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Molecular Cloning and Characterization of Mouse Tspan-3, a Novel Member of the Tetraspanin Superfamily, Expressed on Resting Dendritic Cells

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Dendritic cells (DCs) are the most potent antigen-presenting cells and play an essential role for triggering T-cell-mediated immune responses. In search for novel cell surface molecules expressed on DCs involved in T cell priming by representational differential analysis, we identified a mouse homologue of Tspan-3 (mTspan-3), a novel member of the tetraspanin superfamily. The mTspan-3 consists of four hydrophobic, putative transmembrane regions, forming a small and a large extracellular loop, with short intracellular amino and carboxil tails. Although the mTspan-3 is expressed on a variety of immune cell types including resting DCs, its expression on DCs is downregulated during activation induced by cross-linking CD40 with anti-CD40 monoclonal antibody. These results suggest that mTspan-3 may be involved in the function of DCs in association with T cell stimulation.

Key Words: **tetraspanin; TM-4; Tspan-3; dendritic cell; T cell; antigen-presenting cell.**

Antigen (Ag)-presenting cells (APC) play a central role for triggering of T cell-mediated immune responses. Recognition of MHC-peptide complexes on APC by Ag-specific T cell receptor (TCR) constitutes "signal one" in APC-T cell interaction (1, 2). APC-T cell clustering mediated by several adhesion or costimulatory molecules constitutes "signal two," which is required to sustain T cell activation (3, 4). Dendritic cells (DCs) are the most potent and unique APC for the stimulation of T cells because they are the only ones that are able to induce primary immune responses, thus permitting establishment of immunological memory (5–8). DCs reside as immature cells with high phagocytic capacity in peripheral tissues and migrate to the lymphoid organs following capturing Ag, inducing activation and maturation of DCs to effectively stimulate T cells. Several molecules including CD40, TNF-R, and IL-1R have been shown to activate DCs and to trigger their transition from immature Ag-capturing cells to mature Ag-presenting DCs (9, 10). The maturation process is associated with modulation of cell surface molecules on DCs, including downregulation of endocytic/phagocytic receptors and upregulation of MHC class II and costimulatory molecules CD40, CD58, CD80, and CD86 which are important for effective priming of T cells (11). However, it remained to be determined whether the unique ability of DCs to prime T cells entirely results from the downregulation or upregulation of these molecules.

The tetraspanin superfamily has grown to more than 20 known members in species from *Schistosoma* to humans since the first recognition in 1990 (12, 13). The tetraspanins contain four membrane-spanning domains and short cytoplasmic tails that associate with a variety of molecules, including lineage-specific proteins, integrins and other tetraspanins (14). Some tetraspanins, including CD81, CD82, CD9, and CD63, are expressed in virtually all tissues, whereas the expression of others such as CD37 and CD53 are highly restricted in B cells and lymphoid/myeloid cells, respectively (14, 15). Although their function has been incompletely understood, they may be involved in cell activation, proliferation, differentiation, adhesion and motility.

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In the present study, we searched a novel molecule expressed on resting or activated DCs that is involved in T cell priming by representational difference analysis (RDA), a PCR-based subtractive hybridization, using resting and activated DCs. We describe here the mouse Tspan-3, a novel member of the tetraspanin superfamily, which is expressed on resting DCs and downregulated in association with DCs activation.

MATERIALS AND METHODS

Purification and activation of DCs. DCs were isolated by the method, as described (16), with modification. In brief, splenocytes obtained from C57BL/6 mice were suspended in medium and separated with 13.5% Nycodents (Nyegaard Diagnostics, Oslo, Norway) by centrifugation at 600*g* for 10 min. Cells were then stained with anti-CD11c monoclonal antibody (mAb) (Becton Dickinson, San Jose, CA). Cells expressing high density of CD11c were sorted by flow cytometry (FACS Vantage, Becton Dickinson) and were subjected to experiments as resting DCs. Activated DCs were prepared by stimulation of resting DCs with plate-coated anti-CD40 mAb (Becton Dickinson) for 48 h.

