A novel single-nucleotide substitution, Glu 4 Lys, in the leukotriene C4 synthase gene associated with allergic diseases

KAORI YOSHIKAWA[1], EIKO MATSUI[1], HIDEO KANEKO[1], TOSHIYUKI FUKAO[1], RYOSUKE INOUE[2], TAKAHIDE TERAMOTO[1], SHINJI SHINODA[3], OSAMU FUKUTOMI[4], MINAKO AOKI[1], KIMIKO KASAHARA[1] and NAOMI KONDO[1]

[1]Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1193; [2]Department of Pediatrics, Nishimino Kousei Hospital, 986 Oshikoshi, Yoro-cho, Yoro-gun, Gifu 503-1394; [3]Department of Pediatrics, Gujo City Hospital, 1264 Shimatani, Hachiman-cho, Gujo, Gifu 501-4222; [4]Fukutomi Clinic, 1228 Ajiki, Gifu 501-1109, Japan

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Abstract. Cysteinyl leukotrienes (cysLTs) play important roles in bronchial asthma, and can mediate bronchial smooth muscle constriction and increase mucous secretion, vascular permeability and cellular infiltration. We identified a novel heterozygous single-nucleotide substitution 10G>A (Glu 4 Lys) in the first exon of the leukotriene C4 synthase gene (*LTC4S*). This substitution was detected in 5 of 141 allergic patients, but not in 110 nonallergic subjects. There was a difference in the Glu 4 Lys frequency between the allergic patients and nonallergic subjects (Fisher's exact test, p=0.0460). The five patients with Glu 4 Lys had allergic diseases such as bronchial asthma and/or allergic dermatitis. Furthermore, a familial analysis of Glu 4 Lys revealed a link with allergic diseases. Thus, our results suggest that Glu 4 Lys in the *LTC4S* might be associated with allergic diseases.

Introduction

Susceptibility to allergic diseases, such as bronchial asthma (BA), atopic dermatitis (AD) and allergic rhinitis (AR), is determined by the interaction between an unknown number of genetic and environmental factors (1). Single-nucleotide substitutions in several genes have been reported to be associated with allergic diseases (2-5).

Correspondence to: Dr Kaori Yoshikawa, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
E-mail: kimatan@tj8.so-net.ne.jp

Abbreviations: BA, bronchial asthma; AD, atopic dermatitis; AR, allergic rhinitis; *LTC4S*, leukotriene C4 synthase gene; LTE4, leukotriene E4; 11DTXB2, 11-dehydro-thromboxane B2

Key words: allergic disease, bronchial asthma, cysteinyl leukotriene, leukotriene C4 synthase gene, single-nucleotide substitution, morbidity

The locus of *LTC4S* is on chromosome 5q35(6) and has been associated with allergic diseases on the basis of a genome-wide search (7). Cysteinyl leukotrienes (cysLTs) play important roles in BA and can mediate bronchial smooth muscle constriction and increase mucous secretion, vascular permeability and cellular infiltration (8,9). LTC4 synthase converts LTA4 to LTC4 by conjugation to reduced glutathione. A single-nucleotide promoter polymorphis (A-444C) in *LTC4S* has been associated with aspirin-sensitive asthma in Polish patients (3), although recent studies found no association between this promoter polymorphis and aspirin-sensitive asthma (4). In a small study of 23 severe asthmatics taking oral zafirlukast for 2 weeks, there was a trend (p=0.1) for those who had 1 or 2 copies of the -444C allele to show improvements in lung function (5). We hypothesized that *LTC4S* is one of the candidate genes for BA. Here, we report that a novel single-nucleotide substitution 10G>A (Glu 4 Lys) in *LTC4S* is associated with BA.

Materials and methods

Patients and control subjects. One hundred and forty-one allergic patients (89 males and 52 females; mean age ± 1 SD, 8.5±7.9 years old; 130 BA, 3 AD, 1 AR, 3 BA and AD, and 4 BA and AR) and 110 nonallergic subjects (60 males and 50 females; mean age ± 1 SD, 23.4±11.1 years old) were studied. The allergic patients were selected on the basis of having major allergic diseases such as BA, AD and/or AR. BA was diagnosed according to the criteria of the American Thoracic Society, AD according to the criteria of Hanifin (10) and AR according to the criteria of Skoner (11,12). The nonallergic subjects were healthy and did not have a history of allergic diseases. All of the subjects were randomly selected from patients attending our hospitals. Informed consent was obtained from all subjects or their parents.

Detection of single-nucleotide substitution in the LTC4S. Neutrophils obtained from heparinized blood were stored at -30°C. Genomic DNA was extracted from the neutrophils with a Sepagene kit (Sanko Junyaku, Tokyo, Japan).

For 15 allergic patients, the five coding regions of *LTC4S* (GenBank accession no. U50136) DNA were amplified and

Table I. Sequence of oligonucleotides for PCR.

Primer			Sequence	Position	Size of the amplified fragment (bp)	Annealing temp. (°C)
LTC4S	1	Forward	5'-AGGCTGCTCTTCCTCTCCTG-3'	-96-58	154	62
		Reverse	5'-CTTCCTAGATAGGAACCAGC-3'			
	2	Forward	5'-CCTTAGGGAGAGGACACG-3'	1504-1603	100	62
		Reverse	5'-CTCACTGGGCTCGGTAGACG-3'			
	3	Forward	5'-CACCGCAGGGTGAACTGCAG-3'	1706-1776	71	50
		Reverse	5'-ACCTTCATGAAAGAAGATGCCG-3'			
	4	Forward	5'-TGCGTACCTCTCGCAGGCGC-3'	1861-1942	82	56
		Reverse	5'-CTCACCTGAGCTGCGCGGAGCG-3'			
	5	Forward	5'-GCAGGCTGGCACCGCTGTAC-3'	2173-2429	257	53
		Reverse	5'-GGTCTCGGTCACTAGAAC-3'			

Table II. Distribution of genotype of Glu 4 Lys in *LTC4S*.

Genotype	Nonallergic subjects (n=110)	Allergic patients (n=141)	Total (n=251)	p-value
GAG (Glu)/GAG (Glu)	110 (100%)	136 (96.5%)	246 (98%)	p=0.046[a]
GAG (Glu)/AAG (Lys)	0 (0%)	5 (3.5%)	5 (2.0%)	
AAG (Lys)/AAG (Lys)	0 (0%)	0 (0%)	0 (0%)	

[a]Statistical analyses were performed using Fisher's exact test.

Table III. The clinical features of allergic patients with Glu 4 Lys in *LTC4S*.

Patient	Sex	Age	Allergic diseases	Serum IgE levels (IU/ml)	LTE4 (pg/mg creatinine)	11DTXB2 (pg/mg creatinine)
1	F	15	BA	889.8	ND	ND
2	F	8	BA, AD	755.5	411.0	2049.9
3	F	7	BA	1300.0	259.0	1837.0
4	M	5	BA	171.7	256.0	1532.3
5	F	9	BA	1900.0	215.0	1226.0
Nonallergic subjects[a]						
Mean				19.5	233.0	252.0
Range				6.8-56.2	103.0-389.0	41.0-716.0

[a]They were quoted from ref. 13.

sequenced using the PCR technique and an ABI 3100 DNA sequencer (Applied Biosystems, CA, USA). The conditions for PCR and the primers used are shown in Table I. To detect the Glu 4 Lys substitution, PCR products of the 126 allergic patients and 110 controls were electrophoresed on a 12% polyacrylamide gel following *Mnl 1* (New England BioLabs, MA, USA) digestion at 37°C for 1 h.

Measurement of LTE4 and 11DTXB2. Urine samples were collected from patients with the Glu 4 Lys substitution, 50 randomly selected allergic patients and 7 randomly selected controls. They were stored at -80°C and analyzed within a month of collection.

Urinary leukotriene E4 (LTE4) was measured using the leukotriene C4/D4/E4 enzyme-immunoassay system (Amersham, Buckinghamshire, UK) after purification by high-performance liquid chromatography (HPLC; 486 Tunable Absorbance Detector, Waters, MA, USA) using a C18 reverse-phase column (Cappcell PAC UG 120; Shiseido, Tokyo, Japan). Urinary LTE4 level was expressed as pg/mg creatinine.

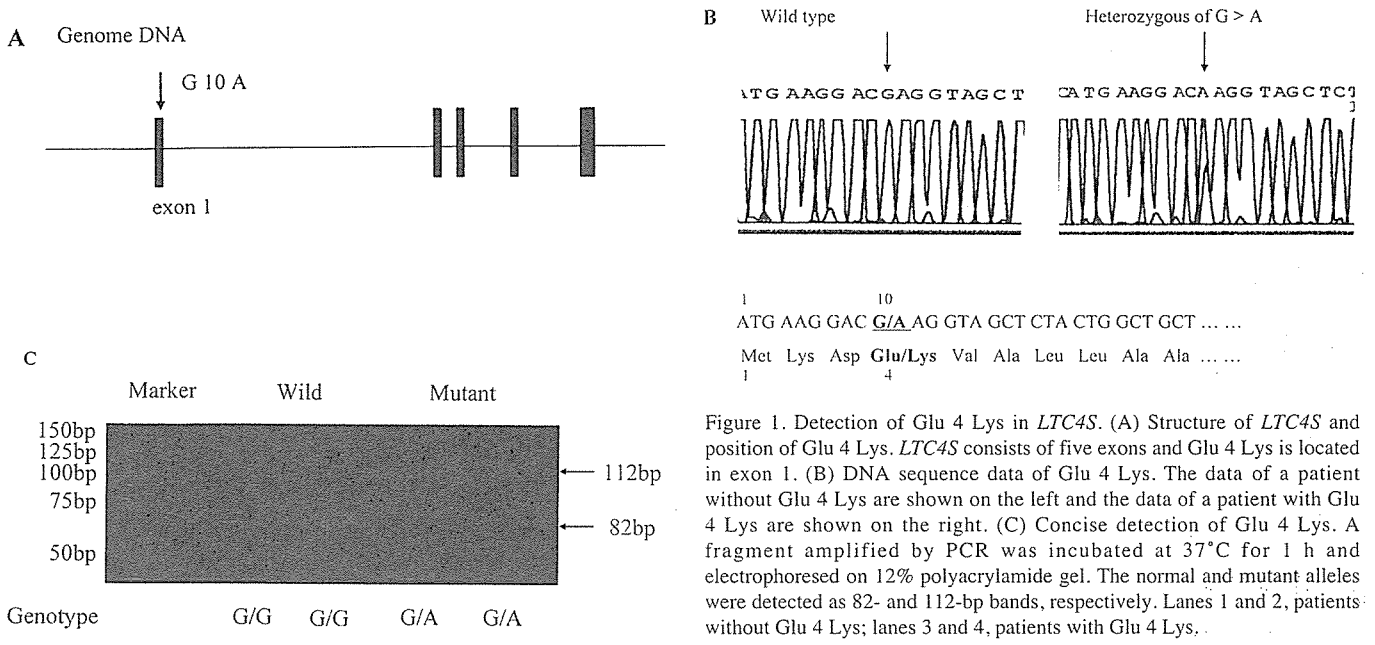

Figure 1. Detection of Glu 4 Lys in *LTC4S*. (A) Structure of *LTC4S* and position of Glu 4 Lys. *LTC4S* consists of five exons and Glu 4 Lys is located in exon 1. (B) DNA sequence data of Glu 4 Lys. The data of a patient without Glu 4 Lys are shown on the left and the data of a patient with Glu 4 Lys are shown on the right. (C) Concise detection of Glu 4 Lys. A fragment amplified by PCR was incubated at 37°C for 1 h and electrophoresed on 12% polyacrylamide gel. The normal and mutant alleles were detected as 82- and 112-bp bands, respectively. Lanes 1 and 2, patients without Glu 4 Lys; lanes 3 and 4, patients with Glu 4 Lys.

