Identification and Characterization of Rat AILIM/ICOS, a Novel T-Cell Costimulatory Molecule, Related to the CD28/CTLA4 Family

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Activation-inducible lymphocyte immuno-mediatory molecule (AILIM) is an inducible cell surface glycoprotein expressed on thymocytes and activated lymphocytes. Specific monoclonal antibody to rat AILIM induced the cell aggregation of a rat thymoma cell line and ConA-activated splenocytes. In the present study, we identified the primary structure of two species of rat AILIM by expression cloning. We also cloned mouse and human AILIM homologues and the predicted amino acid sequences were identical to those of the inducible costimulator ICOS/CRP-1, which belongs to the CD28/CTLA4 family. Although the human and mouse AILIM/ICOS molecule is localized on T-cells, the major population of AILIM/ICOS-positive cells in rat splenocyte was CD45RA-positive B-cells. The expression level of AILIM/ICOS on T-cells was relatively low; however, its expression was drastically induced by the treatment with PMA plus Ca-ionophore or the engagement of CD3 and these costimulatory molecules. Almost all T-cells exhibited potency as to its expression. Functional analysis of AILIM/ICOS demonstrated that AILIM-mediated costimulation was relatively weak compared to that of human.

Key Words: ICOS; CD28; CTLA4; costimulatory molecule.

Direct cellular interactions are thought to play an important role in the regulation of cellular communication like humoral factors. Recent studies revealed the molecular structure that participates in the interaction, such as adhesion molecules and cell surface molecules (1, 2). In the immune system, the cellular interaction between antigen-specific helper T-cells and T-cell-dependent B-cells is necessary and plays an important role in the immunological response (3, 4). The interaction leads to the subsequent proliferation and differentiation of both T- and B-cells, and the secretion of cytokines from T-cells (3, 4).

It is well known that the activation of T-cells requires the two distinct signals: one derived from the interaction between the T-cell receptor and peptide-MHC complex, and the other, designated as the costimulatory signal, derived from the interaction of the costimulatory molecule on T-cells and its counterreceptor on antigen-presenting cells (APCs; 5–8). These reactions lead that at least two different outcomes can result from the interaction between T-cells and APCs: engagement of the T-cell receptor by a specific antigen with costimulatory signals results in T-cell activation including proliferation and cytokine production. On the other hand, the T-cell receptor cognate with the antigen in the absence of the stimulation via the costimulatory molecule leads to anergy to subsequent challenge with the same antigen or results in cell death (5–8).

In recent studies, the molecular basis of the regulation mechanism for T-cell activation was demonstrated (9–15). An important costimulatory signal is delivered to T-cells when CD28 and CTLA4 on T-cells interact with B7 family members, B7-1 and B7-2, expressed on APCs (9–12). A lot of other receptor and counterreceptor interactions have been reported, the costimulatory molecule pairs being CD2-CD58, LFA-1-CD54, CD40L-CD40, and CD4-MHC class II (13–15).

Previously, we generated against monoclonal antibodies to molecules expressed on the rat thymoma cell line, FTL43 cells, and cloned an agonistic antibody, designated as J TT.1, which induced the cell aggregation of FTL43 cells and ConA-activated spleen cells

Abbreviations used: AILIM, activation-inducible lymphocyte immuno-mediatory molecule; ICOS, inducible costimulator. 1 Present address: Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan.

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We also cloned an aggregation blocking monoclonal antibody, designated as JTT.2, which completely blocked JTT.1-induced cell aggregation (16). Immunological studies involving these monoclonal antibodies revealed that they recognized the same molecule, and the antigen expressed on thymocytes and lymphocytes but not on neutrophils. Furthermore, the antigen on lymphocytes was strongly induced by ConA stimulation (16). We designated this antigen as an activation-inducible lymphocyte immuno-mediatory molecule (AILIM).

In this study, we identified the structure of two species of AILIM from ConA-activated rat spleen cells and FTL43. Moreover, we also cloned mouse and human AILIM homologues found using rat AILIM cDNA under low stringent conditions. The predicted amino acid sequences of human and mouse were identical to that of the inducible costimulator, ICOS/CRP-1, which is functionally related to CD28 and CTLA4 (17, 18, 33). The predicted amino acid sequences were conserved at a level of 60–80% identical to each other.

