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ChT1, an Ig Superfamily Molecule Required for T Cell Differentiation$^{1,2}$

Kaisa Katevuo, Beat A. Imhot,$^1$ Richard Boyd,$^3$ Ann Chidgey,$^3$ Andrew Bean,$^3$ Dominique Dunon,$^8$ Thomas W. F. Göbel,$^9$ and Olli Vainio$^*$

The thymus is colonized by circulating progenitor cells that differentiate into mature T cells under the influence of the thymic microenvironment. We report here the cloning and function of the avian thymocyte Ag ChT1, a member of the Ig superfamily with one V-like and one C2-like domain. ChT1-positive embryonic bone marrow cells coexpressing c-kit give rise to mature T cells upon intrathymic cell transfer. ChT1-specific Ab inhibits T cell differentiation in embryonic thymic organ cultures and in thymocyte precursor cocultures on stromal cells. Thus, we provide clear evidence that ChT1 is a novel Ag on early T cell progenitors that plays an important role in the early stages of T cell development.

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The following cDNA sequences have been submitted to the EMBL database with accession numbers Y14063 and Y14064.

Materials and Methods

Animals

H.B2 and H.B15 chickens and chicken embryos from the colonies at the Department of Medical Microbiology, Turku University (Turku, Finland), two ov-Ag congenic H.B19 strains at the Basel Institute for Immunology

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ChT1, A NEW MARKER IN T CELL DIFFERENTIATION

(Gipf-Oberfrick, Switzerland, and the Australphor × White Leghorn hybrids at the Research Poultry Farm, Research, Australia) were used. The two congenic lines, H.B19ov2 and H.B19ov, differ in their expression of the MHC class I, IgM and T cells. The ov-Ag is recognized by mAb 11A9 (15, 21).

Abs and flow cytometry

Abs detecting the ChT1 Ag were TiAg, IgG1 (19), CT1 (IgG1) and CT1a (IgG3) (18), RRS-89 (IgG2b), C3c210 (IgM), MUI-83 (IgG1) (22), and 2-1 (IgG1). Other Abs were kit2c75 (IgG2a) against c-kit (16), 11A9 (IgM) against ov-Ag, 2-6 (IgG1) and 2-35 (IgG2b) against CD3 (23, 11-39 (IgG1), 3-298 (IgG2b) against CD8a (24), MUI-78 (IgG2a) against MHC class II (25), 0-21-25 (IgG1) against the YAC-1.0 TCR (26), MUI-36 (IgG2a), MUI-53 (IgM), MUI-54 (IgM), MUI-70 (IgM), MUI-81 (IgG2a), TCR1 (IgG1) against y8TCR, TCR2 (IgG2) against αβITCR, TCR3 (IgG1) against αβITCR, and CT3 (IgG1) against CD3 were purchased from Southern Biotechnology Associates (Birmingham, AL). Abs were used as ascites, hybridoma supernatant, or purified Ab. Ab purification and conjugation to biotin (biotin-

Sialic acids were digested with 3 U/ml neuraminidase at 37°C for 2 h after the samples in the presence of 50 U/ml enzyme digestions.

37°C or with 0.5% SDS/0.1 M 2-ME for 20 min at 80°C for glycolytic absorbed molecules were eluted with Laemmli sample buffer for 45 min at 4°C. Insoluble nonidet p-40 (Calbiochem, La Jolla, CA) for 45 min at 4°C. Insoluble

temdic isotypes). For three-color analysis cells were blocked with 1% normal Mouse conjugates. For two-color analysis monoclonal Ab against CD4, CD8, and CD3 were detected with PE-conjugated anti-IgG2a with FITC-conjugated anti-IgG1. A double-stained population was scored.

The COS cell expression screening method was used to clone the cDNA encoding for ChT1 Ag from two different chicken cDNA libraries. The thymus cDNA library in pCDM8 vector from an adult RPRL Line 0 animal was a gift from Dr. J. R. Young. The other library was constructed from mRNA of strain H.B19 E13 thymus into vector pcDNA3 (16). The transfection and staining were performed as described by Tregaskes and Young (33). Positively stained cells were picked up from the slide with a Drummond sequencing pipette (Drummond Scientific, Broomall, PA). Proteins were precipitated, after which the DNA was extracted. The plasmid DNA containing the desired cDNA insert was then introduced to Escherichia coli (MC1061/p63 or TOP10/F') by electroporation. For sequencing of the cDNA, the plasmid DNA was extracted using Qiagen spin columns (Qia-gen, Chatsworth, CA) and was sequenced with either dyeodeoxy chain termination (Sequenase version 2.0, U.S. Biochemical Corp., Cleveland, OH) or automated cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer, Norwalk, CT) with an ABI 373A DNA Sequencer (Applied Biosystems). cDNA sequences that have been submitted to the EMBL database with accession numbers Y14063 and Y14064. Sequence data analysis and sequence comparisons were performed using the Wisconsin University software package GCG (Genetics Computer Group, Madison, WI), Lasergene (DNASTar, Madison, WI), and Blast (National Center for Biotechnology Information, Bethesda, MD)