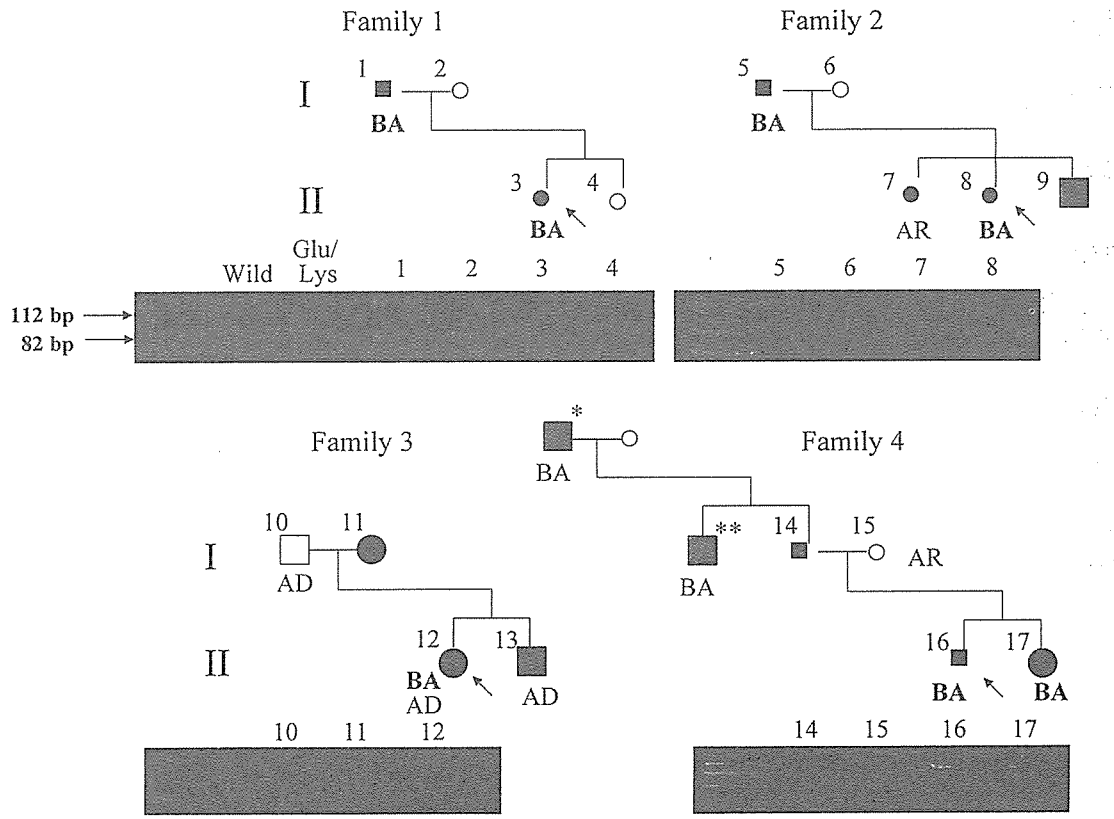

Figure 2. Genetic analysis for Glu 4 Lys in four atopic families. (Family 1) II-3 was the proband with the heterozygous Glu 4 Lys and BA. The father (I-1) had the heterozygous Glu 4 Lys and BA. (Family 2) II-8 was the proband with the heterozygous Glu 4 Lys and BA. The father (I-5) had the heterozygous Glu 4 Lys and BA. The elder sister (II-7) also had the heterozygous Glu 4 Lys and was suffering from AR. We could not get informed consent from the brother (II-9). (Family 3) II-12 was the proband with Glu 4 Lys and BA and AD. The mother (I-11) had the heterozygous Glu 4 Lys without any allergic history. We could not get informed consent from her brother (II-13). (Family 4) II-16 was the proband with Glu 4 Lys and BA. The father (I-14) had the heterozygous Glu 4 Lys without any allergic symptoms. His grandfather (*) and uncle (**) had a history of BA. We could not get informed consent from his sister (II-17).

Urinary 11-dehydrothromboxane B2 (11DTXB2) was measured using an 11-dehydrothromboxane B2 [^{125}I] RIA kit, (Perking-Elmer Life and Analytical Sciences, Boston, MA, USA) after extraction using octadecylsilyl silica (ODS) powder and purification using an open silica mini column (Bond Elute SI; Varian, Palo Alto, CA, USA). The urinary 11DTXB2 level was also expressed as pg/mg creatinine and the urinary creatinine level was determined

using a creatinine test kit (Pure Auto® S CRE-L, Daiichi-kagaku, Tokyo, Japan).

Statistical analyses. The distribution of the genotype of Glu 4 Lys in *LTC4S* was analyzed using Fisher's exact test. The Mann-Whitney unpaired *U* test was performed for allergic patients with Glu 4 Lys and controls, and allergic patients with and without Glu 4 Lys. p-values <0.05 were considered statistically significant.

Results

Glu 4 Lys in LTC4S. We identified a novel heterozygous single-nucleotide substitution 10G>A (Glu 4 Lys) in the genomic *LTC4S* DNA by direct sequencing in some of the allergic patients (Fig. 1A and B). We determined the prevalence of Glu 4 Lys in the *LTC4S* in allergic patients and non-allergic subjects using the restriction enzyme assay (Fig. 1C). The normal and mutant alleles were detected as 82- and 112-bp bands, respectively (Fig. 1C).

Glu 4 Lys was found in 5 (3.5%) of the 141 allergic patients. However, Glu 4 Lys was not detected in any of the 110 nonallergic subjects. There was a difference in Glu 4 Lys frequency between the allergic patients and nonallergic subjects (Fisher's exact test, p=0.0460) (Table II). The clinical features of the allergic patients with Glu 4 Lys are shown in Table III. All of the patients with Glu 4 Lys suffered from BA. The urinary LTE4 levels of patients with Glu 4 Lys (mean, 285.3 pg/mg creatinine; range, 215.0-411.0 pg/mg creatinine) did not show a significant difference from those of allergic patients with wild-type *LTC4S* (mean, 364.0 pg/mg creatinine; range, 86.9-1840.0 pg/mg creatinine) or control subjects (mean, 204.0 pg/mg creatinine; range, 103.0-347.0 pg/mg creatinine). The urinary 11DTXB2 levels of patients with Glu 4 Lys (mean, 1661.2 pg/mg creatinine; range, 1226.0-2050.0 pg/mg creatinine) did not show a significant difference from those of allergic patients with wild-type *LTC4S* (mean, 1294.8 pg/mg creatinine; range, 40.0-6070.0 pg/mg creatinine); however, they were significantly different from those of the control subjects (mean, 596.5 pg/mg creatinine; range, 239.0-1376.0 pg/mg creatinine; p=0.0140) (data not shown).

Genetic analysis of Glu 4 Lys in atopic families. The link between Glu 4 Lys and allergic diseases was investigated in four families (Fig. 2). In family 1, II-3 was the proband with Glu 4 Lys and suffering from BA. Glu 4 Lys was inherited from the father (I-1) who had BA. A younger sister (II-4) without any allergic symptoms did not have this substitution.

In family 2, II-8 was the proband with Glu 4 Lys and suffering from BA. Glu 4 Lys was inherited from the father (I-5) who had BA. An elder sister (II-7) also had the heterozygous Glu 4 Lys and suffered from AR. We could not obtain informed consent from the brother (II-9).

In family 3, II-12 was the proband with Glu 4 Lys and suffering from BA and AD. Glu 4 Lys was inherited from the mother (I-11) who had no allergic history. We could not get informed consent from the brother (II-13).

In family 4, II-16 was the proband with Glu 4 Lys and suffering from BA. Glu 4 Lys was inherited from the father (I-10) who did not have any allergic symptoms. The sister (II-17) also had the heterozygous Glu 4 Lys and suffered from BA.

In families 1 and 2, the probands with Glu 4 Lys (II-3 and -8) all suffered from BA. Glu 4 Lys was inherited from their fathers (I-1 and -5) who had a history of BA in childhood. In families 3 and 4, the proband with Glu 4 Lys (II-12 and -16) also suffered from BA. However, Glu 4 Lys was inherited from the mother (I-11) or father (I-14) who did not have allergic symptoms. Interestingly, the grandfather (*) and uncle (**) in family 4 had a history of BA. In all four families, the subjects with a history of BA also had the Glu 4 Lys substitution.

Discussion

Through linkage and association studies, a number of putative atopy genes have been identified. Studies of the chromosome 5q region, in which the *LTC4S* is located (6), revealed the linkage to atopy and bronchial hyper-responsiveness. Other candidate genes for asthma are located in the 5p region, including the IL-4 cytokine cluster (IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF) and the β_2-adrenergic receptor.

In this study, we identified a novel single-nucleotide substitution 10G>A (Glu 4 Lys) in the first exon of the *LTC4S* gene. All patients who had a Glu 4 Lys substitution suffered from BA or both BA and AD. Furthermore, the familial analysis of Glu 4 Lys revealed a link between allergic diseases and Glu 4 Lys. All patients with BA in the four families we studied had Glu 4 Lys in *LTC4S*. These results showed that Glu 4 Lys in *LTC4S* may be associated with the phenotype of allergic diseases such as BA.

We have reported that the levels of urinary LTE4 and 11DTXB2 are significantly higher in asthmatic children than in control subjects (13). In our present study, the levels of urinary 11DTXB2 were significantly higher in patients with Glu 4 Lys than in control subjects (p<0.05). However, there was no significant difference in the level of urinary LTE4 between the allergic patients with Glu 4 Lys and control subjects. This mutation in the *LTC4S*, which changes the charge from negative to positive, may affect the stability of the *LTC4S* protein. Therefore, this mutation is induced to inhibit the synthesis of cysLTs. Unfortunately, in this study, the urinary prostanoids were measured only in the four patients with Glu 4 Lys. Further studies may be necessary to define the mechanism underlying the activity of *LTC4S* with a Glu 4 Lys substitution.

In conclusion, our study suggested that Glu 4 Lys in *LTC4S* might be associated with BA. Further large-scale clinical studies are required to confirm the effects of the Glu 4 Lys substitution in the *LTC4S* in patients with allergic diseases, together with studies of clustering SNPs on the 5q chromosome affecting BA.

