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The mouse chemerin receptor gene, m*cmklr1*, utilizes alternative promoters for transcription and is regulated by all-*trans* retinoic acid

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Abstract

CMKLR1 (chemoattractant-like receptor 1) is a G-protein-coupled receptor implicated in cartilage and bone development and is expressed in organs like the parathyroid gland, brain, and lung. The receptor is also expressed in dendritic cells and in macrophages where it acts as a co-receptor for entry of HIV/SIV isolates into human $CD4^+$ cells. Recently, a protein named "chemerin" (also known as TIG2) was isolated from human inflammatory fluids and hemofiltrate and found to be the endogenous ligand for CMKLR1. We have previously described the genomic organization of the *cmklr1* gene and characterized its promoter in mouse neuroblastoma NB4 1A3 cells. In the present study we identify a second transcript, *cmklr1*b, in mouse microglia BV2 cells. *Cmklr1*b is transcribed from an alternative promoter with a transcription start site located 6780 bp downstream of the previously identified exon 1 (*cmklr1*a). The *cmklr1*b promoter lacks a TATA box but contains two CCAAT boxes in opposite directions. 5′ Deletion analysis of the promoter region in BV2 cells using a luciferase reporter gene assay indicates two regions, between 623–755 bp and 56–125 bp upstream of transcription start site, to be important for promoter function. The proximal promoter region includes both CCAAT boxes, and site-directed mutagenesis separately within these elements revealed that only the forward CCAAT element was important for transcription. Although the forward CCAAT element is essential for transcription electrophoretic mobility shift and super-shift assays demonstrated that both CCAAT elements actually bind nuclear proteins from BV2 cells and identified the binding factor as NFY. Real-time reverse transcriptase-PCR experiments of *cmklr1*b expression in all-*trans* retinoic acid (ATRA)- stimulated BV2 cells showed strong up-regulation of receptor transcript. Luciferase reporter gene assay of the promoter in ATRA-stimulated BV2 cells confirmed that transcriptional activity of the *cmklr1*b promoter is increased by ATRA. However, deletion analysis could not identify an ATRA-responsive element within the promoter region suggesting that gene activation is likely to occur through alternative mechanisms. The results emphasise a possible role of *cmklr1* in bone modelling.

Keywords: G-protein-coupled receptor; Transcriptional regulation; All-*trans* retinoic acid; Luciferase reporter gene; CCAAT element

1. Introduction

The human chemoattractant-like receptor 1 (hCMKLR1) was first described as a functionally unknown (orphan) G-protein-coupled receptor (GPCR; Gantz et al., 1996). The receptor, also named ChemR23, was shown to be expressed in dendritic cells and macrophages (Samson et al., 1998). It was found to function as co-receptor for select isolates of simian immunodeficiency virus (SIV) and human immunodeficiency virus-1 (HIV-1; Samson et al., 1998) facilitating viral entry into $CD4^+$ cells, thereby assisting the major co-receptors, CCR5 and CXCR4. Recently, Wittamer et al.

Abbreviations: CMKLR1, chemoattractant-like receptor 1; GPCR, G-protein-coupled receptor; EMSA, electrophoretic mobility shift assay; AP-1, activator protein 1; AP-4, activator protein 4; GATA-1/-2/-3, GATA-binding factor -1, -2 or -3; CREB, cAMP-responsive element binding protein; PMA, phorbol 12-mysitate 13-acetat; LPS, lipopolysaccharide; cDNA, complementary DNA; RA, retinoic acid; RARE, RA-responsive element; RAR, retinoic acid receptor; ATRA, all-*trans* retinoic acid; PCR, polymerase chain reaction; RT, reverse transcription; TBE, Tris borate/EDTA; bp, base pair(s); kb, kilobase(s).

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(2003) showed that a (chemotactic) protein isolated from human inflammatory fluids could activate CMKLR1 (Wittamer et al., 2003), and at the same year, Meder et al. (2003) isolated the ligand from a hemofiltrate (Meder et al., 2003). The chemotactic protein, named "chemerin" or "TIG2", is secreted as a precursor, "pro-chemerin," which upon proteolytic cleavage, removing six to nine amino acids in the COOH-terminal, becomes able to activate CMKLR1. Although CMKLR1 thus seems to be identical to the chemerin/TIG2 receptor, its ultimate naming awaits to be established. TIG2 ("tazarotene-induced gene 2") has earlier been described to be implicated in psoriasis and has been reported to be up-regulated by the synthetic RA analogue, tazarotene (Nagpal et al., 1997). The ligand also seems to play an important role in chemotaxis of immature dendritic cells and macrophages (Wittamer et al., 2003) as well as in the mechanisms of bone modelling (Adams et al., 1999).