Representational difference analysis (RDA). Total RNA was isolated using Isogen LS solution (Nippon Gene, Tokyo, Japan) and oligo(dT)-primed double stranded cDNA was synthesized using a cDNA synthesis system (GIBCO BRL, Grand Island, NY) according to the manufacturer's instruction. RDA was performed as described (17), using resting and activated DCs. In brief, the following oligonucleotides were synthesized and used for RDA: R-Bgl-24, R-Bgl-12, J-Bgl-24, J-Bgl-12, N-Bgl-24, and N-Bgl-12 which were previously described (18–20). cDNA was digested with *Dpn*II and ligated to the R-Bgl-12/24 adaptors. Amplicons were made by PCR amplification of the ligated *Dpn*II cDNA fragments for 20 cycles using the R-Bgl-24 as a primer. Driver cDNA was prepared by digesting amplicons with *Dpn*II. Tester DNA was prepared by gel purification of digested amplicons between 150 and 2000 bp followed by ligation to J-Bgl-12/24 adaptors. First subtractive hybridization was performed using a 400-ng tester and a 40-μg driver (tester:driver = 1:100). An aliquot of the hybridization mixture was amplified by PCR for 10 cycles using the J-Bgl-24 as a primer. The PCR products were then digested with mung bean nuclease (New England Biolabs, Beverly, MA) at 30°C for 35 min and further amplified for 18 cycles. These PCR products are the first difference products (DP1). The difference products were digested with *Dpn*II and ligated to a new adaptor, N-Bgl-12/24 (after the first hybridization) or J-Bgl-12/24 (after the second hybridization), and the procedure was repeated twice using tester: driver ratios of 1:800 and 1:4000–40,000 for the second and third round of hybridization, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA were obtained from resting and activated DCs or lineage-specific hematopoietic cells purified by flow cytometry, as described (21), and were subjected to reverse transcription and PCR using mTspan-3-specific primers (5′-ACGTGCTGGTCCGCTACGCGTAGGTTC-3′, 5′-GCACCATGGGCCAATGCGGCATCACCT-3′), HPRT-specific primers (5′-CACAGGACTAGAACACCTGC-3′, 5′-GCTGG-TGAAAAGG-ACCTCT-3′) or GAPDH-specific primers (5′-TGCACCACC-AACTGCTTAG-3′ and 5′-GGATGCAGGGATGATGTTC-3′). Conditions used for PCR were: 35 cycles of 20 s denaturation (94°C), 20 s annealing (55°C), and 60 s extension (72°C). For semiquantitative RT-PCR of hematopoietic cells other than DCs, amounts of cDNA were measured by using an ABI7700 analytical thermal cycler (Perkin–Elmer Applied Biosystem, Foster City, CA). TaqMan Rodent GAPDH Control Reagent (Perkin–Elmer Applied Biosystem) was used to standardize results. For semiquantitative RT-PCR of tissue samples and DCs, sample and HPRT cDNA were serially diluted and subjected to PCR.

TABLE 1

DNA Fragments Generated by RDA: Gene (Accession No.)

Activated DC minus resting DC	Resting DC minus activated DC
Class II MHC (AE0049121)	Unknown
Rat r-RNAs 19 (X51707.1)	
Pseudomonas aeruginosa PA01 (AF004569.1)	
TAP-2 (U60087.1)	

Establishment of transfectants expressing Tspan-3-green fluorescence protein (GFP) fusion protein. Tspan-3 cDNA was subcloned into the pMX-FL retrovirus vector (22) which encodes the fusion protein of Tspan-3 with GFP at the COOH terminus. 293T cells or J774.1 macrophage cell line were transiently transfected with the plasmid using Lipofectamine (GIBCO BRL) according to the manufacturer's instruction.

Proliferation assay. CD8+ T cells were purified from splenocytes of Balb/C mice using MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified CD8+ T cells derived from Balb/C mice were cocultured with total splenocytes or purified resting or activated DCs from C57BL/6 mice were performed in 96-well plates. T cell proliferation was determined by BrdU incorporation using a cell proliferation ELISA kit (Boehringer Mannheim, IN) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Molecular Cloning and Characterization of the Mouse Tspan-3