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Original Article

Characterization of T-cell clones specific to Ovomucoid from patients with egg-white allergy

M. Kondo, K. Suzuki, R. Inoue, H. Sakaguchi, E. Matsukuma, Z. Kato, H. Kaneko, T. Fukao, N. Kondo

Department of Pediatrics, Graduate School of Medicine, Gifu University. Japan

Summary. *Background:* Allergic reactions to foods are specific problems for infants and young children. Ovomucoid (OM) is one of the major allergens found in egg-white. We previously established several T-cell clones (TCCs) specific to OM in non-polarizing conditions from 4 patients (TM and YN are immediate-type, IH and YT are non-immediate-type) with egg-white allergy. We characterized their reactive epitopes, antigen-presenting molecules (HLA class II), and usage of TCR alpha and beta genes and the CDR3 loop sequence.
Objective: The objective of this study was to characterize these seven clones (TM1.3,TM1.4,YN1.1,YN1.5,IH3.1,IH3.3 and YT6.1) for cytokine production patterns and cell-surface-marker phenotypes.
Methods: We measured the production of cytokines, namely interleukin (IL)-4, IL-5 and interferon-γ (IFN-γ) by stimulation with ovomucoid peptides and stained intracellular IL-4 and IFN-γ, and determined cell-surface markers using anti-interleukin-12 receptor (IL-12R) ß1, anti-IL-12Rß2 and anti-interleukin-18 receptor α (IL-18Rα).
Results: Most TCCs secreted both IL-4 and IFN-γ in response to the OM peptide mixture, but the secretion patterns were variable; an IFN-γ dominant pattern was seen in IH3.1 and YT6.1, an IFN-γ >IL-4 pattern in TM1.3 and TM1.4, an IL-4> IFN-γ pattern in YN1.5. In intracellular IFN-γ and IL-4 staining, IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3. All TCCs were IL-12Rß1-positive, and TM1.3, IH3.1, IH3.3 and YT6.1 were both IL-12Rß2- and IL-18Rα-positive. TM1.4 and YN1.1 were both IL-12Rß2- and IL-18Rα-negative. Based on these results, TM1.3 and TM1.4, IH3.1 and YT6.1 had a predominantly Th1 character and YN1.1, YN1.5, and IH3.3 possessed a predominantly Th0 character.
Conclusions: The phenotypes of TCCs were not in accordance with their clinical manifestations. TCCs established from patients with immediate-type hypersensitivity had either the Th1 or Th0 phenotype as well as those with non-immediate-type hypersensitivity.

Key Words: AD (atopic dermatitis), IFN-γ (interferon gamma), IL-12Rß2 (interleukin-12 receptor beta 2), IL-18Rα (interleukin-18 receptor alpha), OM (ovomucoid), TCC (T cell clone), Th (helper T cell).

Abbreviations used:

AD:	atopic dermatitis	PBMC:	Peripheral blood mononuclear cell
FITC:	Fluorescein isothiocyanate	PE:	phycoerythrin
HLA:	Human leukocyte antigen	RAST:	Radioallergosorbent test
IFN-γ:	Interferon gamma	rIL:	recombinant Interleukin
IL:	Interleukin	TCC:	T cell clone
IL-12R:	IL-12 receptor	TCL:	T cell line
IL-18R:	IL-18 receptor	TCR:	T cell receptor
OM:	Ovomucoid	Th:	helper T cell

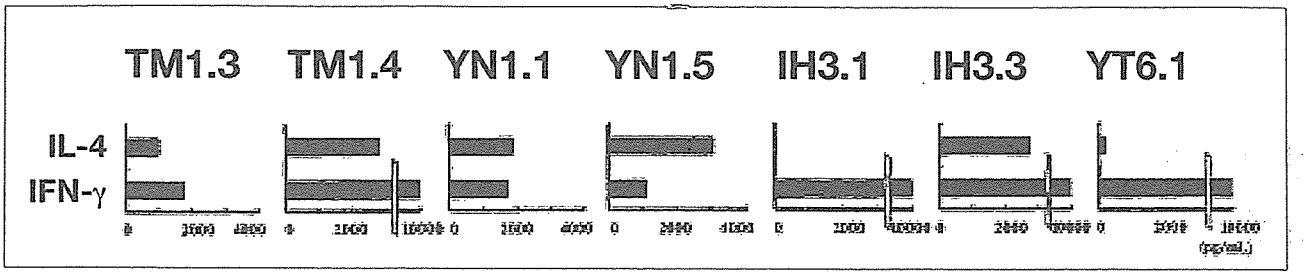

Figure 1. Cytokine production patterns of our TCCs. T-cells were cultured in the presence of an OM peptide mixture (1mM each). After incubation for 56 hours, culture supernatants were collected immediately, and the cytokines were measured by ELISA. Net cytokine concentration in culture supernatants is expressed as the mean value of duplicate cultures.

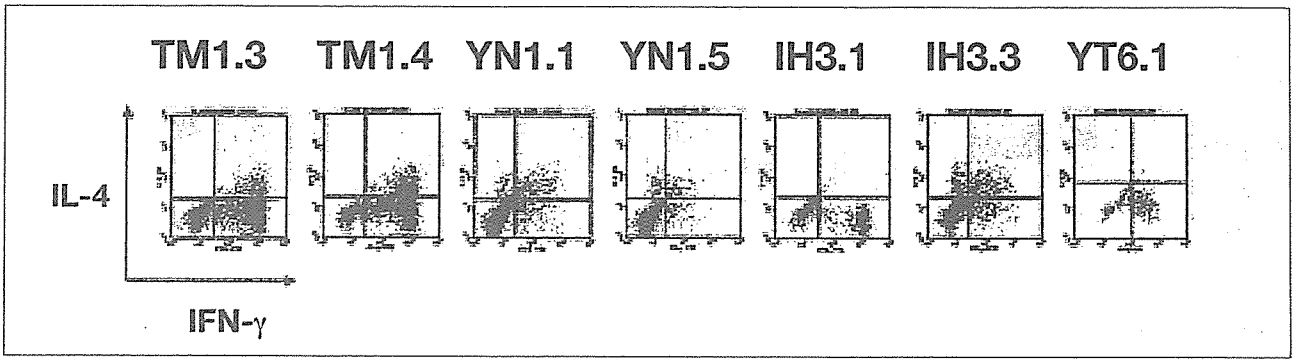

Figure 2. Intracellular IFN-γ and IL-4 staining. TCCs specific to OM were cultured at a density of 2 x 10^6 cells per mL in an RPMI 1640 medium with PMA and ionomycin in the presence of Brefeldin-A for 4 hr at 37°C. After Pma stimulation, the cells were directly stained with a FITC-conjugated anti-CD4 monoclonal antibody. After permeabilization, the cells were stained with IFN-γ FITC/IL-4 PE. Flow cytometric analysis was performed using a FACS Calibur.

Cell-surface-marker phenotypes of TCCs

A FITC-labeled monoclonal anti-IL-12Rß1 antibody (PharMingen, San Diego, CA), a phycoerythrin (PE)-labeled monoclonal anti-IL-12Rß2 antibody (kindly donated by Dr. F Sinigaglia, Italy), anti-IL-18Rα, and anti-human CCR-3 (R&D Systems, McKinley Place, MN) were used to analyze the phenotype of our TCCs. FITC-labeled anti-Leu4/CD3, anti-Leu3a/CD4 (Becton Dickinson), anti-TCR-α/ß-1-WT31 (Becton Dickinson), anti-TCR-γ/δ (Endogen, Woburn, MA), and PE-labeled anti-Leu2a/CD8 (Becton Dickinson) were also used to analyze the phenotypes of our TCCs by double-color staining. The stained cells were analyzed using a FACScan instrument (Becton Dickinson). The forward scatter threshold was set to exclude debris only from the preparation.

Results

Production of IL-4 and IFN-γ in supernatants of TCCs

Cytokine secretion was investigated in the TCCs specific to OM. Since these clones were maintained in the presence of IL-2 and IL-4, they were washed with the culture medium and then cultured with the OM peptide mixture (1 μM) and irradiated autologous PBMC, but in the absence of IL-2 and IL-4, for 56 hr. Cell proliferation was similar among these clones when examined using a 3H-thymidine uptake (data not shown). Most TCCs secreted both IL-4 and IFN-γ in response to the peptide mixture, as well as OM crude protein (data not shown), but the secretion patterns were variable; an IFN-γ dominant pattern (IFN-γ >> IL-4) was seen in IH3.1 and YT6.1, an IFN-γ >IL-4 pattern in TM1.3, TM1.4 and IH3.3, IL-4> IFN-γ pattern in YN1.5, as shown in Figure 1.

Intracellular IL-4 and IFN-γ staining

Since the patterns of IL-4 and IFN-γ secretion were variable among the TCCs, intracellular IL-4 and IFN-γ staining was analyzed. As shown in Figure 2, intracellular IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3.

Together with cytokine secretion data, TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype.

Introduction

Allergic reactions to foods are specific problems for infants and young children and present a wide spectrum of clinical reactions, including cutaneous, gastrointestinal and respiratory symptoms as well as systemic anaphylactic symptoms. It is also known that food allergies are more prevalent in children due to an immature gastrointestinal epithelial membrane barrier that allows more proteins to cross the barrier and get into circulation [1]. Among various food antigens, the hen's egg, particularly its egg-white, is one of the most common causes of food allergy in young children. One of the major components of egg-white, comprising approximately 10% of the total egg-white proteins, is ovomucoid (OM) [2], and it has been reported to play a more important role in the pathogenesis of allergic reactions to egg-white than other egg-white proteins [3,4].

Interferon-γ (IFN-γ) production characterizes the Th1 subset while interleukin (IL)- 4 production characterizes the Th2 subset [5]. The differentiation into either Th1 or Th2 cells is a crucial step that determines the direction of subsequent adaptive immune responses. Interleukin-12 receptor (IL-12R) ß2 mRNA synthesis is restricted to Th1 cells [6,7], and the mRNA expression of Interleukin-18 receptor α (IL-18Rα) was observed only in the Th1 clones [8]. We recently established OM-specific TCCs from four Japanese atopic dermatitis (AD) patients with egg-white allergy [9]. In that study, all the TCCs specific to OM exhibited the $CD4^+$ phenotype. These clones were established in non-polarizing conditions, and it was of interest to see whether they had Th1 or Th2 characters in relation to clinical phenotypes. We determined the cytokine production patterns and cell-surface-marker phenotypes of TCCs specific to OM.

Materials and Methods

Subjects

Clinical information on the four patients whose TCCs have been established was reported previously [9]. The diagnosis of allergy to hen's egg-white was based on clinical symptoms, hen's egg-white challenge test results and CAP-RAST [10] against hen's egg-white and OM. All the patients had AD symptoms. Patient TM and YN presented immediate-type hypersensitivity symptoms, such as systemic urticaria and severe coughing, which occurred within thirty minutes after the antigen challenge, and their levels of total IgE and CAP-RAST for egg-white and OM were high. Patient IH and YT had presented non-immediate symptoms, such as systemic eczema, which occurred more than two hours after antigen challenge, and their levels of total IgE and CAP-RAST against egg-white and OM were low [11].

TCC culture

OM-specific TCC establishment was reported previously [9]. These TCCs were cultured in an RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM of L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 10% pooled A.B, heat-inactivated normal human male plasma in 24-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). The plates were incubated at 37°C in 5% CO_2 humid air. After 7-9 days, irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMCs) (1.5×10^5/well) pulsed with OM peptide mixture (1 mM each for 5 hr), human recombinant interleukin (rIL)-2 (50 U/ml) (Genzyme, Cambridge, MA), and human rIL-4 (10 U/ml) (Biosource International, Camario, CA) were added to the culture wells. Thus, the TCCs were maintained for another 7 days.

Production of IL-4 and IFN-γ in supernatants of TCCs

The TCCs (3×10^4 cells/well in 96-well flat-bottomed culture plates) were cultured in the presence of a soluble OM peptide mixture (1μM) and irradiated autologous PBMC (1.5×10^5/well) for 56 hr. Culture supernatants of the TCCs were collected and stored in aliquots at -80°C until the determination of lymphokine concentrations. Enzyme-linked immunosorbent assay (ELISA) kits for detecting human IL-4 (Biosource Int'l), and IFN-γ (Ohtsuka, Tokyo, Japan) were used for quantification of the lymphokines in the supernatants, according to the manufacturers' instructions.