In contrast to human or mouse AILIM/ICOS molecule which is localized on T-cells, the rat AILIM was detected on not only T cells but B cells in the normal body on flowcytometric analysis. The AILIM/ICOS expression on T-cells was drastically induced by the treatment with PMA plus a Ca-ionophore or the engagement of CD3 plus CD28 or AILIM/ICOS. After these activation of T-cells in vitro, almost of all T-cells expressed AILIM/ICOS on its cell surface and the cells exhibit the ability to express AILIM/ICOS. Functional analysis of AILIM/ICOS using monoclonal antibody demonstrated that rat AILIM/ICOS-mediated costimulation was relatively weak compared to those of human and mouse system. These characteristics indicate that rat AILIM/ICOS has some different features from human AILIM/ICOS.

MATERIALS AND METHODS

cDNA cloning of rat, mouse, human AILIM. A cDNA library of rat splenic ConA-blast cells was constructed in a mammalian expression system. The expected inserts were ligated into the pLPC8 vector (34). The cDNA inserts were inserted into the pLPC8 vector and transformed into E. coli DH5α for sequencing. The putative signal sequence was underlined. The putative transmembrane region is represented by a double underline. Potential N-linked glycosylation sites are indicated in italics. The predicted SH2-binding motif is dotted. (c) Amino acid alignment of rat, mouse and human AILIM/ICOS. Identical residues in rat, mouse, and human are boxed and the conserved residues are shaded. These sequence data have been submitted to the DDBJ/EMBL/GenBank Data Library; the accession numbers were AB023133 (rat AILIM-1), AB023134 (rat AILIM-2), AB023132 (mouse AILIM), and AB023135 (human AILIM).
cDNA Cloning of Rat, Mouse, and Human AILIM

At first, we isolated a cDNA clone encoding rat AILIM, clone T132A7 (AILIM-1) being isolated from a rat splenic ConA blast cDNA library by expression cloning with J TT.1 mAb. AILIM-1 was 835 base pairs (bp) in length and contained a single open reading frame of 650 bp encoding 216 amino acids with a calculated Mr of 24.2 kDa (Fig. 1a). From the results of Northern hybridization with AILIM-1 as a probe, rat AILIM mRNA was found to be 0.9, 1.6, 3.7 kilobase (kb) in length in rat ConA-blast mRNAs (Fig. 3d). For cloning of the full-length cDNA, we screened the FTL43 cDNA library by colony-hybridization with rat AILIM-1 cDNA as a probe, and a cDNA clone f23 (AILIM-2) was isolated. AILIM-2 was 2016 bp in length and contained a single open reading frame of 602 bp encoding 200 amino acids with a calculated Mr of 22.5 kDa (Fig. 1b).

From the results of hydropathy plotting, the AILIM-1 and 2 were found to be a type I transmembrane protein (data not shown). The amino acid region between 1 and 197 of rat AILIM-1 and AILIM-2, comprising a 20 amino acid N-terminal hydrophobic leader sequence, a 122 amino acid extracellular region containing 2 potential N-glycosylation sites, a 21 amino acid hydrophobic membrane-spanning portion, and a part of the cytoplasmic region was identical (Fig. 1c). The cytoplasmic region of AILIM-1 is longer for 16-amino-acid residues than that of AILIM-2. From the results of Northern blot analysis in rat ConA-blast and FTL43 mRNAs, AILIM-1 cDNA of 835 bp in size was derived from 0.9- or 1.6-kb mRNAs and AILIM-2 cDNA of 2016 bp in size was derived from 3.7-kb mRNA (Fig. 3d). We also determined the N-terminal sequence (ELDNLANHRXFXHGDVQI) of AILIM, which was purified from a cell-lysate of FTL43, was identical to the translated sequence of positions 21 to 40.

The mouse AILIM cDNA was obtained by colony-hybridization with clone T132A7 as a probe from a mouse splenic ConA-blast cDNA library. The predicted amino acid sequence of mouse AILIM was 77% identical to that of rat AILIM-1, and 84% to that of rat AILIM-2. The predicted amino acid sequence of mouse AILIM consisted of 200 amino acids with a predicted molecular mass of 22.7 kDa and contained two N-glycosylation sites in the extracellular domain (Fig. 1c).

The human AILIM cDNA encodes a protein of 199 amino acids lacking one amino acid at residue 121 in the extracellular domain of rat AILIM-2 and mouse AILIM, and has three predicted N-glycosylation sites in the extracellular domain. The human AILIM is 62% identical to rat AILIM-1, 67% to rat AILIM-2, and 68% to mouse AILIM in amino acid sequence (Fig. 1c).
the results of the amino acid sequence alignment among rat AILIM-1/AILIM-2, mouse and human AILIM, AILIM-2 would be rat homologue of human and mouse AILIM.