To identify novel molecules differentially expressed during activation of DCs, we performed RDA which is a PCR-based subtractive hybridization, using resting and anti-CD40 mAb-activated DCs. The cDNA from each population is digested with a restriction endonuclease, ligated to adaptors, and then amplified by PCR. To isolate cDNA clones unique to resting or activated DCs, the PCR products of each population were ligated to new adaptors and hybridized to an excess of the PCR products of the other. PCR with primers for the new adaptors preferentially amplified unique cDNA to a population of DCs. After repeating of this process three times, we identified several cDNA clones unique to resting and activated DCs (Table 1). The BLAST search in the database revealed that one of the cDNA fragments identified unique to resting DCs had a significant homology with the members of the tetraspanin superfamily. A full-length cDNA was cloned by RT-PCR using mRNA of resting DCs. The predicted amino acid sequence demonstrated that the protein encoded by the cDNA consists of 253 amino acids (Fig. 1A). The database search demonstrated that this protein was 97% identity with human Tspan-3 (AF054840), which was previously reported as a member of tetraspanin superfamily identified by EST screening (23), suggesting that this is a mouse homologue with human Tspan-3.

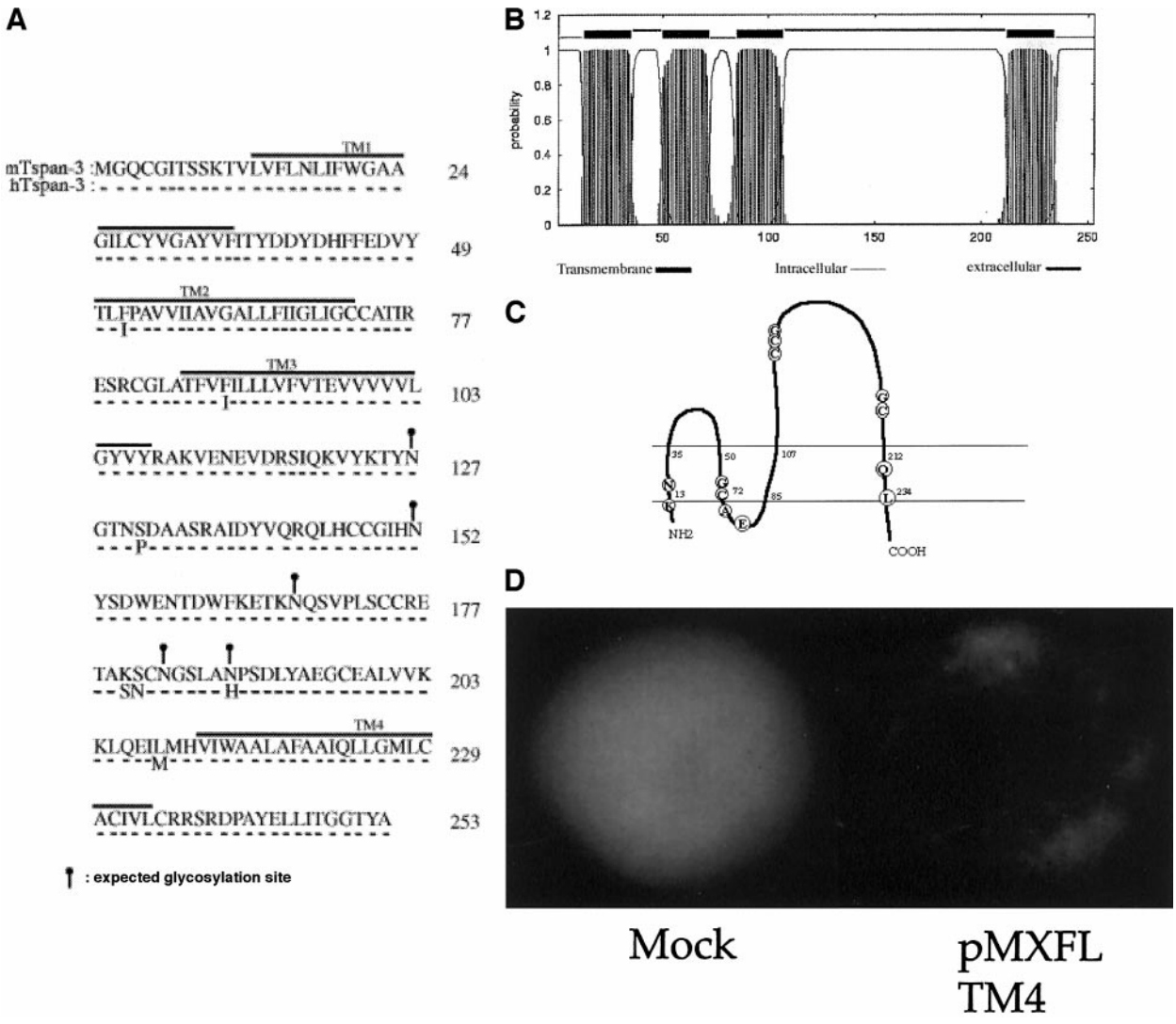

FIG. 1. Molecular characteristics of mTspan-3. (A) Comparison of the predicted amino acid sequences of the human and mouse Tspan-3. The putative transmembrane domains and potential NH_2-linked glycosylation sites in the second extracellular domain are indicated. The cDNA sequence data are available from EMBL/GenBank/DDBJ under Accession No. AF054840 (human) and an accession number to be assigned for mouse. (B) Analysis of the amino acid sequences of the mTspan-3, using TMHMM program (http://genome.cbs.dtu.dk/services/TMHMM/), predicting four-transmembrane hydrophobic domains. (C) Schematic structure of mTspan-3. Amino (NH2) and