Intracellular IFN-γ and IL-4 staining

TCCs specific to OM were cultured at a density of 2×10^6 cells /mL in an RPMI 1640 medium for 4 hr at 37°C. During the 4-hr incubation, the cells were stimulated with a combination of 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (SIGMA) and 2μg/mL of ionomycin (SIGMA) in the presence of 10 ng/mL of Brefeldin-A (SIGMA). Then the cells were directly stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with a FACS Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were preincubated with a FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE IFN-γ FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. The cells were then washed twice and resuspended in a phosphate-buffered saline. Flow cytometric analysis was performed using a FACS Calibur. The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD4 cells.

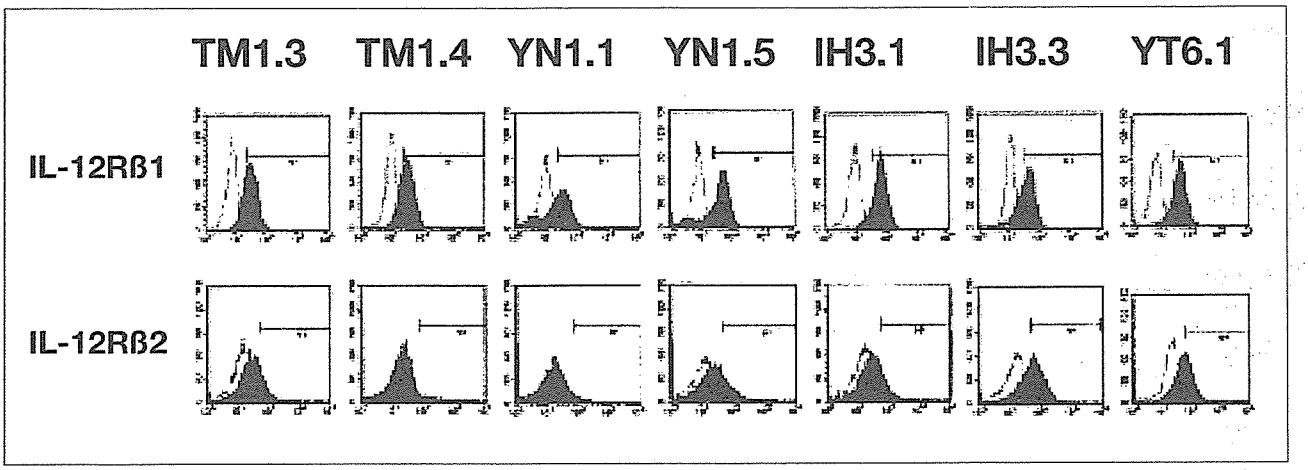

Figure 3. Flow cytometric analysis of our TCCs. FITC-labeled monoclonal antibody, anti-IL-12R ß1, and a PE-labeled monoclonal antibody, anti-IL-12R ß2, were used. Stained cells were analyzed using a FACScan instrument.

Cell-surface-marker phenotypes of TCCs.

All TCCs specific to OM exhibited $CD3^+$, $CD4^+$, $CD8^-$, $\alpha\beta TCR^+$ and $\gamma\delta TCR^-$ phenotypes (data not shown). Moreover, all TCCs were IL-12Rß1-positive (Fig.3). TM1.3, YN1.5, IH3.1, IH3.3 and YT6.1 were IL-12Rß2-positive and TM1.4 and YN1.1 were IL-12Rß2-negative (Fig.3). IL18Rα was co-expressed with IL-12Rß2 in these clones (data not shown).

Discussion

Murine helper T-cells are divided into two subsets, Th1 and Th2 cells. The former produce IL-2 and IFN-γ and the latter produce IL-4 and IL-5 [12]. Such dichotomy is also evident in human cells, albeit to a less polarized extent than in murine T-cells [13]. Th1 cells cause non-immediate-type hypersensitivity reactions whereas Th2 cells promote IgE production leading to immediate-type hypersensitivity.

In our previous study, it was shown that the proliferative responses of PBMCs to Ovalbumin (OVA) in children with AD who are sensitive to hen's eggs were significantly higher than those of healthy children and hen-egg-sensitive children with immediate symptoms. However, in patients with AD there were no significant correlations between the proliferative response to PBMCs and the RAST values [14]. We also reported on the high sensitivity and specificity of proliferative responses of lymphocytes to OVA for the detection of hen-egg allergy in patients with AD [15]. These studies indicate that the molecular basis of non-immediate-type hypersensitivity, as seen in AD patients who are sensitive to hen's eggs is different from that of immediate-type hypersensitivity to hen's eggs.

Chicken OM has been reported to be the most important allergenic protein in egg-white [2,3] and consists of three tandem homologous domains [16]. Hence, we previously established several OM-specific TCCs in non-polarizing conditions from 4 patients with egg-white allergy to investigate the molecular basis of hen-egg allergy [9]. In this paper, we characterized these TCCs with regard to helper T-cell phenotypes. Patients TM and YN presented immediate-type hypersensitivity symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were high. On the other hand, patients IH and YT presented non-immediate symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were low. These clinical manifestations suggested that Th1 and Th2 cells mainly contribute to the pathogenesis of hen-egg allergy in the former two patients and the latter two patients, respectively. All of the twenty-four Der pI-specific TCCs from a patient with severe atopic disease were reported to have the Th2-type character [17]. This may indicate that imbalance between Th1 and Th2 in a patient's condition influences the subtypes of these TCCs. Hence we expected that Th1 clones would be established from IH and YT and that Th2 clones would be from TM and YN.

In the present study, we analyzed IFN-γ and IL-4 production patterns by stimulation with OM, intracellular IFN-γ and IL-4 staining in 7 T-cells clones.

The results were further confirmed by the cell-surface marker of Th1 cells. TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype. IH3.1 and YT6.1 had a Th1-dominant phenotype in accordance with the fact that they were established from patients who presented non-immediate-type hypersensitivity. However, TM1.3 and TM1.4 were established from patient TM who presented immediate-type hypersensitivity symptoms, but both had a Th1-dominant phenotype. YN1.1 and YN1.5 were from patient YN who

also presented immediate-type hypersensitivity symptoms but both had a Th0 phenotype. Our results indicate that the cell phenotype of TCCs does not always reflect clinical manifestations corresponding to immediate or non-immediate hypersensitivity. The phenotypes of TCCs could not be simply predicted, so that establishment of further TCCs and their characterizations are now under way.

Acknowledgements

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Dr. Masashi Kondo

Department of Pediatrics
Yanagido 1-1, Gifu 501-1194, Japan
Phone: +81-58-230-6386; Fax: +81-58-230-6387
E-mail: g2104012@guedu.cc.gifu-u.ac.jp

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The cell cycle associated protein, HTm4, is expressed in differentiating cells of the hematopoietic and central nervous system in mice

Jeffery L. Kutok[1,**], Xing Yang[2,**], Rebecca D. Folkerth[1], Jaime Imitola[3], Khadir Raddassi[3], Yoshihiko Yano[2], Saira Salahuddin[2], Joel Lawitts[2], Hans Imboden[4], Masanobu Chinami[5], Taro Shirakawa[6,7], Helen Turner[8], Samia Khoury[3], Mohamed H. Sayegh[9,10], David Scadden[11] & Chaker Adra[2,10,*]

[1]*Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

[2]*Department of Medicine, Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA*

[3]*Center For Neurological Diseases, Brigham and Women's Hospital, Department of Neurology, Harvard Medical School, Boston, MA 02115, USA*

[4]*Division of Neurobiology, University of Bern, Baltzerstrasse 4, CH 3012 Bern, Switzerland*

[5]*Department of Nutrition, Kyushu Women's University, Jiyugaoka 1-1, kitakyushushi 807-8586, Japan*

[6]*Department of Health Promotion & Human Behavior, Kyoto University Graduate School of Public Health, Kyoto 606-8501, Japan*

[7]*RIKEN SNP Typing Center, Shirokanedai 4-6-1, Tokyo 108-8639, Japan*

[8]*Laboratory of Cell Biology and Immunology, The Queens Medical Center, Honolulu, HI*

[9]*Laboratory of Immunogenetics & Transplantation, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

[10]*Department of Medicine, Children Hospital, Harvard Medical School, Boston, MA 02115, USA*

[11]*Experimental Hematology, AIDS Research Center, Massachusetts General Hospital, Boston, MA 02129, USA.*

*Author for correspondence (e-mail: cadra@caregroup.harvard.edu, cadra@bidmc.harvard.edu)

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Summary

HTm4 is a member of a newly defined family of human and murine proteins, the MS4 (membrane-spanning four) protein group, which has a distinctive four-transmembrane structure. MS4 protein functions include roles as cell surface signaling receptors and intracellular adapter proteins. We have previously demonstrated that HTm4 regulates the function of the KAP phosphatase, a key regulator of cell cycle progression. In humans, the expression of HTm4 is largely restricted to cells of the hematopoietic lineage, possibly reflecting a causal role for this molecule in differentiation/proliferation of hematopoietic lineage cells. In this study, we show that, like the human homologue, murine HTm4 is also predominantly a hematopoietic protein with distinctive expression patterns in developing murine embryos and in adult animals. In addition, we observed that murine HTm4 is highly expressed in the developing and adult murine nervous system, suggesting a previously unrecognized role in central and peripheral nervous system development.

Introduction

HTm4 is a member of a newly defined, extensive family of human and murine proteins. Each member of the MS4 (membrane-spanning four) protein group has a distinctive four-transmembrane structure. To date, few members of this family are well understood, but a diverse functionality is beginning to emerge. These functions include roles as cell surface signaling receptors and intracellular adapter proteins (Hulett *et al.* 2001, Ishibashi *et al.* 2001, Liang *et al.* 2001, Liang & Tedder, 2001, Barnhill *et al.* 2004). We demonstrated that HTm4 is an adapter molecule that regulates the function of the KAP phosphatase, a key regulator of cell cycle progression (Donato *et al.* 2002). HTm4, in humans, is a 214 amino acid protein whose expression is largely restricted to both mature and precursor cells of the hematopoietic lineage, which may reflect a causal role in driving the differentiation/proliferation of hematopoietic lineage-specific cells (Adra *et al.* 1994, 1999, Donato *et al.* 2002, Nakajima *et al.* 2004).

A second important feature of the biology of human *HTm4* is its genetic localization. Human *HTm4* maps to chromosome 11q13.1 (Adra *et al.* 1994), in close

**JLK and XY contributed equally to this work.

proximity to the FcεRI antigen receptor beta chain gene (Ra *et al.* 1989, Ravetch & Kinet 1991) and the gene for CD20 (Tedder *et al.* 1988), both of which are MS4 family members. The 11q13.1 region is linked significantly to human atopic phenotypes (Collee *et al.* 1993, Shirakawa *et al.* 1994, van Herwerden *et al.* 1995, Folster-Holst *et al.* 1998, Adra *et al.* 1999). Specifically, TaqI restriction fragment length polymorphism (RFLP) in the 3rd intron of the human *HTm4* showed a strong association with atopic asthma. This variant showed similar odds ratios to that of intron 2 of the FcεRIβ gene in severe asthma, as well as in severe atopy phenotypes. Interestingly, we found that HTm4 is highly expressed in basophils at both the mRNA and protein levels (Nakajima *et al.* 2004); hence, human *HTm4* is considered an atopy gene candidate on locus 11q13.1 (Adra *et al.* 1999).