Analysis of mRNA expression

Northern blot was performed with mRNA isolated from H.B2 chicken thymus, spleen, liver, bursa, ileum, colon, brain, lung, kidney, testis, and ovary as well as with Marek’s disease virus-transformed MDCD-CU32 (CU32), MDCD-CU36 (CU36) and reticuloendothelial virus-transformed REVCC-RP13 (RP13) chicken cell lines using RNAeasy and Oligotex mRNA Spin column kits (Qiagen). After electrophoretic separation on a 1.2% formaldehyde agarose gel the samples were transferred overnight to nitrocellulose membrane (Hybond-N+, Amersham). The membrane was prehybridized in 1% SDS, 5× SSPE, 0.1% dextran sulfate, and 20 μg/ml ssDNA at 60°C for 6 h. For hybridization the membrane was incubated at 60°C overnight in fresh hybridization solution with total cDNA of ChT1 or a PCR product that encodes for a 1030-bp part of the chicken β-actin cDNA (34) as probes. These were labeled with [α-32P]dCTP (3000 Ci/mM; Amersham) using a Rediprime labeling kit (Amersham). Membranes were hybridized in 2× SSC, 0.1% SDS for 3 h at 42°C, washed in 0.5× SSC/1% SDS at 60°C for 15 min followed by washing in 0.5× SSC/1% SDS at 60°C for 15 min. Signals were visualized by autoradiography.

To study the mRNA expression of ChT1 further by RT-PCR, 0.1 μg of total RNA from spleen, bursa, liver, small intestine, skeletal muscle, and Marek’s disease virus-transformed MDCD-CU20 (CU20), CU36, and RP13 cells was used in each RT-PCR reaction (Titan One Tube RT-PCR Kit, Boehringer Mannheim, Mannheim, Germany). The SMART PCR cDNA Library Construction Kit (Clontech, Palo Alto, CA) was used according to the manufacturer’s instructions to prepare cDNA from E13

To study the mRNA expression of ChT1 further by RT-PCR, 0.1 μg of total RNA from spleen, bursa, liver, small intestine, skeletal muscle, and Marek’s disease virus-transformed MDCD-CU20 (CU20), CU36, and RP13 cells was used in each RT-PCR reaction (Titan One Tube RT-PCR Kit, Boehringer Mannheim, Mannheim, Germany). The SMART PCR cDNA Library Construction Kit (Clontech, Palo Alto, CA) was used according to the manufacturer’s instructions to prepare cDNA from E13
bone marrow mRNA for analysis of ChT1 expression in the bone marrow. One microliter of amplified cDNA was used for PCR in a 50-μl reaction, and the PCR conditions were as follows: 96°C for 2 min; then 35 cycles of 96°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. ChT1-specific oligonucleotides were 5′-GTG ACC GTT CCT GAG AAG- and C3′-GTG ACC GTT 72°C for 5 min. ChT1-specific oligonucleotides were V5′-GTG ACC GTT CCT GAG AAG- and C3′-GTG ACC GTT 96°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. ChT1-specific oligonucleotides were V5′-GTG ACC GTT CCT GAG AAG- and C3′-GTG ACC GTT 96°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. ChT1-specific oligonucleotides were V5′-GTG ACC GTT CCT GAG AAG- and C3′-GTG ACC GTT

In vitro thymic stromal cell and thymocyte precursor coculture

In vitro thymic stromal cell and thymocyte precursor coculture was performed as previously described (36) in medium that contained purified ChT1 in a concentration of 100 μg/ml specific mAb MUI-83 (dialyzed against RPMI 1640 culture medium). Control cultures lacked mAb, contained an irrelevant, isotype-matched control mAb (10-2.16) that was not reactive with chicken tissues, or contained other chicken thymus- or stromal cell-specific mAb (MUI-36, MUI-53, MUI-54, MUI-70, MUI-71, MUI-78, MUI-82). The results were identical for all control conditions; hence, only the one with isotype-matched control mAb has been included herein. The frequency and cell yield of the various thymocyte subsets present in control vs mAb-treated ETOC were statistically compared using unpaired Student’s t test (*, p < 0.05; †, p = 0.01; §, p > 0.001).

In vitro thymic stromal cell and thymocyte precursor coculture

For ETOC, thymus lobes were isolated on E10 and cultured for 6 days as previously described (36) in medium that contained purified ChT1 in a concentration of 6.7 × 10^5 cells/ml (37, 38). T cell precursors were mixed with the stromal cells at a ratio of 5:1 and cocultured as hanging drops in inverted Terasaki plates at 40°C in 10% CO2. Cells were harvested on day 5 and were stained with anti-ov mAb 11A9 followed by staining with anti-CD4 and anti-CD8 mAb. Cells were analyzed by flow cytometry using a FACScan (Becton Dickinson).