A comparison of the predicted amino acid sequence of AILIM with the GenBank database demonstrated that AILIM is a member of the CD28/CTLA4 family (Fig. 2). The rat AILIM-2 exhibits 19% identity with that of CD28, and 13% identity with CTLA4. Cysteine residues, which are important for the formation of a single immunoglobulin V-like domain and a disulphide bridge, are highly conserved in CD28/CTLA4/AILIM. The YMFM motif in the cytoplasmic tail, which is predicted to bind to the src-homology region 2 (SH2) domain of PI3-kinase (22), is conserved. However, the motif MYPPPY, which is required for the ligation of CD28/CTLA4 and B7-1/B7-2 (23, 24), and the SH3-binding motif (PxxP) observed in CD28 (25) are not conserved in AILIM. The predicted amino acid sequence of human AILIM is identical with that of ICOS, and mouse AILIM is identical with CRP-1, which were recently reported to be an inducible T-cell costimulator (17, 18, 33).

Expression of AILIM/ICOS mRNA in Lymphoid Tissues

To examine the expression of AILIM/ICOS in vivo, polyA+ mRNA from various kinds of tissues was analyzed by Northern blot analysis. As shown in Fig. 3a, rat AILIM-2 mRNA was detected as a strong band corresponding to 3.7 kb in size in spleen and lung. Slight expression was also observed in liver, kidney, and testis. Mouse AILIM/ICOS was expressed at relatively low level only in spleen, as a band 3.7 kb in size (Fig. 3b). Human AILIM/ICOS mRNA was slightly expressed in thymus, lung, lymph node, and peripheral blood leukocytes (PBL) (Fig. 3c). Unlike the rat and mouse ones, the size of human AILIM mRNA was 2.8 kb, different from that of other species. By ConA-stimulation, only AILIM-1 mRNA of 0.9 or 1.6 kb in size was upregulated in rat splenocytes (Fig. 3d), however, only a human 2.8-kb transcript correspond to rat AILIM-2 was upregulated in peripheral blood mononuclear cells (PBMC: data not shown).

Population of AILIM/ICOS-Positive Cells

To characterize the cell populations of the AILIM/ICOS-positive cells, we isolated AILIM/ICOS-positive cells from rat spleen cells, lymph node cells, and thymocytes by magnetic cell sorting. Figure 4 shows the results of flow cytometric analysis of AILIM/ICOS-positive cells, which were isolated from rat various lymphoid tissues, stained with various antibodies against lymphocyte-surface molecules. Among rat
spleen, over 90% of the anti-AILIM-sorted cells were CD45RA^+ B-cells, and the content of CD4^+ T-cells and CD8^+ T-cells in AILIM-positive cells were 4.3 and 4.9%, respectively. Among lymph node cells 57.2% were CD45RA^+ B-cells, 32.7% were CD4^+ T-cells and 3.1% were CD8^+ T-cells. In thymus, 98.1% of anti-AILIM-sorted cells were CD4CD8^+double positive T-cells, only 0.3% were CD4^+CD8^-cells and 1.5% were CD4^-CD8^+cells. The expression of rat AILIM was relatively low in the steady-state in vivo. Without stimulation, the contents of AILIM-positive cells among rat spleen cells, lymph node cells, and thymocytes were 0.4, 0.2, and 0.7%, respectively (data not shown).

**Induction of the Expression of AILIM/ICOS on Rat T-Cells**

Next, we investigated the induction of AILIM/ICOS on T-cells isolated from lymph node by flowcytometric analysis. The stimulation with PMA plus Ca-ionophore induced the expression of AILIM/ICOS on T-cells (Fig. 5a). ConA (2 μg/ml) weakly stimulated the cell surface expression of AILIM/ICOS (data not shown). Interestingly, the expression of rat AILIM/ICOS was drastically induced in T-cells by the engagement of CD3, and CD28 or AILIM (Fig. 5b). The expression of AILIM/ICOS was detected and induced in each CD4^+ and CD8^- T-cells (data not shown). Almost all T-cells have the ability of the AILIM/ICOS expression. The expression level of AILIM/ICOS by costimulation was high level rather than that by the treatment with PMA plus Ca-ionophore.