In this report, we present data on the genetic sequence, genomic organization, protein structure prediction, biochemistry and tissue expression pattern of the murine HTm4 homologue. Our data show that, like the human homologue, murine HTm4 is also predominantly a hematopoietic protein with distinctive expression patterns in developing murine embryos and adults. We also report the unexpected finding that murine HTm4 is expressed in the developing murine nervous system. Moreover, we demonstrate that the functional interaction with the KAP phosphatase, previously demonstrated by our group in human cells, is also present in the murine system. Our work suggests a conservation of structure and function for HTm4 between the mouse and human systems.

Materials and methods

Cloning of murine HTm4 cDNA

Rapid Amplification of cDNA Ends (RACE)-PCR was used for the cloning of murine *HTm4* cDNA. Marathon-Ready BALB/c mouse spleen cDNA library (Clontech Laboratories Inc, Palo Alto, CA) was used as the template. Two gene specific primers were derived from a single EST sequence that was highly homologous to human *HTm4*. A gene specific antisense primer, 5′-CAT TCG TGT GGG GTT TCT CCT GC-3′, was paired with the 5′ primer supplied by the manufacturer to generate PCR product that would cover the 5′ end region of cDNA of interest, murine *HTm4*. In the same manner, a gene specific sense primer, 5′-GTT TAC AAC ACG TGT CCC ACC ACT TCA G-3′, was used to generate PCR product that would cover the 3′ end region of murine *HTm4* cDNA, when paired with the 3′ primer from the manufacturer. PCR products from both experiments were sequenced and compared. The longest contiguous murine *HTm4* cDNA sequence was generated through the merging of largest 5′ and 3′ PCR fragments at the overlapping region that was flanked by the aforementioned gene specific primers. Based on this sequence, a pair of murine *HTm4* specific primers was generated for the procurement of a full-length cDNA. The sequences for this pair of primers were 5′-CAA AGA AGA GAT TCG TTA AGC CTG AGG-3′, the sense primer, and 5′-CGC CTA AGG AAA TAT ATT TAT TCA TCC AGA TC-3′, the antisense primer. The final full-length murine *HTm4* cDNA was cloned into the pGEM-T vector (Promega, Madison, WI) and analyzed by DNA sequencing.

Cloning and sequencing of the murine HTm4 genomic sequence

A BAC genomic library of murine 129/SvJ DNA (Genome Systems Inc., St. Louis, MO) was screened by PCR using primers designed for the 5′ and 3′ ends of the gene. The positive clone was isolated, and its DNA purified, restricted and sub-cloned in the pBluescript vector (Stratagene, CA). The mouse genomic DNA insert (accession number: AY258288) was sequenced by direct BAC sequencing and also by sequencing the sub-clones using the BigDye Terminator Cycle Sequencing Kit and ABI prism-377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Murine HTm4 protein modeling

Murine HTm4 protein modeling was predicted and compared to human HTm4 by program 'SOSUI' (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) which was made for prediction of existence of transmembrane helices and determination of transmembrane helical regions in the proteins based on physicochemical properties of amino acid sequences such as hydrophobicity and charges. A 'primary' transmembrane helix is composed of the highly hydrophobic and successive region of the AA sequence by a hydropathy profile based on the Kyte–Doolittle hydropathy index (Kyte & Doolittle 1982), while a 'secondary' transmembrane helix is composed of less hydrophobic and some polar AA sequences of which parameters are calculated with amphiphilicity index of polar amino acids (Mitaku *et al.* 1995).

Generation of a polyclonal antiserum directed against murine HTm4

A peptide derived from the amino-terminus of murine HTm4 (amino acids 4–19: EETGGSVYQPLDESRH) was coupled to keyhole limpet hemocyanin and used to generate a rabbit polyclonal antiserum. We produced amino- and carboxyl-terminal fusion proteins (aa 4–19 and aa 197–213) of murine HTm4 with the GST protein using pGEX6P1 vector (Amersham

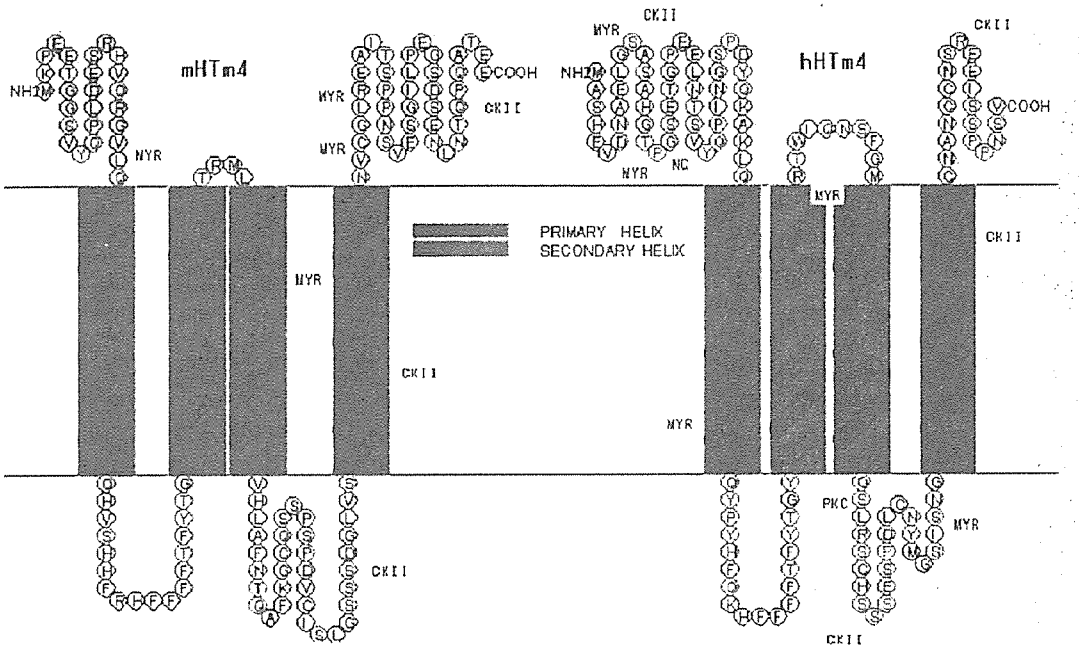

Figure 1. Comparison of the predicted model of murine HTm4 and human HTm4 proteins. The predicted murine (m)HTm4 (left panel) and human (h)HTm4 (right panel) protein structures were compared. Each figure shows topology with AA sequence from outer to inner side through membrane portion. Two outside loops are predicted for each protein as 'primary' transmembrane helixes (dark green loops), while two inside loops are predicated as 'secondary' transmembrane helixes (light green loops).

Pharmacia Biotech, Piscataway, NJ) in order to screen the reactivity of these antibodies. Affinity purification was performed by standard methods using the immunizing peptide.

Immunoprecipitation and western blotting

Samples consisted of either 5 μg murine HTm4 GST fusion proteins, or 20 μg proteins derived from BALB/c mouse spleen cells lysed in a standard SDS buffer. Protein samples were boiled for 8 min before loading. Cells were pelleted in a microcentrifuge (2000*g*, 2 min) and washed once in 1 ml ice cold phosphate buffered saline to remove serum proteins. 10^7 cells were lysed on ice for 30 min in 350 μl of the following buffer: 50 mM HEPES pH 7.4, 75 mM NaCl, 20 mM NaF, 10 mM iodoacetamide, 0.5% (w/v) Triton X-100, 1 mM phenlymethylsulfonyl fluoride (PMSF), 500 mg/ml Aprotinin, 1.0 mg/ml Leupeptin and 2.0 mg/ml Chymostatin. Lysates were clarified by microcentrifugation (10,000*g*, 5 min). Supernatants were transferred to clean tubes and either mixed with 1.4 volumes acetone and placed at −20 °C for 1 h (to pellet total protein) or tumbled at 4 °C for 2 h with the indicated antibody. Acetone precipitates were harvested by centrifugation at 10,000*g* for 5 min. Immunocomplexes were captured using 15 μl/tube Protein G-sepharose beads and washed using two rounds of microcentrifugation and re-suspension in 1 ml lysis buffer. Beads and protein pellets were dried by aspiration and then incubated at 95 °C for 8 min in a reducing SDS polyacrylamide gel electrophoresis (PAGE) loading buffer. After 10% SDS–PAGE, the resolved proteins were transferred to PVDF membrane by electroblotting and the membrane was blocked for 1 h using 5% BSA bovine serum albumin (Figure 3B) or 5% non-fat milk (Figure 5) in PBS pH 7.0. The membrane was then probed for either 2 h at RT (Figure 3B) using 1:1000 dilution of murine HTm4 antiserum, or 16 h at 4°C (Figure 5) using 1:2000 dilution monoclonal anti-KAP (BD Pharmingen, San Diego, CA.). After extensive washing (4 × 5 min in PBS/0.5% Tween-20) a developing antibody was applied (1 h at RT, 1:10,000 dilution of donkey anti-rabbit (Amersham, Piscataway, NJ) or sheep anti-mouse (Amersham, Piscataway, NJ) IgG coupled to HRP). After further washing bands were visualized using ECL according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Northern blotting procedures

Northern blot filters of Poly (A)+ RNA from normal mouse tissues were purchased from BD Biosciences, CLONTECH, Palo Alto, CA. The filters were probed with 32p-labelled full-length murine cDNA according to the published protocols (Adra *et al.* 1994, 1997). The blots have been normalized to beta-actin by the manufacturer.

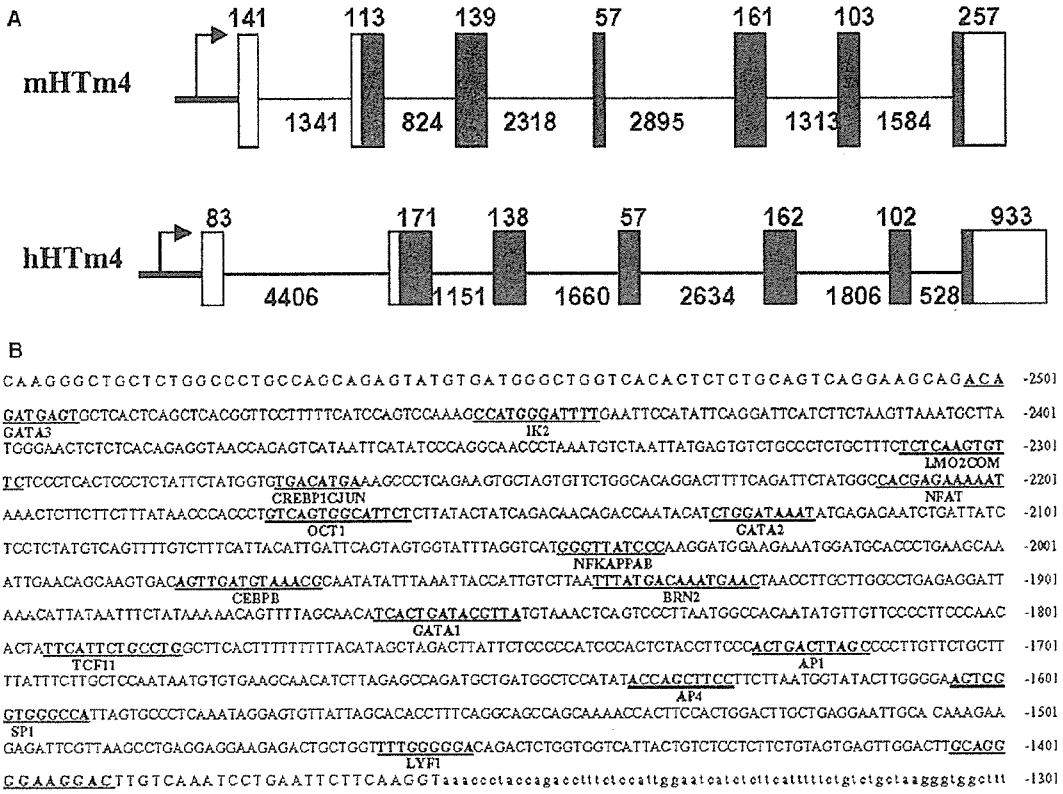

Figure 2. Structure and 5′ nucleotide sequence of the murine HTm4 gene. (A) Comparison of the complete gene structure of mouse HTm4 and human HTm4. Exons are shown as boxes and introns are connecting lines. The coding region is shaded. Exon and intron lengths are marked. Arrows are used to indicate the direction of transcription. (B) 5′nucleotide sequence of the murine HTm4 gene.
Accession number is AY258288. Sequence analysis started up to 1 kb upstream from the ATG (+1). The potential transcription factor binding sites were searched using MatInspector V2.2 (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl) (Quandt *et al.* 1995) and are shown in boldface and underlined.