The expression of the mouse orthologue mCMKLR1, designated DEZ by Methner et al. (1997), was shown to be differentially regulated during embryonic development, displaying high expression in cartilage and bone, whereas the main expression in the adult mouse was seen in the parathyroid glands, lung, and brain (Methner et al., 1997). In order to clarify the molecular mechanisms underlying the mCMKLR1 expression we previously isolated the m*cmklr1* gene, mapped its genomic structure, and characterized its promoter (Mårtensson et al., 2004). In this study we present a new splicing variant of m*cmklr1*, m*cmklr1*b, which has an identical coding sequence compared to *cmklr1*a but differs in its non-coding region and is transcribed by an alternative promoter. In view of the effect of tazarotene, a RAR-specific retinoid (Chandraratna, 1996), on chemerin/TIG2 ligand expression (Nagpal et al., 1997), we also investigated whether the m*cmklr1* gene is similarly regulated. This was performed by studying the effects of the RAR-specific activator, ATRA, on m*cmklr1* gene function.

2. Materials and methods

2.1. Cell culture

The mouse microglia cell line, BV2, was kindly provided by Prof. Adriano Fontana (Department of Internal Medicine, University Hospital Zürich, Switzerland) and the mouse embryonic fibroblast cell line, 3T3 clone A31, was obtained from ECACC (no. 86110401). Both cell lines were grown in Dulbecco's modified Eagle's medium with Glutamax I (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were incubated at 37 °C in 7% CO_2.

2.2. Northern blot analysis

Northern blot analysis was performed on mRNA from the mouse cell lines BV2, NB4 1A3, and 3T3 clone A31. Total RNA was isolated from the cells by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987), mRNA was selected using a commercial kit (Amersham Biosciences), and 6 μg was used for the blot. Northern blotting was also performed on mRNA isolated from BV2 cells treated for 20 h with different concentrations of ATRA, 100 nM PMA, and 500 ng/ml LPS all from Sigma. Total RNA and mRNA were isolated as above and 4 μg was used for the blots. A 1.2 kb *Eco*RI/*Bam*HI-fragment containing the complete coding region of m*cmklr1* was radioactively labelled with [α ^{32}P] dCTP (NEN) using the Megaprime DNA labelling kit (Amersham Biosciences). The blots were hybridized and washed according to standard procedures (Sambrook et al., 1989) and exposed to X-ray film (KODAK) overnight at −70 °C.

2.2.1. RNA isolation and RT reaction for real-time PCR

BV2 cells (1.2×10^5/well), NB4 1A3 cells (2.3×10^5/well), and 3T3 clone A31 cells (1.4×10^5/well) were seeded into 24-well plates. The day after, 5 μM ATRA was added to some wells and the plates were incubated for 4 h and 16 h, respectively. Cells were lysed in 200 μl lysis buffer (100 mM Tris–HCl pH 8, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, and 5 mM DTT in each well) and the cell lysate was further processed with a QIAshredder (Qiagen) to reduce viscosity. PolyA$^+$ RNA was captured from the cell lysate with 50 μg Seramag Oligo dT_{14} paramagnetic beads (Serva). The beads were washed twice in 200 μl washing buffer I (100 mM Tris–HCl pH 8, 150 mM LiCl, 1 mM EDTA, and 0.1% lithium dodecyl sulfate) and once in 200 μl washing buffer II (100 mM Tris–HCl pH 8, 150 mM LiCl, and 1 mM EDTA). PolyA$^+$ RNA was eluted in 10 μl water, and first-strand synthesis was carried out by RT for 50 min at 50°C in a final volume of 20 μl containing 20 mM Tris–HCl pH 8.4, 50 mM KCl, 10 mM DTT, 5 mM $MgCl_2$, 0.5 mM dNTP, 0.5 μg Oligo $(dT)_{12–18}$, 30 units RNaseOUT (Invitrogen), and 50 units Superscript III RT (Invitrogen). To generate first strand cDNA from mouse tissues (skeletal muscle, spleen, brain, kidney, liver, lung, and heart), total RNA was isolated as described in Section 2.2. Approximately 10 μg was mixed with 100 μg Seramag Oligo dT_{14} paramagnetic beads in 100 mM Tris–HCl pH 8, 500 mM LiCl, 1 mM EDTA, and subsequent PolyA$^+$ RNA isolation and first strand synthesis was carried out as described above for cell culture-derived samples.

2.2.2. Real-time PCR

Real-time quantitative PCR was performed in a Light-Cycler system (Roche) using the Sybr Green I detection method. The reactions were performed in a total volume of 10 μl containing 2 μl of diluted (1:20) cDNA or external PCR standard, 50 mM Tris-HCl pH 8.3, 10 mM KCl, 50 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μg/μl BSA, 1:30 000 dilution of SYBR Green I, 0.5 μM of each primer (Table 1), and 0.5 units FastStart Taq DNA polymerase (Roche). Following denaturation at 95 °C for 10