Results

Tissue distribution and ontogeny of ChT1

Flow cytometric analysis of adult lymphoid tissues showed that approximately 90% of the thymocytes expressed ChT1; in the spleen 5–20% (n = 12; mean ± SD, 11.3 ± 4.0%) of cells were positive, and in the peripheral blood 5–15% (n = 13; mean ± SD, 8.3 ± 5.3%) of cells were positive (Fig. 1A). In the embryo, ChT1+ cells are already present in the thymus on E10 (mean ± SD in three experiments, 4.7 ± 1.9%; data not shown). By E14 expression had reached its adult level, demonstrating that this Ag appears very early on differentiating T-lineage cells (18–20). ChT1-specific mAb 2-1 and MUI-83 also stained a subpopulation (n = 7; mean ± SD, 4.0 ± 3.2%) of embryonic bone marrow cells (Fig. 1B). These cells did not express CD3/TCR complex (data not shown). As expected from the large number of ChT1-expressing cells in the thymus, the Ag was found on thymocytes at all stages of differentiation, defined by the expression of CD4 and CD8 (20).

FIGURE 1. Tissue distribution of ChT1. A. Thymocytes, PBL, and spleen lymphocytes from a young adult chicken were stained with anti-ChT1-specific mAb RRS-89 followed by FITC-conjugated anti-mouse-Ig. The figure shows a representative example of each tissue. The closed histogram shows the expression of ChT1; the open histogram is the negative control for each sample. The percentage of positive cells is shown in the upper right corner and is marked by a vertical bar. B, E13 thymocytes and bone marrow cells were stained with anti-ChT1 mAb 2-1 and were detected by anti-mouse IgG1-FITC. The closed histogram shows the expression of ChT1; the open histogram is the negative control for each sample. The percentage of positive cells is shown in the upper right corner and is marked by a vertical bar. C, Spleen lymphocytes from a young animal were stained with anti-ChT1 mAb RRS-89 and with mAb detecting different TCR subpopulations followed by FITC- and PE-conjugated anti-isotype Ab. The percentage of positive cells in each quadrant is shown in the upper corner.

Embryonic thymus organ culture

For ETOC, thymus lobes were isolated on E10 and cultured for 6 days as previously described (36) in medium that contained purified ChT1 in a concentration of 100 μg/ml specific mAb MUI-83 (dialyzed against RPMI 1640 culture medium). Control cultures lacked mAb, contained an irrelevant, isotype-matched control mAb (10-2.16) that was not reactive with chicken tissues, or contained other chicken thymus- or stromal cell-specific mAb (MUI-36, MUI-53, MUI-54, MUI-70, MUI-71, MUI-78, MUI-82). The results were identical for all control conditions; hence, only the one with isotype-matched control mAb has been included herein. The frequency and cell yield of the various thymocyte subsets present in control vs mAb-treated ETOC were statistically compared using unpaired Student’s t test (*, p < 0.05; †, p = 0.01; §, p > 0.001).

In vitro thymic stromal cell and thymocyte precursor coculture

In vitro thymic stromal cell and thymocyte precursor coculture was performed as previously described (37). E13 T cell precursors were obtained from ov† congenic chicks. The thymocyte suspensions were labeled with anti-CD4 and anti-CD8 mAb and then sorted for CD4+ CD8− lymphoid cells on a FACStar Plus (Becton Dickinson). The sorted CD4+ CD8− cells were prepared at a concentration of 3.4 × 10^6 cells/ml. Where relevant, purified anti-ChT1 (MUI-83) and anti-c-kit (kit2c75) Abs were added at a concentration of 100 μg/ml. Several control mAbs that were reactive with either chicken thymocytes or stromal cells were also used. Stromal cells from adult chickens (ov†) were prepared by enzymatic digestion of lymphocyte-depleted thymi using 0.15% collagenase/0.1% DNase (Boehringer Mannheim). Stromal cells were enriched by elutriation and resuspended at a concentration of 6.7 × 10^6 cells/ml (37, 38). T cell precursors were mixed with the stromal cells at a ratio of 5:1 and cocultured as hanging drops in inverted Terasaki plates at 40°C in 10% CO2. Cells were harvested on day 5 and were stained with anti-ov mAb 11A9 followed by staining with anti-CD4 and anti-CD8 mAb. Cells were analyzed by flow cytometry using a FACScan (Becton Dickinson).
ChT1 is expressed on cells that have a capacity to differentiate into thymocytes and prethymic stage. We analyzed these two cell populations from E13.5 thymocytes by staining with anti-ChT1 and anti-c-kit mAb. The cells were sorted into two populations (region 1 and region 2) for intrathymic injection of 1000 ChT1+ cells with mean chimerism of 13.7% and 6.8%, respectively. To confirm the specificity of mAb and to show the start of T cell development (data not shown).