**Rat AILIM/ICOS Acts as a Costimulatory Molecule for T-Cell Activation**

Next, we investigated whether or not the engagements of CD3 and costimulatory molecules have co-

![FIG. 3. Expression of AILIM/ICOS mRNAs in various tissues. Northern blot analysis of rat tissues (a), mouse tissues (b), human tissues (c), rat thymoma cells and ConA-blast (d) was performed. The RNA molecular sizes (kb) are shown on the left. The sizes of AILIM/ICOS mRNAs were also indicated on the right. Tissues are indicated at the top of each figure. The method used is described under Materials and Methods.](image-url)
FIG. 4. Phenotypic characterization of the AILIM/ICOS-positive cells isolated from rat tissues. AILIM/ICOS-positive cells were prepared from spleen cells, lymph node cells and thymocytes as described under Materials and Methods. The unfractionated cells and sorted cells were stained with FITC- or PE-conjugated mAbs, such as CD3, CD4, CD8, and CD45RA. Cells located in region R1 in the plots of forward and side scatter were analyzed. The mAbs used are indicated at the left and the bottom of the plots.
stimulatory activity for T-cell proliferation. The AILIM-specific mAb, clone JTT.2, did not exhibit direct mitogenic activity in the absence of anti-CD3 antibodies. However, the proliferation of rat T cells was induced by the cross-linking with the combination of JTT2 and anti-CD3 mAbs in a dose-dependent manner (Fig. 6). The maximal costimulatory activity of rat AILIM/ICOS was less than 50% of that of CD28 in combination with anti-CD3 mAb.

DISCUSSION

It is well known that T cells require both a TCR generated signal and a costimulatory signal for their full activation. Recent studies demonstrated that CD28 provides a costimulatory signal through a signal transduction pathway distinct from that generated through TCR (26). CTLA4 is also a T-cell costimulator, which is structurally related to CD28. Although these molecules belong to the same family from the viewpoint of their structures, CD28 and CTLA4 have opposite effects on the T-cell responses. CD28 plays important roles in the activation, the normal immune response and T-cell development (5). CTLA4 exhibits inhibitory activity toward T-cell activation (27, 28). However, studies involving CD28-deficient mice have shown that CD28 is not required for all of the T-cell responses in vivo, and also suggested that an alternative costimulatory pathway mediated by some novel costimulatory molecule may exist (29, 30).

In the present paper, we described the molecular structure, expression, and function of a novel costimulatory molecule, AILIM, which is structurally related to the CD28/CTLA4 family, and showed some new findings about rat AILIM/ICOS, different from human and mouse ones. First, we isolated two kinds of alternatively spliced variants of rat AILIM, designated as AILIM-1 and AILIM-2. The predicted amino acid sequence of AILIM-2 encoded by the open-reading frame...
is that of a protein with a molecular weight of 22.5 k, which was similar to the molecular weight (20 kDa) of the deglycosylated native AILIM protein (16). Transfection experiments involving COS cells with the cloned AILIM revealed a disulfide-linked homodimer on the cell surface like CD28 and CTLA4 (data not shown). Furthermore, we also cloned not only rat AILIM but also human and mouse AILIM and those deduced amino acid sequences were highly conserved to rat AILIM-2. Recently, several groups reported the identification of a novel costimulator, designated as an inducible costimulator, ICOS (17, 31) or CRP-1 (18). Those predicted amino acid sequences of human and mouse were identical to those of AILIM. The signaling pathway following CD28-stimulation has been elucidated (22, 25, 26). The CD28-mediated activation signal is delivered through the binding of the SH2-domain of PI3-kinase and GRB-2/SOS to the pYMM motif. On the contrary, CTLA4 functions as a negative regulator through the binding of tyrosine phosphatase to the YVFM motif (32). The cytoplasmic tail of AILIM/ICOS contains the YXXM motif, which was predicted to bind to the SH2-domain of PI3-kinase. We showed that AILIM-1, a novel species of AILIM/ICOS, was thought to be an alternatively spliced form in rat. Because the motif is conserved in AILIM-1, it would be able to transduce the signals mediated by the binding between AILIM/ICOS and its counter-receptor. From the results of Northern blot analysis, the 0.9 or 1.6-kb AILIM-1 mRNA was drastically upregulated by ConA activation. AILIM-1 might work as a costimulatory molecule mainly in the activation of T-cells. However, its roles in vivo are not elucidated.

