adenylate cyclase, are detected in several normal and malignant tissues and cell lines. The presence of putative calcitonin receptors on C cells has been suggested as exogenous calcitonin inhibits its own secretion by normal [4] or malignant C cells [5]. The sequence of the calcitonin receptor is now established in pig [6], human [7] and rat [8]. We have searched, using the polymerase chain reaction [9] and specific primers, for the presence of this receptor in the TT cells [10]. This cell line is derived from human medullary thyroid carcinoma (MTC). We report here that an isoform of the calcitonin receptor is expressed in TT cells. We also detected the presence of this receptor in tissues removed at surgery from two MTC cases. Thus an autocrine process of regulation could be implicated in the secretion of calcitonin by the malignant cells in vitro or in vivo.

*Corresponding author. Fax: (33) 49 95 84 52.

Abbreviations: CTR, calcitonin receptor; H-CTR, human calcitonin receptor; P-CTR, pig calcitonin receptor; R-CTR, rat calcitonin receptor; MTC, medullary thyroid carcinoma; SDS, sodium dodecyl sulfate; SSC, sodium saline citrate; RT-PCR, reverse transcriptase polymerase chain reaction.

2. Materials and methods

2.1. Cell culture

TT cells were cultured in RPMI, 10 mM HEPES, 6 mM glutamine, supplemented with 10% fetal calf serum. The medium was changed every two days and the cells were harvested at confluence.

2.2. RNA extraction

Total RNA was extracted from TT cells or from MTC tissues by a guanidine thiocyanate chloride method [11]. All extracts were immediately frozen at −80°C or stored at −20°C in 75% ethanol until used.

2.3. RT-polymerase chain reaction

cDNA was synthesized from 5 μg of total RNA. The reaction mixture had a final volume of 20 μl, and contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, nuclease-free BSA (1 mg/ml), 20 U of RNAsin, 200 U of reverse transcriptase superscript (BRL), 1 mM of each dNTP and 50 pmol of a 3′ oligo-dT primer. Annealing was performed during 10 min at 23°C and primer extension during one hour at 37°C. The reaction mixture was then completed to 95 μl with Taq polymerase buffer containing 2 units of Taq polymerase (Eurobio). After heating to 90°C (hot-start), 50 pmol of each specific primer in 5 μl were added. Amplification was performed during 30 cycles: 1 min at 95°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (extension).

Oligonucleotide primers spanning the entire coding sequence of the human calcitonin receptor were used (Fig. 1). RNA samples pretreated with RNAase A were also amplified as controls.

2.4. Analysis of PCR products

Amplified products were analyzed by electrophoresis on 1.2% agarose gels, visualized by ethidium bromide, transferred to membranes (GeneScreen NEN). The amplified cDNA was hybridized with a CTR specific cDNA probe radiolabelled with ^{32}P using a random priming method (specific activities $\geq 10^8$ cpm/μg DNA were obtained). After prehybridization for 4 h at 42°C in 50% deionized formamide, 1% SDS, 2 × SSC, 10% dextran sulfate, the membranes were hybridized at 42°C in the same buffer containing the specific CTR cDNA probe and were washed at 55°C, in 2 × SSC for 10 min, twice 2 × SSC containing 0.1% SDS for 20 min, and twice 1 × SSC containing 0,1% SDS for 20 min. Autoradiography was performed at −80°C with intensifying screens overnight.

0014-5793/94/$7.00 © 1994 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(94)00256-U

2.5. Cloning and DNA sequencing

After precipitation of PCR products, cDNA fragments were cloned into plasmid pCRII using the TA cloning kit (Invitrogen, Inc). Positive clones were selected with a radiolabelled CTR specific probe. Both strands of cDNA fragments were sequenced with the ds cDNA cycle sequencing system kit (Gibco BRL) using M13 reverse and M13–40 primers labeled with [γ-^{33}P]ATP and Taq DNA polymerase. Amplification was carried out for 20 cycles (30 s at 95°C, 30 s at 55°C, 1 min at 70°C) followed by 10 cycles (30 s at 95°C, 1 min at 70°C).