ChT1 cDNA cloning and protein characterization

To clone the cDNA encoding the ChT1 Ag, we screened COS cells transiently transfected with adult RPRL Line 0 thymus cDNA library with various mAb. Clone p10.6 (1114 bp) was isolated with mAb T10A9. The specificity of other mAb (e.g., 2-1, RR5-89, MUI-83, CT1, CT1a) for ChT1 was confirmed by their reactivity with p10.6-transfected COS cells. Another independent clone, pc210 (1087 bp), was isolated with mAb cF3c210 from a cDNA library made from E13 H.B19 thymus mRNA. The complete nucleotide sequence from both strands of these two clones was then determined (Fig. 3). The cDNA clones p10.6 and pc210 consist of a 1008-bp open reading frame encoding a 21-aa leader peptide, a 28-bp and an 8-bp, respectively, 5'-untranslated region, a 212-aa extracellular region, a 24-aa transmembrane region, and a 78-aa cytoplasmic part followed by a 78-bp and a 5'-untranslated region that does not contain a poly(A) tail. The coding region sequences of the two clones are identical except for one nucleotide difference in the transmembrane region at position 765. Internal peptides were obtained by partial microsequencing of the purified protein of 46 kDa resulted in a 30-residue sequence, VVTVTPKETNVKGGGx-ATLXXTYTSSQPL, which completely matched the predicted amino acid sequence. Internal peptides were obtained by partial digestion with an endoproteinase Lys-C. These peptides were sequenced, and two sequences, residues 87–96 (DRITAATSPG) and contents were measured by flow cytometry with the ov-allotransplantation-specific mAb 11A9 at 2 wk after injection. A clear thymus reconstitution was obtained by 1000 or even 100 ChT1+ cells with mean chimerism of 13.7 and 6.8%, respectively. In contrast, the injection of ChT1+ cells resulted in only 2.9 and 0.9% chimerism, respectively (Table I and Fig. 2B). The chimeric cells were analyzed for their capacity for thymus reconstitution by the surface expression of CD4, CD8, and TCR. The majority of the donor cells expressed both CD4 and CD8, and only a small fraction of the injected cells remained at the CD4+CD8− stage. The bone marrow progenitors also developed into CD4+CD8+ single-positive thymocytes (Fig. 2C). Differentiated thymocytes expressed both γδ- and αβ-TCR, indicating that the ChT1+ embryonic bone marrow precursors have a capacity to mature along all pathways of T cell development (data not shown).

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<table>
<thead>
<tr>
<th>Injected Cell Population</th>
<th>No. of Injected Cells/Thymus Lobe</th>
<th>ov° Cells/Animal (%)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChT1+ c-kit+</td>
<td>1000</td>
<td>13.8, 14.7, 12.7</td>
<td>13.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.6, 9.4, 6.5</td>
<td>6.8 ± 2.4</td>
</tr>
<tr>
<td>ChT1+ c-kit+</td>
<td>1000</td>
<td>1.1, 2.0, 2.9</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.7, 1.1, 1.0</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Table I. Thymus reconstitution capacity of ChT1+ c-kit+ and ChT1+ c-kit− cells

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FIGURE 2. Thymus reconstitution capacity of ChT1+ c-kit+ embryonic bone marrow cells. A, E13.5 bone marrow cells from ov° strain were stained with anti-ChT1 and anti-c-kit mAb. The cells were sorted into ChT1+ c-kit+ (region 1) and ChT1+ c-kit− (region 2) populations for intrathymic injections into ov− congenic chickens. B, Two weeks after the intrathymic injection of E13.5 bone marrow cells, the donor cells in the recipient thymi were analyzed by staining with anti-ov-specific mAb 11A9. The histogram shows a representative example of the reconstitution capacity of 1000 ChT1+ c-kit+ cells from three experiments. The horizontal bar marks the ov° population. C, The ov° cells from B were gated and analyzed for CD4 and CD8 expression to study the T cell differentiation of the injected cells.

ChT1+ thymocytes and peripheral T cells included γδ T cells as well as both Vβ1- and Vβ2-expressing αβ T cells (Fig. 1C). Together these results show that ChT1 is highly expressed on most thymocytes and prethymically on a subpopulation of embryonic bone marrow cells as well as on a subset of peripheral T cells.

A ChT1-positive population of embryonic bone marrow cells contains T cell progenitors

The E13.5 bone marrow cells can be divided into ChT1+ c-kit+ and ChT1+ c-kit− populations (Fig. 2A). To determine whether ChT1 is expressed on cells that have a capacity to differentiate into T cells in vivo, we sorted these two cell populations from E13.5 bone marrow of congenic H.B19ov° animals and injected the cells intrathymically into 14-day-old H.B19ov° recipients. Thymus reconstitution was measured by flow cytometry with the ov-allotransplantation-specific mAb 11A9 at 2 wk after injection. A clear thymus reconstitution was obtained by 1000 or even 100 ChT1+ c-kit+ cells with mean chimerism of 13.7 and 6.8%, respectively. In contrast, the injection of ChT1+ c-kit+ cells resulted in only 2.9 and 0.9% chimerism, respectively (Table I and Fig. 2B). The chimeric cells were analyzed for their capacity for thymus reconstitution by the surface expression of CD4, CD8, and TCR. The majority of the donor cells expressed both CD4 and CD8, and only a small fraction of the injected cells remained at the CD4+CD8− stage. The bone marrow progenitors also developed into CD4+CD8+ single-positive thymocytes (Fig. 2C). Differentiated thymocytes expressed both γδ- and αβ-TCR, indicating that the ChT1+ embryonic bone marrow precursors have a capacity to mature along all pathways of T cell development (data not shown).