Secondly, we examined the tissue distribution of AILIM/ICOS mRNA and the expression of AILIM/ICOS on lymphoid cells in rat, and compared to those in human (17) or mouse (18, 31). Under physiological conditions, the expressions of mouse and human AILIM/ICOS mRNA were detected slightly in lymphoid tissues or lymphocyte-containing organs. Exceptional case was observed in rat. The expression level of AILIM/ICOS mRNA in rat spleen was drastically high compared to those in human and mouse spleen. As judged from the results of flowcytometric analysis, over 90% of human AILIM/ICOS positive cells in PBMC were CD4+ T-cells (33). In contrast, 89.2% of rat AILIM/ICOS-positive cells were CD45RA+ B-cells. Rat AILIM might to be an immuno-mediatory molecule not only for T-cells but also B-cells. In rat T-cells, the expression of AILIM/ICOS was highly regulated. AILIM/ICOS only appeared on a small portion of CD4+ T-cells and CD8+ T-cells, and its expression was drastically induced by the treatment with PMA plus Ca ionophore, like in the case of CTLA4 (33). Interestingly, AILIM/ICOS expression was induced by the costimulations, such as the engagement of not only CD3 and CD28 but CD3 and AILIM/ICOS.

Thirdly, we indicated that rat AILIM/ICOS acts as a costimulatory molecule for T-cell activation. In human T-cells, the engagement of CD3 and AILIM/ICOS drastically induced the T-cell proliferation, and its level of [3H]thymidine uptake was similar to that induced by the engagement of CD3 and CD28 (17, 33). However, in rat T-cells, the effect on the T-cell proliferation by the engagement of CD3 and AILIM/ICOS relatively low level in comparison with that by the stimulation of the engagement of CD3 and CD28. In our previous study, AILIM/ICOS was isolated as a possible cell adhesion molecule from the activity of JTT.1 and JTT.2 mAbs (16). The cloned agonistic antibody, JTT.1, drastically induced the cell aggregation of FTL43 cells and ConA-activated spleen cells, JTT.2 completely blocked the JTT.1-induced cell aggregation (16). We speculate that AILIM/ICOS would have some another functions through the activity, such as a adhesion molecule, and could regulate a immune response not only for T-cells but also B-cells. The expression of AILIM/ICOS on CD45RA+ B-cells also suggested this possibility.

We noticed the function of AILIM/ICOS in the development of T-cells in thymus. We revealed the expression of AILIM/ICOS in the process of T-cell development using rat thymus. The flowcytometric analysis showed that almost of all AILIM/ICOS-positive cells were enriched in CD4CD8-double positive cells and CD4+CD8low- and CD4low CD8+ cells. CD28 was expressed in the almost all population of thymocytes and slight expression of CTLA4 was also observed in CD4CD8-double positive cells, CD4- and CD8-single positive cells (34, 35). The analyses of CD28 or CTLA4 knockout mice revealed that the roles of CD28/CTLA4 for the development of T-cells in thymus is quite different (29, 30). Although T-cell development was normal in the CD28-knockout mice (29), CTLA-4 knockout mice exhibited a marked decrease of CD4CD8-double positive cells and a relative increase in CD4- and CD8-single positive T-cells (30). The expression profiles of AILIM/ICOS on thymocytes was different from those of CD28/CTLA4, and shows the possibility that AILIM might play an other important role for the maturation or selection of T cells in thymus.

Further studies for the elucidation of the roles of AILIM/ICOS, and the classification of the roles of AILIM/ICOS compared to those of another costimulatory molecules, such as CD28 or CTLA4, should provide valuable information on the immunological responses of lymphoid cells and T-cell development in vivo.

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Contributors: K. Tezuka and T. Tsui wrote the paper. K. Tezuka contributed to the cDNA cloning of rat, mouse, human AILIM/ICOS, Northern hybridization, cell surface marker analysis, and costimulation studies. T. Tsui supervised the cDNA cloning of human AILIM/ICOS, expression analysis, and functional analysis of AILIM/ICOS in the course of this study. D. Hirano contributed to the functional assay of AILIM/ICOS. T. Tamatani generated the monoclonal antibodies against rat AILIM/ICOS and purified rat AILIM/ICOS. K. Sakamaki supervised the expression cloning of rat AILIM/ICOS cDNA. Y. Kobayashi supported the preparation of monoclonal antibodies to rat AILIM/ICOS and functional assay of AILIM/ICOS. T. Tamatani generated the monoclonal antibodies against human AILIM/ICOS. D. Hirano contributed to the cloning studies. T. Tsuji supervised the cDNA cloning of human AILIM/ICOS.

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