Molecular Immunology 62 (2014) 1–9

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Identification of two regulatory elements controlling *Fucosyltransferase 7* transcription in murine CD4$^+$ T cells

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ARTICLE INFO

Article history:
Received 4 March 2014
Received in revised form 7 May 2014
Accepted 9 May 2014

Keywords:
T cell
Migration
Fucosyltransferase
Gene regulation
Immunology

ABSTRACT

Fucosyltransferase VII encoded by the gene *Fut7* is essential in CD4$^+$ T cells for the generation of E- and P-selectin ligands (E- and P-lig) which facilitate recruitment of lymphocytes into inflamed tissues and into the skin.

This study aimed to identify regulatory elements controlling the inducible *Fut7* expression in CD4$^+$ T cells that occurs upon activation and differentiation of naive T cells into effector cells.

Comparative analysis of the histone modification pattern in non-hematopoetic cells and CD4$^+$ T cell subsets revealed a differential histone modification pattern within the *Fut7* locus including a conserved non-coding sequence (CNS) identified by cross-species conservation comparison suggesting that regulatory elements are confined to this region.

Cloning of the CNS located about 500 bp upstream of the *Fut7* locus, into a luciferase reporter vector elicited reporter activity after transfection of the αβ-WT T cell line, but not after transfection of primary murine CD4$^+$ Th1 cells. As quantification of different *Fut7* transcripts revealed a predominance of transcripts lacking the first exons in primary Th1 cells we searched for an alternative promoter. Cloning of an intragenic region spanning a 1 kb region upstream of exon 4 into an enhancer-containing vector indeed elicited promoter activity. Interestingly, also the CNS enhanced activity of this intragenic minimal promoter in reporter assays in primary Th1 cells suggesting that both elements interact in primary CD4$^+$ T cells to induce *Fut7* transcription.

1. Introduction

E- and P-lig mediate rolling of leukocytes on inflamed vessels and by that promote the entry of leukocytes into inflamed tissues. Moreover, E- and P-ligs are required for homing of T cells into the skin. Most selectin ligands are *O*-type glycoproteins which require specific carbohydrate modifications, the sialyl Lewis^x tetrasaccharide and related structures (Ley and Kansas, 2004; Lowe, 2002; Vestweber and Blanks, 1999). Several glycosyltransferases are involved in the generation of these oligosacchrides (Lowe and Marth, 2003).

In myeloid cells, E- and P-ligs are constitutively expressed while in CD4$^+$ T cells, E- and P-ligs are only induced upon TCR activation of naive T cells in the presence of appropriate co-stimulatory signals. Once induced, P-ligs are stably expressed on subsets of CD4$^+$ effector/memory T cells (Jennrich et al., 2007).

Induction of the selectin ligands in CD4$^+$ T cells is mainly attributed to and controlled by the induction of two glycosyltransferases, namely the fucosyltransferase (FucT)-VII and core 2 ß1,6-N-acetylglucosaminyltransferase-I (C2GlcNAcT-I) (Homeister et al., 2001; Smithson et al., 2001; Snapp et al., 2001). Whereas C2GlcNAcT-I is dispensable for E-lig generation, FucT-VII is required in CD4$^+$ T cells for both E-and P-lig generation (Snapp et al., 2001). Thus, FucT-VII$^{-/-}$ CD4$^+$ effector cells lack E- and P-ligs and show strongly impaired homing to sites of inflammation (Doebis et al., 2008).

However, the transcriptional and molecular regulation of FucT-VII is still poorly defined. *In vitro*, selectin ligands and expression of FucT-VII and C2GlcNAcT-I are predominately induced upon Th1 differentiation (Austrup et al., 1997; Lim et al., 1999; Wagers and

Abbreviations: FucT, fucosyltransferase; E- and P-lig, E- and P-selectin ligands; CNS, conserved noncoding sequence; C2GlcNAcT-I, Core2 ß1,6-N-acetylglycosaminyltransferase-I Trichostatin A (TsA).