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To further confirm the specificity of mAb and to draw the start of the mature polypeptide, the ChT1 protein was purified from thymus lysates by an immunofluorescence column using mAb RR5-89. N-terminal microsequencing of the purified protein of 46 kDa resulted in a 30-residue sequence, VVTVTPKETNVKGGGx-ATLXXTYTSSQPL, which completely matched the predicted amino acid sequence. Internal peptides were obtained by partial digestion with an endoproteinase Lys-C. These peptides were sequenced, and two sequences, residues 87–96 (DRITAATSPG) and...
residues 130–150 (xVIVNVLVKPSKPFxKIEGTP), were assembled. Both internal sequences showed 100% identity to the deduced amino acid sequence and spanned the first half of the extracellular region.

The anti-ChT1-specific mAb immunoprecipitated four bands from a surface-labeled thymocyte lysate as analyzed by SDS-PAGE: a major band of 63 kDa and its putative dimer of 138 kDa as well as minor bands of 46 and 121 kDa, which are probably degradation products of the monomer and putative dimer bands, respectively (Fig. 4). A similar pattern was present under reducing, nonreducing, and mild detergent (digitonin) conditions (data not shown). The material that was precipitated with mAb RR5-89 was digested with V8 protease, and SDS-PAGE analysis of the digestion products revealed a similar peptide pattern for each band (data not shown). This suggests that the complex immunoprecipitation pattern is composed of a single polypeptide. The glycosylation of ChT1 was studied by removing N- and O-linked carbohydrates. After treatment with N-glycanase, the core protein of the major band measured about 46 kDa. Additional treatments with neuraminidase and O-glycanase did not change the migration of the protein (data not shown).

Analysis of the mRNA expression of ChT1

Northern blot analysis with a probe containing the total cDNA of ChT1 revealed two mRNA species, one major band of 4.7 kb and a fainter band of 4.0 kb from the thymus, but no ChT1-specific mRNA was detected in any other organ or cell line tested (Fig. 5A and data not shown). Using a more sensitive RT-PCR method we were also able to show ChT1 mRNA in 4-wk-old chick spleen and E13 bone marrow (Fig. 5B). After hybridization of the PCR products with a ChT1-specific oligonucleotide probe, bursa, liver, and small intestine also showed a very low amount of ChT1 mRNA, most likely indicating the presence of a few ChT1+ T-cells in these tissues (Fig. 5B).

ChT1 is an IgSF member consisting of two Ig domains

Structural analysis of the deduced ChT1 protein sequence identifies the molecule as a member of the IgSF consisting of two Ig domains: an N-terminal V-like domain and a membrane-proximal C2-like domain. Both represent typical Ig domains, as all cysteines forming intradomain disulfide bridges are conserved in the B and Fβ strands, and the tryptophans were conserved at positions 58 and 173 in the C strands. The cysteines at positions 144 and 221 are located in the A and G strands in the C2-like domain, so that during protein tertiary structure formation they may come close enough to form an additional intradomain disulfide bridge. At the end of the V-like domain the sequence AGQSQKSVIVNVLV resembles a J-like segment characterized by features of a diglycine bulge (39, 40) (Fig. 3). However, the J-like segment in ChT1 is modified so that the second glycine is replaced by a serine. The extracellular part of ChT1 contains four possible N-glycosylation sites at aa positions 38, 97, 199, and 218 (Fig. 6). The putative hydrophobic transmembrane region is followed by a long cytoplasmic tail rich in glutamic acids. The carboxyl-terminal end of ChT1 was studied by removing N- and O-linked carbohydrates.
the molecule (residues 309–333) probably forms an α-helical structure. In the cytoplasmic region, Ser265 and Ser266 belong to consensus motif R/KX2/S/T for cAMP phosphorylation. Ser266, Ser278, and Ser307 are potential casein kinase II phosphorylation sites (motif SX2E; Fig. 3).

**ChT1 is homologous to CTX**

Comparative sequence analysis indicated that ChT1 is most homologous to the *Xenopus* thymocyte Ag CTX (41). Overall identity to CTX is 41% (identical amino acids/total amino acids in ChT1), and similarity is 60%. Homologies between the V- and C2-like domains are 48 and 47%, respectively. The transmembrane region shows the highest identity at 54%. In contrast, homology of the cytoplasmic portion is lower, only 23%. All N-glycosylation sites as well as the two extra cysteines in the C2-like domain forming a putative disulfide bridge within the domain are fully conserved between ChT1 and CTX (Fig. 6).