carboxyl (COOH) termini are indicated. The first and last amino acids of each transmembrane domain are shown in numbers. Highly conserved amino acids are shown in circles. (D) 293T cells were transfected with the plasmid of the mTspan-3-GFP fusion protein. The fusion protein localized at the cell surface of transfected 293T cells (left panel). In contrast, 293T cells transfected with the mock control vector showed the diffuse staining pattern with green fluorescence in the cytoplasm (right).

Mouse Tspan-3 (mTspan-3) consists of four hydrophobic, putative transmembrane regions, forming a small and a large extracellular loop, with short intracellular amino and carboxil tails (Figs. 1B and 1C), consistent with the characteristics of the tetraspanin superfamily. There are five potential N-linked glycosylation sites in the second extracellular domain (Fig. 1A). Importantly, it contains a few highly conserved polar, hydrophobic and charged residues in transmembrane domains, as demonstrated in Fig. 1C, which may be important for the stability of protein assembly or association with other proteins. Presently, it is uncertain whether mTspan-3 associates with other proteins. However, several lines of evidences indicated that tetraspanins physically associate with integrins, coreceptor molecules or other tetraspanins (24–33). We also observed conserved CCG and CG motifs in the second extracellular domain (Fig. 1C), which might be involved in disulfide bonding of the mTspan-3, as observed in other member of tetraspanin superfamily such as CD53, CD81 and sm23 (14, 34, 35).

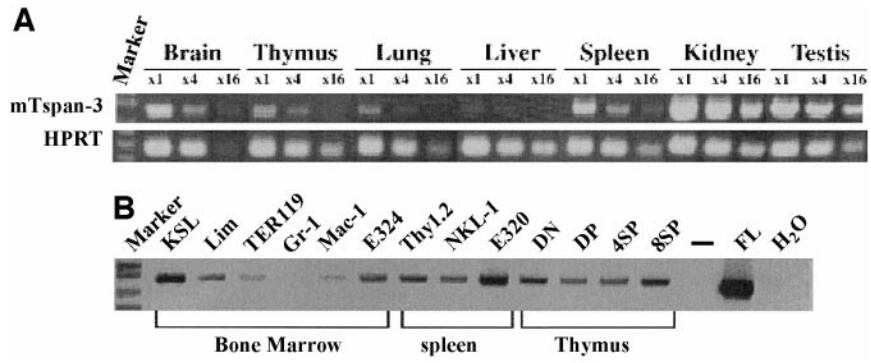

FIG. 2. Analysis of mTspan-3 expression by RT-PCR. cDNA, adjusted to comparable quantities using a HPRT (A) or a GAPDH (B) control as described under Materials and Methods, were prepared from tissues or FACS-purified lymphohematopoietic progenitor (KSL) and lineage-committed cells in the bone marrow, thymus, and spleen. These cDNA were diluted with water, as indicated, and were used as templates for RT-PCR.