Immunohistochemical studies for murine HTm4

All staining was performed by standard immunoperoxidase methods. Briefly, either embryonic murine tissue slides from day 8-day 16 or adult murine BALB/c tissue (Novagen, Madison, WI) were deparaffinized and pre-treated in 10 mM sodium citrate, pH 6.0 using a pressure cooker system (Decloaking chamber, Biocare Medical, Walnut Creek, CA as per manufacturer's instructions). All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with Peroxidase Block (DAKO, Carpinteria, CA) for 5 min to quench endogenous peroxidase activity, and a 1:5 dilution of goat serum in 50 mM Tris-Cl, pH 7.4, for 20 min to block non-specific binding sites. Affinity purified rabbit anti-murine HTm4 antibody (1:100 dilution in 50 mM Tris–Cl, pH 7.4 with 3% goat serum) was applied at room temperature for 1 h. After washing in 50 mM Tris–Cl, pH 7.4, secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Envision detection kit, DAKO, Carpinteria, CA) was applied for 30 min. After further washing, immunoperoxidase staining was developed using a DAB chromogen kit (DAKO, Carpinteria, CA), per manufacturer's instruction, and counterstained with hematoxylin. To assure specificity, murine HTm4 antibody was pre-incubated with the immunizing peptide in most experiments and applied to identical tissues, revealing complete blocking of staining.

Bone marrow cell preparation for flow cytometry analysis

C57/BL6 mice were used. Either femurs or tibias were harvested from mice without exsanguinations following cervical dislocation. The bone marrow contents were flushed with 4 °C PBS using a 26G hypodermic needle, thoroughly resuspended by gentle pipetting several times, and filtered through 30-μm-nylon mesh to remove remaining particulate material. Bone marrow cells were then washed twice with PBS buffer, pelleted by centrifugation at 1000 rpm for 10 min at 4 °C, resuspended, and counted. Cells were permeabilized with the Fix & Cell Permeabilization Kit, as described

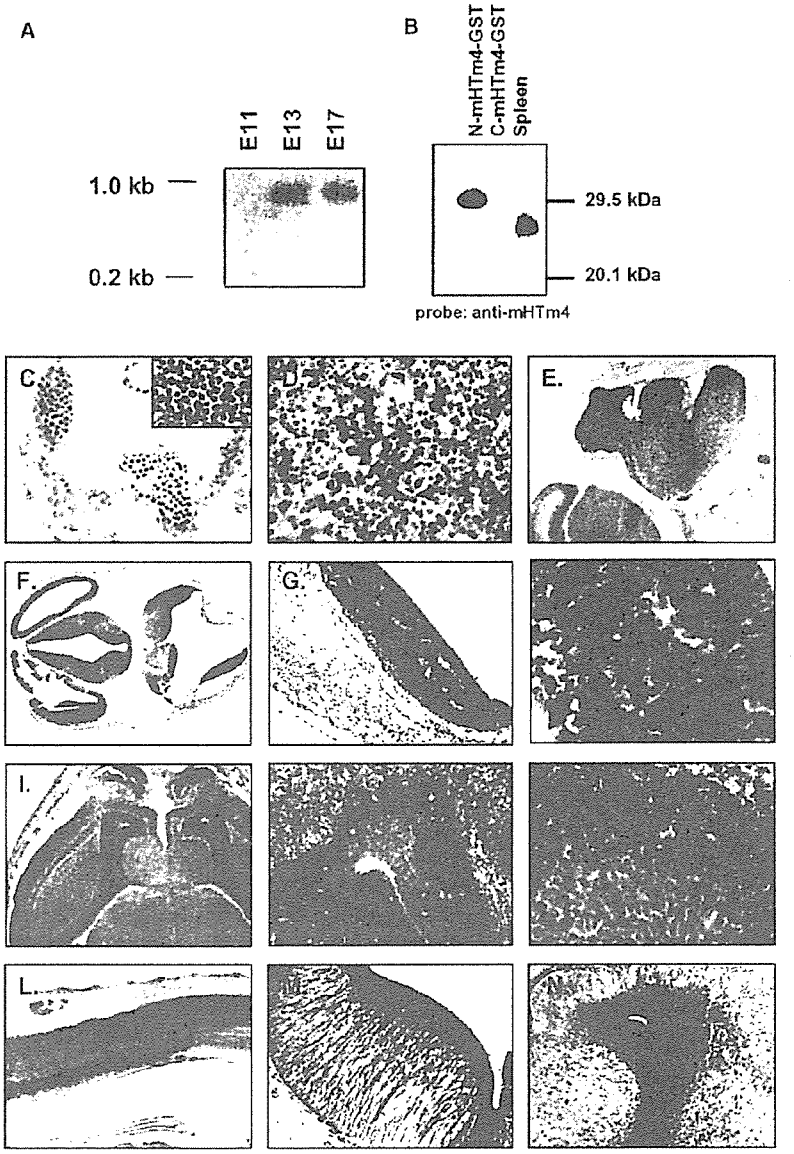

Figure 3. Murine HTm4 expression patterns in the embryonic mouse. (A) Northern blot analysis of HTm4 transcription during mouse embryogenesis. Poly A^+ RNA (approximately 2 μg/lane) was prepared from mouse embryos at the indicated stage of development (The Northern membrane was probed as described in Methods to detect the 1.0 kb HTm4 transcripts, which are detectable by this technique after day E11. This commercial blot has been normalized to beta-actin by the manufacturer (BD Biosciences, CLONTECH, Palo Alto, CA). (B) Validation of anti-murine HTm4 antibody by specific western blot. Polyclonal anti-murine HTm4 was generated against N-terminal amino acids 4-19 in rabbit as described in Methods. Protein samples corresponding to 5 μg/lane GST fusion proteins of either the amino- or carboxyl-terminal cytoplasmic tails of murine HTm4 (N-murine HTm4-GST, C-murine HTm4-GST), or total lysate from $1x10^6$ murine spleen cells, were resolved by 10% SDS–PAGE. After electrotransfer to PVDF membrane, western analysis was performed with 1 μg/ml anti-murine HTm4. Note the presence of approximately 25 kDa band in murine spleen and immunoreactivity for only the N-murine HTm4-GST fusion protein, which contains the immunogenic peptide. (C–N) Immunohistochemisty for murine HTm4 in embryonic murine hematopoietic and neural tissues. Representative sections of formalin-fixed paraffin embedded embryonic (E) murine tissues were stained with anti-murine HTm4 antibody. Positive staining (DAB chromogen with hematoxylin counterstain) was restricted to either developing hematopoietic cells within the blood islands (C; E8; 400× original magnification; inset 1000×) and liver (D; E14; 400× original magnification), or neural derived cells within the developing brain (E for E15, sagittal section, 40× original magnification; F–H for E12, cross-section, 20×, 100×, or 400× original magnifications, respectively; and I–K for E16, cross section, 20×, 100×, or 400× original magnifications, respectively). Strong expression of murine HTm4 was also detected within the spinal cord and dorsal and ventral ganglia starting at E14 (L; representative E15 embryo, 40× original magnification). Nestin staining is shown in panel M (E12, 40× original magnification) and panel N (E16 original magnification, 100×). Note the distinct patterns of murine HTm4 and nestin expression (panel G versus M for E12 and panel H versus N for E16) indicating diminished murine HTm4 protein expression in the more primitive periventricular cells of the developing brain compared to nestin.

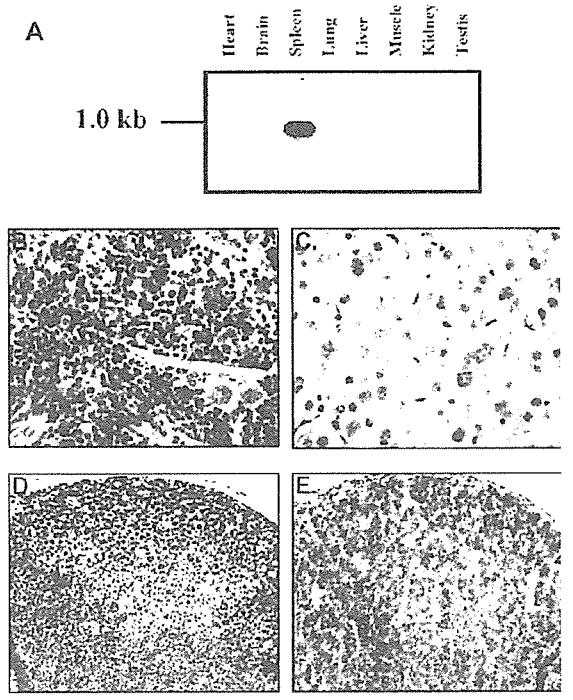

Figure 4. Murine HTm4 expression patterns in the adult mouse. (A) Northern analysis of HTm4 transcript distribution in adult mouse. Adult mouse multiple tissue Northern blot was probed with 32P labeled full length HTm4 cDNA. Of the tissue RNA extracts present on this membrane, only the spleen sample displayed significant hybridization. Visualized band corresponds to the approximately 1 kb murine HTm4 transcript. This commercial blot has been normalized to beta-actin by the manufacturer (BD Biosciences, CLONTECH, Palo Alto, CA). (B–E). Immunohistohemical staining of adult murine spleen, brain and lymph node for murine HTm4. Representative sections of formalin-fixed paraffin embedded adult murine spleen, lymph node, or brain were stained with anti-murine HTm4 antibody as described in Methods. Positive cytoplasmic staining of cells (DAB chromogen with hematoxylin counterstain) was restricted to either hematopoietic cells within the red pulp of the spleen (B; 400× original magnification) or neurons of the cerebral cortex of the adult murine brain (C; 400× original magnification). Germinal center B-lymphocytes within secondary lymphoid follicles also express murine HTm4 (D; 200× original magnification). The pattern of expression is identical to that of the murine HTm4 interacting protein KAP (E; 200× original magnification). (F) Flow cytometric analysis of HTm4 expression in cell populations derived from adult murine bone marrow. Mouse bone marrow cells were isolated as described in Methods. Aliquots of cells were co-stained with either control rabbit IgG or anti-murine HTm4 and antibodies to specific surface markers for various bone marrow subpopulations. Cell-type specific co-stains were as follows: B-lymphocytes (CD19), T-lymphocytes (CD3), monocytes (CD11b), nucleated red blood cells (Ter119), and granulocytes (GR-1).

by the manufacturer (Caltag Laboratories Inc., Burlingame, CA, USA). Cells were labeled with 2 μg/ml of polyclonal anti-murine HTm4 for 20 min, followed by the incubation with anti-rabbit IgG-FITC (Fab) (Caltag, Burlingame, CA), along with CD19-PE (BD Pharmingen, San Diego, CA), CD3-PE (BD Pharmingen), Ter119 (BD Pharmingen), Gr1-PE (BD Pharmingen) and CD11b-PE (Caltag). The flow cytometry analyses were performed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, CA). In the control group, bone marrow cells were labeled with 2 μg/ml of Rabbit IgG (Sigma, St. Louis, MO) for 20 min, followed by the incubation with anti-rabbit IgG-FITC (Fab) (Caltag).