Other proteins that were closely related to ChT1 included HCAR/MCAR (24% identity; human and mouse coxsackie and adenovirus receptors) (42), human A33 Ag (23% identity) (43), myelin P0 protein, and Ig heavy and λ-chains of different species. In contrast to the extracellular regions the cytoplasmic part did not show significant homology to any known proteins in the databases.

**Anti-ChT1 mAb inhibits thymocyte differentiation**

ChT1 is one of the earliest markers expressed during T cell development; therefore, its potential functional role was investigated by the addition of purified anti-ChT1 mAb to ETOC. Chicken ETOC provides a structurally intact thymic microenvironment that allows both lymphoid and stromal cells to develop much as they would in the intact embryo. As for mammals, it thus represents a valid model for functional studies (36, 44). Thymic lobes were carefully removed on E10 and were cultured in the presence (100 μg/ml) of purified anti-ChT1-specific mAb MUI-83, isotype-matched control mAb, or no mAb. Penetration of cultured lobes by MUI-83 and its binding to the target thymocytes was verified by immunohistology and flow cytometry using anti-mouse Ig-FITC (data not shown). In cultures treated with isotype-matched control mAb and a panel of other mAbs against lymphoid and thymic stromal cells, there was no effect on T cell development. The treatment with anti-ChT1 mAb for 6 days, however, had multiple effects on thymopoiesis. It caused a significant (p \( \leq 0.001 \)) decrease in ChT1 high cells from >60% in control cultures to approximately 5% (data not shown). The total number of viable thymocytes per lobe was also decreased to 39% of that in control cultures, from \( 2.6 \times 10^5 \) to \( 1 \times 10^5 \) (p \( \leq 0.001 \)). Such a marked decrease in cell number was reminiscent of mouse fetal thymic organ cultures treated with anti-CD3, which induced apoptosis of immature thymocytes (45). However, electron microscopic examination of MUI-83-treated thymus lobes showed no evidence of extensive cell death, in contrast to lobes that were cultured with both anti-CD3 or ionomycin (data not shown).

The effect of anti-ChT1 on thymocyte subsets, defined by the expression of CD3, CD4, and CD8, was also examined (Table II). The major effect was a block in development downstream from the precursor CD3+CD4+CD8− cells (which were proportionally increased relative to the control cultures), involving markedly reduced CD3+CD4+CD8− immature intermediates (25% of control cultures), CD3+CD4−CD8+ cells (13% of control cultures), and CD3−CD4+CD8+ cells (31% of control cultures). The effect was not absolute, however, as some phenotypically mature T cells did develop, presumably through positive selection of the low proportion of CD3−CD4+CD8+ cells. One explanation for the incomplete inhibition could have been the difficulty in saturating the ChT1 determinant that is expressed in high levels on the surface of the thymocytes. Flow cytometry of the MUI-83-treated thymocytes with directly conjugated exogenous MUI-83 showed a slight increase in staining over that of anti-mouse Ig-FITC-stained cells, which revealed in situ bound Ab, supporting this possibility (data not shown).

To investigate the functional role of ChT1 further, we used a recently developed in vitro model for T cell differentiation involving coculture of freshly isolated thymic stromal cells with intrathymic precursor cells (37, 38). In this system mAb can be added...
directly to the precursor cells from the very onset of coculture, saturating the determinants before contact with the stroma and hence differentiation induction. Sorted CD4 \textsuperscript{+}CD8 \textsuperscript{−} E13 thymocytes from ov-alloantigen-positive embryos and isolated adult thymic stromal cells (ov −) were cocultured for 4 days in the presence of anti-ChT1, anti-c-kit, no mAb, or a variety of control mAb to thymic stromal or lymphoid molecules. After 4 days the cultures were harvested, the total cell yield was counted, and the expression of CD3, CD4, and CD8 was analyzed on ov − cells. Treatment with anti-ChT1 was comparable to that with anti-c-kit, so that in both cases the frequency of CD4 \textsuperscript{+} cells was decreased from 33.7% in control cultures to approximately 3.5% of double-negative cells partially differentiated to CD4 \textsuperscript{+}CD8 \textsuperscript{+} stage (Table III). There was no alteration numerically or proportionally in the control cocultures. These in vitro data emphasize the functional role of ChT1 during the proximal stages of thymocyte differentiation.

Discussion

Searching for molecules involved in early T cell differentiation, we isolated a cDNA encoding the avian thymocyte Ag ChT1, a member of the IgSF with two extracellular domains, V and C2. The expression of ChT1 on peripheral T cells defines recent thymic emigrants (20). We show here that the ChT1 “c-kit” subpopulation of embryonic bone marrow cells is enriched for T cell progenitors, as assayed by intrathymic cell transfer to congenic animals. Anti-ChT1 mAb also blocks T cell differentiation in vitro in thymic organ cultures and thymocyte precursor cultures on stromal cells. These results demonstrate clearly that ChT1 plays an important role in the early stages of T cell development.