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http://dx.doi.org/10.1016/j.molimm.2014.05.005
0161-5890/

Kansas, 2000; White et al., 2001). IL-12 dependent STAT4 signals control C2GlcNAcT-1 expression whereas FucT-VII expression is induced by a strong TCR signal and enhanced by IL-12 in a STAT4 independent fashion (White et al., 2001; Barry et al., 2003; Zisoulis and Kansas, 2004). However, according to our data, IL-12 and T-bet are dispensable for *Fut7* induction in murine T cells as we observed high induction of *Fut7* mRNA under non-polarizing conditions which could be attributed to IL-2 (Schroeter et al., 2012). In addition, TGFβ has been shown to induce FucT-VII in a p38-dependent manner (Wagers and Kansas, 2000).

In vivo, E- and P-lig expression is not restricted to Th1 cells but also found on Th2 cells, IL-10 secreting T cells and on T regulatory cells, further indicating the existence of IL-12 independent induction pathways (Kretschmer et al., 2004; Siegmund et al., 2005). *In vivo* studies also revealed the impact of microenvironment-dependent factors on the regulation of E- and P-lig expression. E- and P-lig are preferentially induced in peripheral lymph nodes and guide the cells back to inflamed sites notably within skin tissue. In contrast, in mesenteric lymph nodes E- and P-lig induction is suppressed mainly due to the action of retinoic acid, which on the other hand promotes induction of the integrin α4β7 and CCR9 supporting recruitment to the gut (Campbell and Butcher, 2002; Iwata et al., 2004). Retinoic acid suppresses induction of selectin ligands mostly *via* suppression of *Fut7* (Schroeter et al., 2012; Iwata et al., 2004).

Concerning the molecular regulation of FucT-VII which is encoded by the *Fut7* gene, little is known. The human *Fut7* gene was first identified by Sasaki et al. (1994) and Natsuka et al. (1994). Later, the murine *Fut7* gene was identified in a hybridization screen probed with the catalytic domain of the human α(1,3/1,4) fucosyltransferase-III in a murine genomic DNA phage library (Smith et al., 1996). In that study, four different transcripts were identified in a T cell line; the transcriptional start site was defined according to the longest mRNA transcript.

Further studies showed control of the human *Fut7* gene by a cyclic adenosine monophosphate (cAMP)-responsive element (CRE) located in close proximity of the transcriptional start site of the human gene (Hiraiwa et al., 1997, 2003). A complex consisting of CBP/p300 and T-bet also seems to drive expression of FucT-VII in human T cells by binding to a putative promoter region of the human *Fut7* gene (Chen et al., 2006a). This T-bet driven transactivation was abrogated by GATA-3.

Apart from the molecular studies mentioned above, regulatory elements within the *Fut7* gene are poorly defined. Only one recent publication defined the functional boundaries of the *Fut7* gene to a 12.7 kb genomic region of the murine *Fut7* locus that contains all relevant *cis*-acting regulatory elements for expression as transgenic expression of this region yielded expression of the transgene (Ebel and Kansas, 2014).

The aim of this study was to further specify regulatory elements of the *Fut7* gene that control expression in $CD4^+$ T cells. We used 5′ Rapid Amplification of cDNA Ends (RACE)-PCR which confirmed the expression of several *Fut7* transcripts in primary $CD4^+$ T cells. Analysis of the pattern of histone modifications across the *Fut7* locus revealed distinct differences in the histone modification pattern in the *Fut7* locus including a conserved non-coding region (CNS) located about 500 bp upstream of the first exon of the *Fut7* gene. Cloning of this region and transfection of a T cell line or primary Th1 cells revealed a context-dependent promoter or enhancer activity. Moreover, we identified an intronic minimal promoter that most likely acts as an alternative promoter in primary $CD4^+$ T cells and the activity of which is enhanced by the CNS acting as an enhancer in primary T cells. Thus, this study identified *cis*-regulatory regions controlling *Fut7* expression in $CD4^+$ effector cells.