Results

cDNA, genomic and protein sequence of murine HTm4

Our database analysis identified a murine expressed sequence tag (EST) that was highly homologous to the human *HTm4* cDNA sequence. This enabled the design of a specific PCR primer that was used to isolate a clone from a murine cDNA library. After several rounds of RACE-PCR and DNA sequence analysis, we obtained a single contiguous cDNA corresponding to murine *HTm4*. The cDNA and translated protein sequence of murine HTm4 was deposited in Gene Bank (accession number AY184359).

Murine HTm4 is a 213 amino acid protein with a predicted molecular weight of approximately 25 kDa. Proscan analysis reveals that murine HTm4 has no obvious enzymatic or protein–protein interaction domains, but does contain a single consensus sequence for *N*-glycosylation. Human HTm4 is similarly devoid of amino acid sequence clues to its functional role, and also contains a single *N*-glycosylation site and a PKC substrate motif.

We compared the protein sequences of murine HTm4 and human HTm4 using ClustalW alignment (Oxford Molecular and MacVector, Accelrys, San Diego, CA). Across the ~220 amino acid sequence, 119 identities (55%) and 27 functional similarities (12%) were found. Murine HTm4, like its human counterpart, has four putative transmembrane domains resulting in very similar predicted structures (Figure 1). Both murine HTm4 and human HTm4 proteins can be defined as members of the MS4A protein family, on the basis of their four-transmembrane structure and sequence homology. Murine HTm4 is designated MS4A3.

We have previously assigned the chromosomal location of human *HTm4* to 11q13.1 (Donato *et al.* 2002). Location of the murine gene for *HTm4* was assigned using genomic BLAST against the available mouse genome (NCBI BLAST facility). Probes were murine *HTm4* genomic sequence and human *HTm4* cDNA sequence, which gave identical results. Using this analysis, the murine *HTm4* gene was localized to chromosome 19A. We note that the murine *HTm4* gene is clustered in this location with the *MS4A2* gene, corresponding to the mouse *FcεRIβ* subunit and the gene for *MS4A6D*. This cluster is highly reminiscent of that

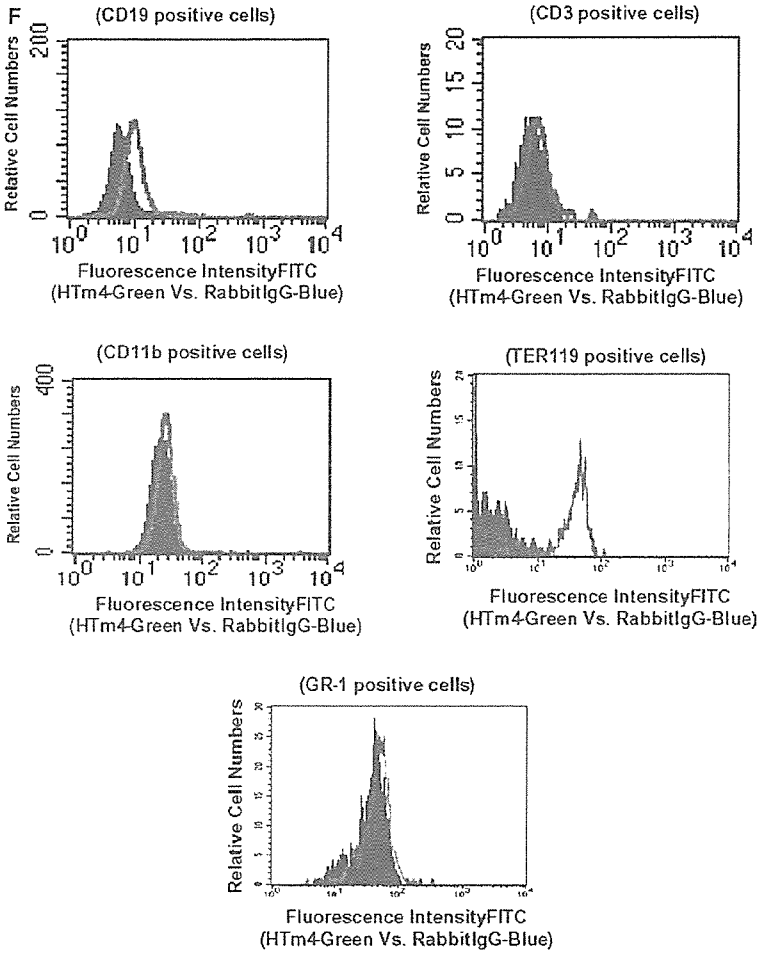

Figure 4. (Continued).

located in humans at chromosome 11q13.1, which has been linked to atopic/allergic disorders (Adra *et al.* 1999). Other members of the MS4A family are located at more distal locations also on the mouse chromosome 19, including *MS4A10, MS4A8 and MS4A7* (Ishibashi *et al.* 2001, Liang *et al.* 2001, Liang and Tedder, 2001).

Further analysis of the murine genomic sequence revealed that the murine *HTm4* gene has the same six-intron and seven-exon gene structure as the human HTm4 gene (Figure 2A). In addition, the full length of murine *HTm4* gene (Gene Bank accession number AY258288) spans approximately 11 kb, which is also comparable to the human *HTm4* gene (Adra et al., 1999). Sequence analysis up to 1 kb upstream from the ATG (Figure 2B) showed that the putative promoter region contains many potential binding sites for transcription factors that are involved in hematopoiesis, immune response and cell proliferation, such as AP1 (Mathas *et al.* 2002), CEBPB (Akira *et al.* 1990), IK1-2 (Georgopoulos *et al.* 1994) and GATA1-3 (Tsai *et al.* 1994, Pandolfi *et al.* 1995, McDevitt *et al.* 1997).

Murine HTm4 expression pattern in developing mouse embryos reveals restriction to hematopoietic and neural tissues

One of the most notable features of the human HTm4 protein is its relatively restricted tissue expression pattern. Current data suggest that human HTm4 is expressed, in adult tissues, in cells of the hematopoietic lineage. In our characterization of murine HTm4, we asked if this tissue specificity is also a feature of the mouse system. We first probed Northern blots for the presence of murine *HTm4* transcripts in RNA extracts from whole murine embryos at defined stages of development (Figure 3A). Sufficient levels of a ~1.0 kb murine HTm4 transcript are present for detection by at least day 13 and continue to be expressed at day 17. Northern analysis provides a valuable, but low-resolution, picture of the expression levels of a given transcript during development. In order to localize expression of murine HTm4 within specific tissuetypes, we performed immunohistochemistry on tissue from various embryonic stages. Initially, we validated the

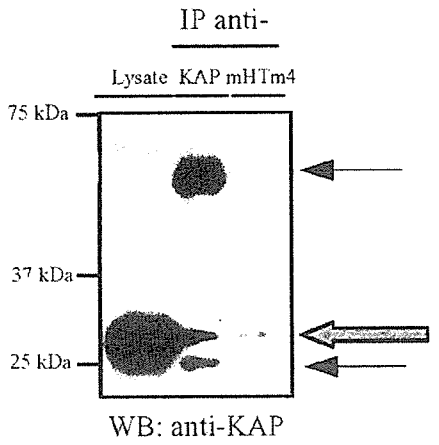

Figure 5. Co-immunoprecipitation of HTm4 and KAP proteins in murine leukocytes. P815 mouse mast cells (10^7 cells per lane) were lysed as described in Methods. Total protein precipitates (lysate) were produced by acetone precipitation or immunoprecipitation was performed using either 2 μg/lane anti-KAP (mouse monoclonal) or 11 μg/lane anti-murine HTm4 (rabbit polyclonal). Samples were resolved by 10% SDS-PAGE and visualized using anti-KAP western blot. KAP appears as a 32 kDa band in lysate from these cell (open arrow). KAP is also present in anti-KAP and anti-murine HTm4 immunocomplexes. Filled arrows mark presence of antibody heavy (55 kDa) and light (26 kDa) chains in immunoprecipitations using mouse anti-KAP and visualized with the same antibody.

specificity of our anti-murine HTm4 antibody raised against the N-terminal amino acids 4-19 via western blot. Figure 3B shows that our anti-murine HTm4 antibody specifically recognizes a 25-kDa protein from adult murine spleen cells (right lane). Importantly, anti-murine HTm4 also recognizes the amino-terminus of murine HTm4 when expressed as a glutathione *S*-transferase fusion protein (Figure 3B, left lane). A carboxy-terminal murine HTm4 fusion protein is not recognized (center lane). These data confirm the specificity of the antiserum for the amino-terminus of murine HTm4.

Immunohistochemical analysis was performed for murine HTm4 expression in formalin-fixed, paraffin-embedded day 8–16 embryonic murine tissues. As anticipated, hematopoietic expression was confirmed in murine development. We observed that from embryonic days (E) 8–10, murine HTm4 expression appears in small numbers of hematopoietic cells resident in blood islands and vessels (Figure 3C top left panel and inset). By E12–13, murine HTm4 expression is seen within the hematopoietic cells now resident in the liver, where mononuclear cells show positive staining (Figure 3D). Morphologically, these cells are compatible with both erythroid and myeloid precursors, with some staining also noted in megakaryocytes. At E14, expression persists in the liver hematopoietic cells and appears in splenic red pulp hematopoietic cells for the first time (data not shown). Staining was not present within non-germinal center B-cells of the spleen. Scattered cortical thymocyte staining was also noted, later in development (E15–16) (data not shown).