Table II. Effect of anti-ChT1 treatment on ETOC

<table>
<thead>
<tr>
<th>Thymocyte Population</th>
<th>Isotype Control ETOC</th>
<th>Anti-ChT1-Treated ETOC, 100 ( \mu )g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive cells (%)</td>
<td>Cell number (\times 10^7)</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>10.4 ± 2.7</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>1.4 ± 1.3</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>20.2 ± 5.0</td>
<td>3.1 ± 1.8</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>19.0 ± 6.2</td>
<td>2.9 ± 1.4</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>14.1 ± 6.0</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>1.2 ± 0.7</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>15.5 ± 4.3</td>
<td>2.3 ± 1.0</td>
</tr>
<tr>
<td>CD3 \textsuperscript{−}CD4 \textsuperscript{−}CD8 \textsuperscript{+}</td>
<td>23.2 ± 4.9</td>
<td>3.3 ± 0.8</td>
</tr>
</tbody>
</table>

\* E10 thymus lobes were isolated and cultured for 6 days in the presence of anti-ChT1 mAb or an isotype-matched control mAb 10-2.16 (anti-mouse I-A\textsuperscript{k}). After culture the lymphocytes were isolated, and recovered cells were counted, stained, and analyzed for the expression of CD3, CD4, and CD8 by flow cytometry. Results are expressed as mean ± SD of six experiments. Unpaired Student’s \( t \) test was used to test for significance: \( ^{\ast} p < 0.05, ^{\ast\ast} p < 0.01, ^{\ast\ast\ast} p < 0.001 \).

\* All subsets were defined by discrete nonoverlapping gates; cells outside of these gates, including CD4\textsuperscript{−}CD8\textsuperscript{low} apoptosing cells (present to some degree in all cultures), were excluded.
The earliest intrathymic T cell precursors in the mouse have been defined as CD3^+ CD4^- CD8^- CD117^- CD44^+ CD25^- cells in which the TCR loci are still in germline configuration (8, 46). In addition, several other markers, e.g., Sca-1/2, HSA, Thy-1, and IL-7R, have been used to characterize early T cell precursors either in the thymus or fetal bone marrow (1, 3, 47, 48). However, none of these markers is T-lineage specific. Since ChT1 is the earliest T-lineage marker to be defined to date, being present on thymocytes on E10 of embryogenesis, well before CD4, CD8, and the CD3/TCR complex, and since its expression is developmentally regulated, we explored the functional role of ChT1 in embryonic thymocyte differentiation. In ETOC the treatment with anti-ChT1-specific mAb resulted in a significant decrease in total cell yield. The Ab treatment most significantly perturbed the differentiation step from the CD4^-CD8^- to the CD4^+CD8^- stage. The immature CD3^- thymocyte subpopulations were affected to a much larger extent than the CD3^- populations. ChT1 mAb also inhibited T cell differentiation in in vitro coculture of precursors with thymic stromal cells. Thus, in these in vitro assays convincingly show that ChT1 has an important function in the early stages of T cell differentiation. A similar effect on T cell development has been reported in reaggregate fetal thymic organ cultures with an anti-E-cadherin-2 mAb (100 μg/ml) (40, 53), without affecting the dimerization function of the motif. Even though CTX does not form dimers spontaneously, it can be induced to dimerize by cross-linking (54). Indeed, earlier reports of ChT1 or of an Ag that most likely is ChT1 have proposed that ChT1 is able to form spontaneous homodimers (18, 55), and our results in this study support this idea. Because the migration of all bands was increased equally after N-deglycosylation, we suggest that the bands are composed of similar proteins and carbohydrates. However, as we were not able to dissociate the proposed dimers by reduction there are other possibilities, in addition to dimerization, to interpret the immunoprecipitation pattern obtained. It may be that the subunits of the protein complex are linked by forces that are not breakable by the reducing agent used. Cross-reactivity of anti-ChT1 mAb with an unrelated protein is still another possibility to produce this result, but since we have used many different anti-ChT1 mAb with identical results this seems to be an unlikely solution. In addition to diglycine bulge, the ChT1 sequence contains an extra cysteine residue, approximately in the B/C loop of the C2-like domain, which could mediate dimerization by forming an intermolecular disulfide bridge. Thus, there is a possibility that ChT1 functions as a lymphocyte-specific receptor during T cell differentiation.

The second common feature with CTX is that the C2-like domain of the ChT1 harbors a conserved extra disulfide bridge, which has been found in the same position in other molecules that are obviously ChT1/CTX relatives, e.g., A33 (43) and HCAR/MCAR (42). Third, the intracellular tail is highly polar, containing