3. Results

Fig. 1 shows the three sets of primers (upper panel) spanning the coding region of the calcitonin receptor (lower panel) we used. When RNA extracted from TT cells was amplified using these primers, agarose gel electrophoresis of the amplified products revealed the presence of a single ethidium-stained band with each set of primers used. These bands were not detected if the samples were pretreated with RNase A. However the band, amplified with primers (II) spanning transmembrane regions I and II was shorter (529 bp) than the awaited size (577 bp) by approximately 50 bases (Fig. 2a). All the bands obtained with the other two sets of primers had the expected length (results not shown). After transfer of the amplified products to a GeneScreen membrane and hybridization with a specific calcitonin receptor cDNA probe, a strong hybridization signal was consistently observed (Fig. 2b). Sequencing of the products amplified with the three sets of primers allowed us to deduce the complete coding sequence of the molecular species present in the TT cells. This sequence was identical to the one reported by Gorn et al. [7], with the exception of 16 amino acids which were missing between transmembrane domains I and II (Fig. 3) and the substitution of a serine for lysine at position 175 of the amino acid sequence. A strong signal was also observed when RNA extracted from two cases of MTC was reverse-transcribed, amplified, transferred and hybridized to the calcitonin receptor probe (Fig. 4). No difference in size between the amplification products of MTC and TT cells was observed with primers II and III (results not shown).

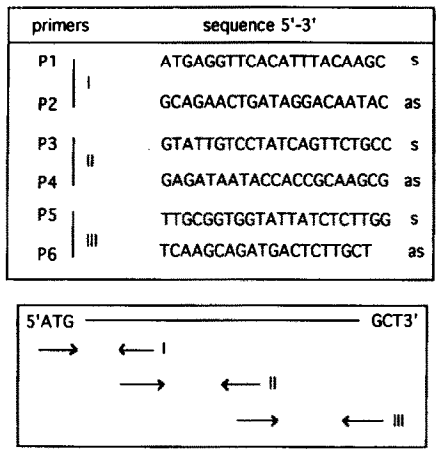


Fig. 1. Upper panel: sequence of primers used to amplify the H-CTR coding sequence. Lower panel: sequencing strategy. Arrows represent the position of the set of primers used and the direction of amplification and sequencing.

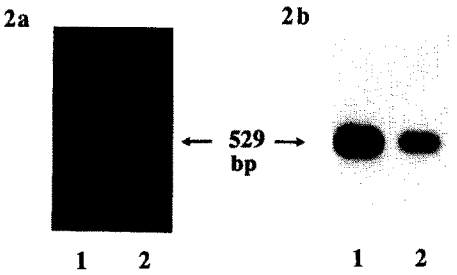


Fig. 2. (a) Agarose gel electrophoresis of amplification products (using primer set II) of RNA extracted from TT cells stained with ethidium bromide. Lanes (1,2) Amplification products from two different pools of TT cells harvested at confluence. (b) Autoradiography of the Southern blot of the above amplification products hybridized to a ^{32}P-labelled CTR probe.

4. Discussion

The calcitonin receptor [6] is a seven transmembrane receptor coupled to adenylate cyclase pathway and belonging to a new family of transmembrane receptors which includes the parathyroid [12] and secretin [13] receptors. In the species so far studied a single form is reported in human and pig while two isoforms exist in rat. The human receptor differs from the other known receptors (porcine, murine) by the existence of an insert of 16 amino acids in the intracellular loop separating the first and second transmembrane domains.

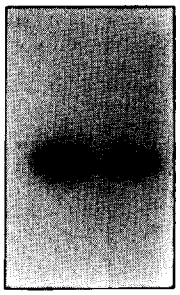
Our results clearly demonstrate that a mRNA expressing a calcitonin like receptor molecule is present in the TT cells. This messenger is functional as we used an oligo-dT primer and reverse transcriptase to obtain the first strand before amplification. The sequence of this

```
Frag. H-CTRb   HPENNRTWSN YTMCNAFTPE KLKNAYVLYY LAIVGHSLSI FTLVISLGXF
Frag. H-CTRa   HPENNRTWSN YTMCNAFTPE KLKNAYVLYY LAIVGHSLSI FTLVISLGIF
Frag. R-CTRa,b HPDSNRTWSN YTLCNAFTPD KLHNAYVSYY LALVGHSMSI AALIASMGIF
Frag. P-CTR    HPESNISWSN YTMCNAFTPD KLQNAYILYY LAIVGHSLSI LTLLISLGIF

Frag. H-CTRb   VFFRSL---- ---------- --GCQRVTLH KNMFLTYILN SMIIIXHLVE
Frag. H-CTRa   VFFRKLTTIF PLNWKYRKAL SLGCQRVTLH KNMFLTYILN SMIIIIHLVE
Frag. R-CTRa,b LFFKNL---- ---------- --SCQRVTLH KNMFLTYILN SIIIIIHLVE
Frag. P-CTR    MFLRSI---- ---------- --SCQRVTLH KNMFLTYVLN SIIIIVHLVV

Frag. H-CTRb   VVPNGELVRR DP
Frag. H-CTRa   VVPNGELVRR DP
Frag. R-CTRa,b VVPNGDLVRR DP
Frag. P-CTR    IVPNGELVKR DP
```

Fig. 3. Deduced amino acid sequence of the first two transmembrane domains of the isoform present in TT cells compared to the corresponding regions of the human, rat and porcine receptors. Amino acid identities in the four sequences are shown by a vertical line (|).