2. Materials and methods

2.1. Mice

8–12 weeks old BALB/c and C57BL/6 mice were purchased from the Bundesinstitut fuer Risikobewertung (Berlin, Germany) and BALB/c ex breeder mice from Charles River (Sulzfeld, Germany). All animal experiments were performed in accordance with institutional, state and federal guidelines.

2.2. Antibodies, magnetic beads, culture media and cytokines

The following antibodies were produced in our laboratory or the Deutsche Rheuma-Forschungszentrum (Berlin, Germany): anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IL-4 (11B11), anti-CD4-FITC (GK1.5). Anti-CD4-FITC (L3T4), anti-CD4-PerCP (L3T4), anti-CD45RB-FITC (16A). Streptavidin(SA)-Cy7 and -APC were purchased from BD Biosciences (Franklin Lakes, USA); biotinylated anti-α4β7 (DATK32), anti-CD62L eFluor 450 (MEL-14), anti-CD44-PE (IM7) and anti-CD4-PE (L3T4) from eBioscience (Hatfield, UK) and PE-and Cy5-conjugated $F(ab')_2$ donkey anti-human IgG antibody from Jackson ImmunoResearch (West Grove, USA). All microbeads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-FSP1 was obtained from Santa Cruz Biotechnology. Anti-H3K4me2 and anti-H3K27me3 were obtained from Millipore (Billerica, USA).

2.3. Isolation of different $CD4^+$ T cell subsets and fibroblasts

Naive $CD4^+$ T cells were purified from pooled spleens, peripheral and mesenteric lymph nodes of BALB/c mice by direct isolation of $CD4^+$ cells using anti-CD4–FITC (GK1.5) mAb and anti-FITC multisort–magnetic cell separation (MACS) beads with further positive selection of $CD62L^+$ fraction by anti-CD62L microbeads.

In vitro-polarized P-lig^+ and P-lig^- Th1 cells were sorted from day 5 Th1 cultures. *In vivo* generated P-lig^+ and P-lig^- memory $CD4^+$ T cell subsets were isolated from pLN, mLN and spleens of old exbreeder BALB/c or C57BL/6 (for ChIP) mice. BALB/c: $CD4^+$ T cells were enriched by MACS depletion of macrophages, B-cells and $CD8^+$ cells using anti-B220, anti-Mac1 and anti-CD8 rat mAb and anti-rat-IgG MACS beads. For further enrichment of effector/memory T cells, $CD45Rb^{high}$ cells were depleted using anti-CD45Rb-FITC and anti-FITC MACS beads. The remaining cell population was stained with P-lig-IgG, anti-human-IgG-Cy5, anti-CD4-PerCP, and anti-α4β7-Bio/SA-PE-Cy7. $CD4^+CD45Rb^{low}$ cells were sorted into P-lig^+ $\alpha4\beta7^-$ and P-$lig^-\alpha4\beta7^+$ T cells by FACS. C57BL/6: $CD4^+$ T cells were enriched by anti-CD4-FITC and anti-FITC beads based magnetic sorting. The enriched fraction was stained with P-lig-IgG, anti-human-IgG-PE, anti-α4β7-Bio/SA-APC, anti-CD62L eFluor 450 and anti-CD44-PE-Cy7. $CD4^+CD44^{high}$ cells were sorted into P-lig^+ $\alpha4\beta7^-$ and P-$lig^-\alpha4\beta7^+$ T cells by FACS.

Lung fibroblasts were acquired from C57BL/6 mice as described by Schuler and Blankenstein (2003). In brief, lungs were minced, treated with 1 mg/ml collagenase D (Roche Diagnostics), separated into single-cell suspension using the gentleMACS™ Dissociator and subsequently pushed through a 70-μM nylon filter. After hypotonic erythrocyte lysis, cells were cultured in DMEM plus 10% FCS, penicillin/streptomycin (100 U/ml), glutamax (2 mM), and sodium pyruvate (1 mM) for 24 h. Nonadherent cells were removed and adherent cells were passaged every 3–5 days. Cells were used for ChIP analysis after 5 passages. 95% the cells expressed the fibroblast-specific protein 1 (Santa Cruz) classifying them as fibroblasts.