mTspan-3 Is a Transmembrane Protein

To examine whether mTspan-3 is indeed a transmembrane protein, 293T cells were transfected with the plasmid of the mTspan-3-GFP fusion protein, which was constructed in pMX-FL expression vector containing multiple cloning sites followed by the GFP at the COOH terminus. As demonstrated in Fig. 1D, the fusion protein localized at the cell surface of transfected 293T cells, as detected by green fluorescence under fluorescent microscopy. In contrast, 293T cells transfected with the mock control vector showed the diffuse staining pattern with green fluorescence in the cytoplasm. These results indicated that mTspan-3 is a transmembrane protein.

Expression of mTspan-3 in Tissues

RT-PCR analyses demonstrated that mTspan-3 is expressed in various organs, including brain, thymus, lung, spleen, kidney and testis, but not in liver (Fig. 2A), consistent with the previous report of human Tspan-3 (23). To further analyze the expression of the mTspan-3 on hematopoietic cells, RNA was obtained from FACS-purified lymphohematopoietic progenitor and lineage-committed cells in the bone marrow, thymus and spleen. The mTspan-3 transcripts were expressed in all these hematopoietic cell fractions except Gr-1+ granulocytes (Fig. 2B). These results suggest that mTspan-3 may be involved in fundamental functions commonly required in a variety of cell types.

mTspan-3 Is Downregulated in Activated DCs

The mTspan-3 was cloned from resting DCs by RDA using anti-CD40 mAb-stimulated DCs, suggesting that the mTspan-3 is downregulated during activation process. To confirm this, DCs were purified from splenocytes by gradient centrifugation using Nycodents, followed by FACS-sorting of population expressing high density of CD11c that defines DCs. Reanalysis by flow cytometry demonstrated that more than 95% of the sorted cells were strongly positive for CD11c (data not shown). These purified DCs significantly stimulated

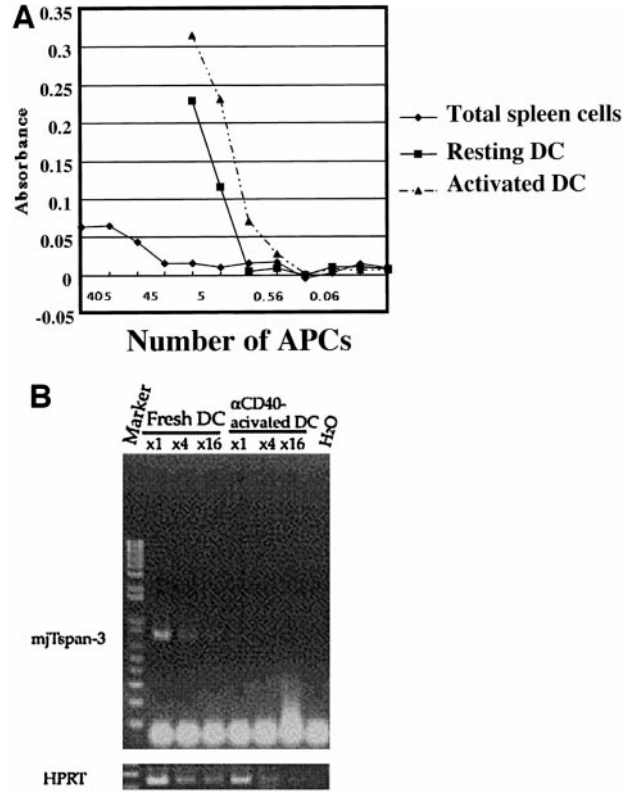

FIG. 3. Downregulation of mTspan-3 transcript during process of DC activation. (A) Purified CD8+ T cells from Balb/C mice were cocultured with total splenocytes or purified resting or anti-CD40 mAb-activated DCs from C57BL/6 mice. Proliferation of T cells were determined as described under Materials and Methods. Data are representative from several independent experiments. (B) cDNA, adjusted to comparable quantities using a HPRT, were prepared from FACS-purified DCs before and after activation with anti-CD40 mAb. These cDNA were diluted with water, as indicated, and were used as templates for RT-PCR. H_2O was used as a template for negative control.