1 2

Fig. 4. Autoradiography of a Southern blot of amplification products (using primer set I) of RNA extracted from TT cells (lane 1) and MTC (lane 2) hybridized to a ^{32}P-labelled CTR probe.

isoform is identical to that reported for human calcitonin with the exception of a gap of 48 nucleotides similar to the gap found in the messengers coding for the porcine receptor and in the two isoforms of the murine receptor. Thus an isoform of the human receptor (H-CTRb) exists which has a shorter intracellular loop (13 amino acids) than the form (H-CTRa) reported by Gorn et al. (29 amino acids). We also detected the presence of this messenger in the tissues of patients suffering from medullary carcinoma of the thyroid. In the two cases, in which we have detected the receptor mRNA, the species present corresponded in size to that detected in TT cells and thus could express a receptor molecule with a truncated intracellular loop.

The expression of the calcitonin receptor gene in C cells could explain the results reported previously on the inhibition of calcitonin secretion by the addition of exogenous hormone in both normal [4] and tumoral C cells [5]. This inhibition could be due to the hormone acting through the putative receptor we report here. In this case an autocrine or paracrine system regulating calcitonin secretion could exist in tumoral C cells.

Further studies are necessary to establish if this subtype of the human receptor is restricted to tumoral C cells or is expressed in normal tissues and if it is functional. The presence of this receptor in medullary carcinoma tissue raises the question of the potential role of this receptor during oncogenesis and its role as a tumoral marker.

Acknowledgements: We thank Dr. F. Lasmoles for constant advice and help. Dr. R. Cohen kindly provided us with the medullary thyroid carcinoma RNA samples. J.-L. Frendo and F. Pichaud are the recipients of a grant from the French Ministry of Research and Education.

References

[1] Hirsch, P.F., Gauthier, G.F. and Munson, P.L. (1963) Endocrinology 73, 244–252.
[2] Copp, D.H., Cameron, E.C., Cheney, B.A., Davidson, A.G.T. and Henke, K.G. (1962) Endocrinology 70, 638–649.
[3] Marx, S.J., Woodward, C.J. and Aurbach, G.D. (1972) Science 178, 999–1001.
[4] Morimoto, S., Birge, S.J., Fausta, A. and Avioli, L.V. (1984) Endocrinology 115, 60–64.
[5] Morimoto, S., Miyauchi, A. and Fukuo, K. (1986) Clin. Sci. 70, 333–338.
[6] Lin, H.Y., Harris, L., Flannery, M.S., Aruffo, A., Kaji, E.H., Gorn, A., Kolakowski, L.F., Lodish, H.F. and Goldring, S.R. (1991) Science 254, 1022–1024.
[7] Gorn, A.H., Lin, H.Y., Yamin, M., Auron, P.E., Flannery, M.R., Tapp, D.R., Manning, C.A., Lodish, H.F., Krane, S.M. and Goldring, S.R. (1992) J. Clin. Invest. 90, 1726–1735.
[8] Sexton, P.M., Houssami, S., Hilton, J.M., O'Keeffe, L.M., Center, R. J., Gillespie, M. T., Darcy, P. and Findlay, D.M. (1993) Mol. Endocrinol. 7, 815–821.
[9] Saiki, R., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H. and Arnheim, N. (1985) Science 230, 1350–1354.
[10] Leong, S.S, Horoszewicz, J.S, Shimoaka, K., Friedmann, M., Kawinski, E., Song, M.J., Ziegel, R., Chu, T.M., Baylin, S. and Mirand, E.A. (1981) in: Advances in Thyroid Neoplasia, pp. 95–108 (M. Andreoli, F. Monaco and J. Robbins, Eds.) Rome, Field Educational Italia.
[11] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[12] Jüppner, H., Abou-Samra, A.B., Freeman, M., Kong, X.F., Shipani, E., Richards, J., Kolakowski, L.F., Hock, J., Potts, J.T., Kronenberg, H.M. and Segre, G.V. (1991) Science 254, 1024–1026.
[13] Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, K., Takahashi, T. and Nagata, S. (1991) EMBO J. 10, 1635–1641.