In addition to staining of murine embryonic hematopoietic cells, embryos from E8 through 16 stained with murine HTm4 show a developmental pattern of expression in the central nervous system (CNS) (Figure 3E–L). In general, murine HTm4 is expressed only in regions outside the ventricular zone, suggesting upregulation as cells begin to differentiate (Figure 3F–K). Specifically, at E8 and E9, the CNS is negative. By E10, however, expression is visible at the periphery of the neural tube (i.e., away from the ventricular zone neuropithelium) at all levels. Sagittal sections at E11–12 show expression that is somewhat stronger, particularly within the dorsal prosencephalon, mesencephalon, isthmal plate, cerebellar plate, and pontine plate, and along the ventral aspect of the spinal cord. Faint expression of murine HTm4 is noted in the trigeminal and paravertebral ganglia. At E12, faint expression appears in the anterior prosencephalon, and at E13, additional positivity is noted in the neocortex rostrally, the diencephalon ventral to the neuroepithelium, and along the peripheral (subpial) regions caudally, corresponding to dorsal and ventral gray matter columns. At this stage, the peripheral nerve roots are now faintly positive. At E14, sagittal sections demonstrate strong immunoreactivity in the ventral telencephalon and diencephalon, tegmentum of the mesencephalon, anterior and posterior pons, medulla, and ventral spinal cord, but absence of staining in the ventricular zone neuroepithelium. There is also staining in the peripheral nerves and paravertebral ganglia (Figure 3L). The tectum and the eye are negative. E15 sagittal sections show staining as in the E14 embryo, additionally with faint staining in the pretectum and dorsal root ganglia, and in the ganglion cell layer of the retina (Figure 3E). The ependyma of the fourth ventricle and the myenteric plexus of the gut are also positive. E16 sagittal sections additionally revealed staining in the olfactory bulb (a structure not identified in earlier age sections). To determine whether the HTm4-expressing cells were undifferentiated neural progenitor cells, serial sections were stained for nestin at E12 and E16 (Figure 3M and N). In these sections, nestin highlights ventricular zone neuroepithelial cells and radial (possibly glial) cell processes, but do not stain the presumed differentiating cells in the primitive neocortex and ventral diencephalon (at E12 and E16), the caudoputamen, anterior and posterior pontine nuclear groups, superior tectal neuroepithelium, superior central raphe and reticular formation of the midbrain and pons, or the subventricular zone (at E16) that are positive on HTm4 immunostaining (Figure 3F–K) Thus, an apparently non-overlapping pattern of expression is noted with these two antibodies. For all ages studied, appropriate negative controls (sections incubated with preimmune serum and/or

peptide, or with the secondary antibody only) showed no staining.

Murine HTm4 is expressed in cells of the hematopoietic lineage and brain in adult mouse

We analyzed the expression pattern of murine *HTm4* mRNA in adult animals using Northern blot. Assaying multiple tissues revealed that of the tissue RNA preparations probed in this experiment, only the spleen extract contained sufficient RNA for detection with the murine *HTm4* probe (Figure 4A). Transcript size of approximately 1 kb is consistent with the human *HTm4* mRNA. We also performed a dot blot analysis on adult mouse tissues and observed a similar degree of restriction in *HTm4* expression pattern (data not shown).

Immunohistochemical analysis of adult murine tissues yielded similar patterns to that seen in developing embryos. Strong cytoplasmic staining for murine HTm4 was noted within maturing hematopoietic cells of the spleen (Figure 4B) and in lymphoid cells (presumably B-lymphocytes) within germinal centers of lymphoid tissue of lymph nodes (Figure 4D) and Peyer's patches of the gut (not shown). As was previously demonstrated in human lymphoid tissue (Donato *et al.* 2002), murine HTm4 co-localized within germinal centers with mKAP protein (Figure 4E), a putative functional regulator of HTm4 activity. Additionally, in adult murine brain, murine HTm4 expression is identified in cells morphologically consistent with neurons in the hypothalamus, thalamus, cerebral cortex (Figure 4C), and, more faintly, in the hippocampus.

To further characterize the specific lineages of hematopoietic cells expressing murine HTm4, we isolated cells from murine bone marrow and spleen and assessed their expression of intracellular murine HTm4 by flow cytometry. Staining patterns from isolated hematopoietic cells were identical in both tissues. Representative data from bone marrow studies is shown in Figure 4F. Murine HTm4 expression was noted in Ter119 positive erythroid precursors and a smaller fraction of CD19-positive B-lymphocytes. Minimal murine HTm4 detection was observed in GR-1 positive granulocytic forms, CD11b-positive monocytic elements, or CD3 positive T-lymphocytes.

Association of murine HTm4 with the KAP phosphatase

In the human, we have proposed that HTm4 is involved in cell cycle regulation in hematopoietic cells, based on the association between HTm4 and the KAP phosphatase (Hannon *et al.* 1994, Poon & Hunter 1995) in human hematopoietic cells (Donato *et al.* 2002). In murine germinal centers, the cell populations that express murine HTm4 and KAP co-localize as assessed by immunohistochemical staining for these proteins (Figure 4D and E), raising the possibility that these proteins may be functionally associated. We asked if murine HTm4 is also physically associated with KAP. We identified a murine leukocytic line, P815, with expression of murine HTm4 and KAP. Co-immunoprecipitation using the P815 cell line demonstrates that immunocomplexes isolated using either anti-KAP or anti-murine HTm4 antibodies contain KAP protein (Figure 5). These data indicate that in the murine system, as in human cells, murine HTm4 and KAP form a physiological complex.

Discussion

In this study, we asked whether the cell cycle-associated protein HTm4, previously characterized by us in the adult human (Adra *et al.* 1994; Adra *et al.* 1999, Donato *et al.* 2002; Nakajima *et al.* 2004), shared similar structural, biochemical and functional features, in the developing and adult mouse. To that end, we undertook the cloning, genomic organization, and expression analysis of murine HTm4, and demonstrated its interaction with a protein known to interact with the human homologue of HTm4.

In our previous work, we cloned the human *HTm4* gene and assigned two key features to the protein. First, the expression of human HTm4 appears largely restricted to the hematopoietic cell lineages (Adra *et al.*, 1994). Second, a function for human HTm4 as an adapter protein that contributes to cell cycle progression was supported (Donato *et al.* 2002). Murine HTm4 is highly similar to human HTm4 at the DNA and amino acid level, and both proteins display a similar predicted four-transmembrane structure (Figure 1). Comparison to two reported HTm4 cDNA sequences (Hulett *et al.* 2001; Liang *et al.* 2001) revealed two amino acid sense variants (Ser162Ala and Ser211Thr). CLUSTAL alignment analysis and chromosomal grouping places murine *HTm4*, like human *HTm4*, in the MS4 protein superfamily (Hulett *et al.* 2001, Ishibashi *et al.* 2001, Liang *et al.* 2001, Liang & Tedder 2001). A similar genomic structure between the two genes is also identified with both genes containing a six-intron and seven-extron arrangement. Several promoter regions for transcription factors important for hematopoiesis, immune response and cell proliferation, such as AP1, CEBPB, IK1-2, and GATA1-3 are also identified. The presence of the GATA promoter regions is particularly interesting given the critical role of these transcription factors in erythopoiesis (Tsai *et al.* 1994, Pandolfi *et al.* 1995, McDevitt *et al.* 1997). The high level of expression of murine HTm4 in erythroid progenitors suggests GATA-regulated transcriptional regulation of HTm4 and a potential role for HTm4 in erythroid differentiation. Additionally, GATA-3 appears to be critical for neural development

as well (Pandolfi *et al.* 1995). Overall, we have found a high degree of conservation of structure and function of this gene and its protein across species, suggesting a fundamentally conserved biological function.

In addition to similarities at the genomic and protein level, the tissue expression pattern of murine HTm4 also has similarities to that seen in the human. Immunohistochemical techniques reveal expression of murine HTm4 within early hematopoietic cells of the blood islands starting at E8. Hematopoietic cell expression persists as hematopoiesis moves to the liver then spleen later in embryonic life. Immunohistochemical analysis of adult murine tissues confirms that murine HTm4 appears to continue to be largely hematopoietically restricted. Clusters of predominantly mononuclear cells present within the spleen react with antibody directed against murine HTm4. These cells morphologically appear most consistent with nucleated erythroid, myeloid and lymphoid elements. To confirm this morphologic impression and define more specifically the hematopoietic cell lineages expressing murine HTm4, multicolor flow cytometric analysis was performed on permeabilized, disaggregated spleen and bone marrow cells. These studies reveal that the majority of Ter119-expressing nucleated erythroid precursors display positive staining for murine HTm4. Very weak expression was noted within cells co-expressing the myeloid markers GR-1 and CD11b by flow cytometry. In addition, murine HTm4 co-expression was also noted in CD19-positive B-lymphocytes within the marrow. Further evidence for expression in the B-lymphoid population comes from the strong immunoreactivity for murine HTm4 antibody in germinal center B-cells in lymph nodes and Peyer's patches. The predominance of staining in germinal center cells as opposed to surrounding mantle or marginal zone cells suggests an upregulation of murine HTm4 during the germinal center reaction. Minimal staining was seen in the T-lymphoid cell population by immunohistochemistry or flow cytometry.

In contrast to previous human studies in which analysis of expression was restricted to mature tissues, the current work includes extensive study of both embryonic and adult murine tissues. Examination of the developing mouse has expanded the tissue distribution of expression from being solely hematopoietic, as in adult human tissues, to include prominent expression in the developing and mature murine nervous system, a novel and unexpected finding. By E10, immunohistochemical staining can detect murine HTm4 expression within cells that are morphologically consistent with differentiating neuronal and glial cells of the developing central nervous system, and by E15–16, prominent expression is seen as well in the peripheral nervous system. Importantly, there is no overlap in the expression of murine HTm4, most highly expressed in more mature neuronal and glial cells, and nestin, a protein that is associated with primitive neural cells. The strong expression of murine HTm4, and non-overlapping pattern of expression with nestin, in the developing brain suggests a role of murine HTm4 in CNS development and neural cell differentiation. Once a site expresses HTm4, it appears to retain expression into adulthood. Further analysis of the specific role of murine HTm4 expression in CNS cells is needed. These data also suggest that a CNS-based phenotype, in addition to an effect on hematopoiesis, may be a feature of experimental murine HTm4 deficient mice.

Our previous work on human HTm4 has suggested that this is an intracellular protein. Unlike several other MS4 family members (Tedder *et al.* 1988, Ravetch & Kinet, 1991), HTm4 is not present on the cell surface, but rather resides in the perinuclear compartment within the ER/Golgi apparatus. We have shown that human HTm4 is part of a complex containing the KAP phosphatase (Donato *et al.* 2002). Immunoprecipitation studies and western blotting confirm a similar biochemical association between murine HTm4 and KAP protein in a murine leukocyte cell line, P815, suggesting that murine HTm4 plays a similar role in mouse and human leukocytes. Although we have already detected HTm4 expression in primary human mast cells, basophils, eosinophils (Adra *et al.* 1994, Nakajima *et al.* 2004), and also in murine P815, another group did not detect HTm4 mRNA in P815 by PCR (Hulett *et al.* 2001). This could be due to a very low copy number of mRNA that nevertheless corresponds to a very high level or protein. It is also possible that HTm4 mRNA may not have been detectable in their experiments. We have not yet been able to conduct similar studies on CNS cells that express murine HTm4, and thus we do not know if murine HTm4 in the embryonic CNS cells also physically interacts with KAP. Interestingly, preliminary data (not shown) using immunohistochemical staining suggest that murine HTm4 and KAP may co-localize in developing CNS, but proof of this interaction will require co-purification from dissociated embryonic CNS cells.

In summary, we have examined the distribution of murine HTm4 in murine tissues. Murine HTm4 distribution in adult mice closely resembles the highly restricted pattern seen in adult human tissues. In studying the murine embryo, we have revealed a potentially exciting facet of HTm4 biology and function, namely, the strong expression in the developing CNS, and apparent persistence in a subset of adult neural cells. Other aspects of HTm4 biology are closely parallelled between murine and human systems. In hematopoietic cells from both systems, HTm4 binds with KAP and therefore, likely functions as a component of the cell cycle machinery. These data will aid in the creation and analysis of a murine HTm4 deficient