dands as dimers and are thought to serve as receptors (39, 40). Although in ChT1 the second glycine of the diglycine bulge is replaced by a serine residue, similar modification has been observed in other molecules, such as human CD28 (51), mouse B cell specific glycoprotein B29 (52), and Xenopus σ locus light chain (40, 53), without affecting the dimerization function of the motif. Even though CTX does not form dimers spontaneously, it can be induced to dimerize by cross-linking (54). Indeed, earlier reports of ChT1 or of an Ag that most likely is ChT1 have proposed that ChT1 is able to form spontaneous homodimers (18, 55), and our results in this study support this idea. Because the migration of all bands was increased equally after N-deglycosylation, we suggest that the bands are composed of similar proteins and carbohydrates. However, as we were not able to dissociate the proposed dimers by reduction there are other possibilities, in addition to dimerization, to interpret the immunoprecipitation pattern obtained. It may be that the subunits of the protein complex are linked by forces that are not breakable by the reducing agent used. Cross-reactivity of anti-ChT1 mAb with an unrelated protein is still another possibility to produce this result, but since we have used many different anti-ChT1 mAb with identical results this seems to be an unlikely solution. In addition to diglycine bulge, the ChT1 sequence contains an extra cysteine residue, approximately in the B/C loop of the C2-like domain, which could mediate dimerization by forming an intermolecular disulfide bridge. Thus, there is a possibility that ChT1 functions as a lymphocyte-specific receptor during T cell differentiation.

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### Table III. In vitro coculture of embryonic CD4^-CD8^- ov^- thymocytes on ov^- stromal cells

<table>
<thead>
<tr>
<th>mAb (100 μg/ml)</th>
<th>Total cell recovery</th>
<th>CD4^-CD8^-</th>
<th>CD4^-CD8^-</th>
<th>CD4^-CD8^-</th>
<th>CD4^-CD8^-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUI-83 (anti-ChT1)</td>
<td>17.3</td>
<td>10.0</td>
<td>0.6</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>kit2c75 (anti-c-kit)</td>
<td>22.4</td>
<td>9.3</td>
<td>0.7</td>
<td>12.2</td>
<td>0</td>
</tr>
<tr>
<td>MUI-82 (antithymic stroma)</td>
<td>19.8</td>
<td>3.9</td>
<td>8.5</td>
<td>5.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* E13 ov^- thymocytes were sorted for CD4^-CD8^- cells and prepared at a concentration of 3.4 × 10^6 cells/ml. Adult ov^- stromal cells were prepared by enzymatic digestion of lymphocyte-depleted thymus. Stromal cells were enriched by elutriation and resuspended at a concentration of 6.7 × 10^5 cells/ml. Two cell populations were mixed at a ratio of 5:1 and cocultured in the presence of anti-ChT1, anti-c-kit, control Ab or no mAb as hanging drops in inverted Terasaki plates at 40°C, 10% CO_2 for 4 days. Results for MUI-82 are representative of 50 control Abs reactive to thymic stromal cells and lymphoid markers which included isotype-specific controls.
several glutamic acid residues. The carboxyl-terminal end of both ChT1 and CTX can be predicted to form an α-helical structure where the negatively charged amino acids would gather close to one side of the helix and thus possibly interact with other polar intracellular molecules. A similar cytoplasmic tail, rich in glutamic acids, has been found in A33 and HCAR/MCAR. However, even though ChT1 and CTX are clearly homologues, their cytoplasmic regions are quite different. There are several potential serine phosphorylation sites in the ChT1 sequence, none of which is present in CTX. Thus, in addition to a possible extracellular receptor function, ChT1 may function in signal transduction. Determining whether ChT1, CTX, and other products of the ChT1/CTX family of genes mediate similar functions awaits additional experiments in different species.

In addition, to being a T-lineage marker in embryonic thymus, ChT1 expression together with c-kit expression define a prethymic T cell precursor population in embryonic bone marrow. After adoptive intrathymic cell transfer, the ChT1 "c-kit" cell population gave rise to γδ as well as CD4+ and CD8+ mature T cells. Recently, the existence of a common lymphoid progenitor population, Lin-IL-7R-Thy-1-Sca-1low-c-kitlow, has been described in the mouse (9). Whether the ChT1 "c-kit" bone marrow subpopulation contains T-lineage-restricted, common lymphoid, or multipotent hematopoietic progenitors remains to be clarified. In vivo ChT1 interaction with its putative ligand might result in a maintenance or viability signal that would allow thymic selection events to occur. Interestingly, CTX has been shown to mediate a cellular growth inhibition signal in Xenopus T cell tumors in agreement with the idea that ChT1/CTX is involved in the regulation of cell proliferation (54). According to our data from ETOC and precursor coculture with thymic stromal cells, we suggest that ChT1 is required for thymocyte precursors to develop to the CD4+CD8- stage; again, this could operate at the level of cell proliferation. It may be that when the cells have matured to the CD3+CD4+CD8- stage, ChT1 would no longer be crucial for T cell differentiation. However, ChT1 expression would continue for some time after the cell has emigrated from the thymus, thus marking the recent thymic emigrants (20). We hypothesize that the signal received from ChT1 Ag interaction with its ligand is necessary for developing thymocytes to proceed to the stage of positive (and negative) selection. The results presented in this study demonstrate that ChT1 Ag is an early T-lineage-specific marker on embryonic thymus and bone marrow cells and emphasize its importance in the first phases of T cell development.

Acknowledgments

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References