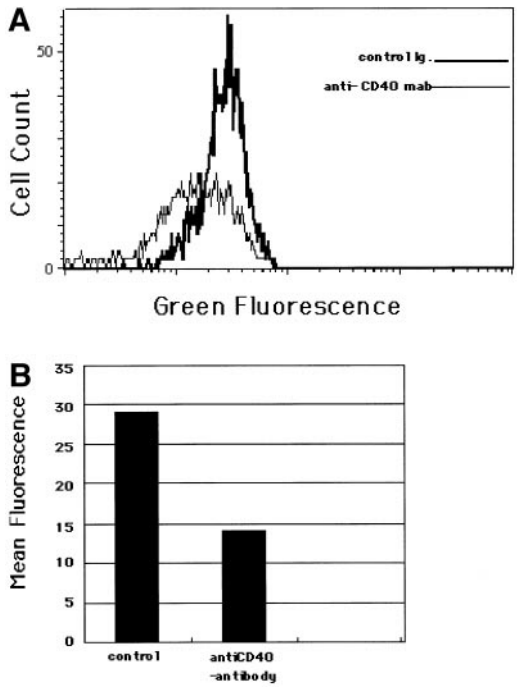

FIG. 4. Downregulation of mTspan-3 protein during activation of J774.1 macrophage cell line. J774.1 cells were transiently transfected with the plasmid encoding mTspan-3/GFP fusion protein. The transfectants were stimulated with anti-CD40 mAb or control Ig and analyzed by flow cytometry. The expression of GFP was downregulated after stimulation with anti-CD40 mAb, as demonstrated in a profile of flow cytometry (A) and in mean fluorescence level of GFP (B). Data are representative from several independent experiments.

allogeneic CD8+ T cells, as determined by T cell proliferation, whereas total splenocytes had little stimulatory effect (Fig. 3A), indicating that the purified DCs used in the present studies were functionally distinct. DCs activated with anti-CD40 mAb, however, were able to stimulate allogeneic CD8+ T cells more efficiently than resting DCs (Fig. 3A), which may be caused by modulation of cell surface molecules on activated DCs that interact with T cells. Indeed, we observed upregulation of MHC class II molecule on activated DCs by RDA (Table 1) compared with resting DCs. RT-PCR analysis using mRNA from each population of DCs demonstrated that mTspan-3 was expressed in resting, but not activated, DCs (Fig. 3B), indicating that the mTspan-3 transcript was downregulated during activation process of DCs.

We further examined whether the mTspan-3 protein is also downregulated in association with cell activation. J774.1 macrophage cells were transfected with the plasmid of the mTspan-3-GFP fusion protein, followed by stimulation with anti-CD40 mAb or control Ig. Analysis by flow cytometry demonstrated that the transfectants stimulated with anti-CD40 mAb showed green fluorescence significantly lower than those stimulated with control Ig (Figs. 4A and 4B). These results indicate that mTspan-3 protein as well as the transcript is downregulated during cell activation. Many tetraspanins are associated with cellular activation. For example, CD9, CD53, CD63 and CD82 are upregulated on activated cells, whereas CD37 and CD53 are downregulated in association with growth arrest (36). However, the regulation of tetraspanin gene transcription and the physiological relevance of the protein expression have little been understood. Future studies should be required to clarify the regulatory mechanism of mTspan-3 expression and its physiological role in cell function.

CONCLUDING REMARKS

In search for a novel molecule on DCs involved in T cell priming, we have identified a mouse homologue of Tspan-3, a novel member of the tetraspanin superfamily, which is downregulated in association with cell activation. During the preparation of this manuscript, Bronstein and colleagues have reported a novel tetraspanin molecule OAP-1 which associates with oligodendrocyte-specific protein/claudin-11 primarily expressed in oligodendrocytes of the central nervous system and Sertoli cells of testis in adult mouse (37). The putative amino acid sequence of OAP-1 is identical to the mTspan-3 described here except two amino acid residues. Presently, it is uncertain whether or not mTspan-3 and OAP-1 are the same gene product. Nonetheless, modulation of mTspan-3 expression on DCs during activation process suggests that mTspan-3 may be involved in DCs function in T cell stimulation. Further studies should be required to clarify the role of mTspan-3 in immune system.

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