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Mouse MD-1, a Molecule That Is Physically Associated with RP105 and Positively Regulates Its Expression[1]

Kensuke Miyake,[2]* Rintaro Shimazu,* Jun Kondo,[†] Tamotsu Niki,[†] Sachiko Akashi,* Hirotaka Ogata,* Yoshio Yamashita,* Yoshihiro Miura,* and Masao Kimoto*

RP105 is a leucine-rich repeat molecule that is expressed on mouse B cells and transmits a growth-promoting signal. An anti-RP105 Ab precipitated additional molecules as well as RP105. These molecules were found to be a mouse homologue of chicken MD-1. Chicken MD-1 was previously isolated as a *v-myb*-regulated gene, since its transcription increases rapidly after *v-myb* induction. Mouse MD-1, when transiently expressed as an epitope-tagged protein, is secreted in culture fluid but tethered to the cell surface by coexpressed RP105. An association of these molecules was confirmed by immunoprecipitation with the anti-RP105 Ab and subsequent probing of the epitope tag on MD-1. Moreover, MD-1 has an effect on the expression of RP105. In transient transfection of RP105, the percentage of RP105-positive cells increased more than twice with the coexpression of MD-1. The stable expression of MD-1 conferred approximately a sevenfold increase in cell surface RP105 on a cell line that expresses RP105 alone. Thus, MD-1 is physically associated with RP105 and is important for efficient cell surface expression. *The Journal of Immunology,* **1998, 161: 1348–1353.**

Leucine-rich repeat (LRR)[3] is a protein motif that has been implicated in protein-protein interaction. It is present in a number of proteins with diverse functions and cellular locations (reviewed in Ref. 1). Some members have a role in the innate immune system. The tomato resistance genes *cf-2* and *cf-9* are transmembrane proteins that possess LRR in their extracellular domains (2, 3). They recognize specific pathogen molecules by LRR and activate plant defense responses (reviewed in Ref. 4). Similarly, the Toll receptor, another LRR molecule of Drosophila, elicits defense responses against fungal pathogens (5). A human homologue of Toll was recently cloned (6) and is expressed on lymphocytes and monocytes. Thus, LRR molecules seem to have a basic role in the immune system among diverse species.

RP105 is another LRR molecule that is expressed on B lymphocytes. It was first identified by a mAb that protects spleen B cells from irradiation-induced apoptosis (7). The RP105 molecule, when cross-linked by an Ab, transmits an activation signal that leads to massive B cell proliferation as well as resistance against apoptosis. Interestingly, these activated and proliferating B cells arrest their growth and undergo apoptosis upon a signal from the AgR (8). RP105 may be involved in the regulation of B cell growth and Ag-induced death. RP105 is a type I transmembrane protein of 105 kDa with LRR on the extracellular domain (9). In the course of biochemical studies, we conducted transfection of the RP105 cDNA, but RP105 was always poorly expressed on the cell surface. The expression of RP105 by transient transfection is significantly lower than that seen with other molecules. We speculated that the LRRs of RP105 might be associated with another molecule that is required for efficient expression. In the present study, we sought such a molecule and identified it as a mouse homologue of chicken MD-1. Chicken MD-1 was first isolated as a *v-myb*-regulated gene from chicken myeloblasts that had been transformed with the avian myeloleukemia virus. Burk et al. (10) used the estrogen-dependent *v-myb* expression system to show that MD-1 expression rapidly increases after *v-myb* induction. It was predicted that MD-1 was a secretory molecule, because it has only one hydrophobic stretch at the amino-terminus. It does not have any homology to other molecules, and no functional study has been reported since. This report has rediscovered MD-1 as a B cell surface molecule. We also describe a role of MD-1 in cell surface expression of RP105.

Materials and Methods

Cells and Abs

The B cell lines M12.4, CH12, A20, and WEHI231 were obtained from Drs. D. Mckearn (Mayo Clinic, Rochester, MN), R. J. Hodes (National Cancer Institute, Bethesda, MD), T. Kurosaki (Kansai Medical School, Osaka, Japan), and N. Sakaguchi (Kumamoto University, Kumamoto, Japan), respectively. A human kidney epithelial cell line, 293T, was provided by Dr. T. Hirano (Osaka University, Osaka, Japan). BaRP30 was established previously by transfecting the IL-3-dependent line Ba/F3 with an expression vector encoding RP105 (9). BaRP30 was further transfected by electroporation with another expression vector coding for MD-1flag. Cells that express MD-1flag were selected by cell surface staining of the flag epitope and referred to as BaRPMD. The rat anti-mouse RP105 Ab RP/14 was established in our laboratory (7). The mouse anti-flag mAb M2 was purchased from Eastman Kodak (New Haven, CT).

Microsequencing of N-terminal aa

The purified anti-RP105 mAb (10 mg RP/14) was coupled to the *N*-hydroxy-succinidyl-activated HiTrap column according to the manufacturer's instructions (Pharmacia Biotech Japan, Tokyo, Japan). Up to 2×10^{10} cells from the B cell line M12.4 were collected. Lysis buffer was added to create the cell lysate at 5×10^7 cells/ml. Lysis buffer consisted of 50 mM

Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor (Wako Pure Chemical Industries, Osaka, Japan), and 5 mM EDTA. After a 1-h incubation on ice, lysate was cleared by centrifugation and loaded onto an anti-RP105-coupled column. The column was washed with buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 0.02% sodium azide. Bound proteins were eluted with buffer containing 50 mM glycine/HCl (pH 2.6) and 0.1% Triton X-100. Each fraction was neutralized immediately with 1 M Tris/HCl (pH 8.0) and subjected to SDS-PAGE analysis. Peak fractions were combined, dialyzed against 0.1% Triton X-100 and 10 mM NaCl, and lyophilized. Purified proteins were resolved with SDS-PAGE, blotted onto a Immobilon P membrane (Nihon Millipore, Tokyo, Japan), and visualized with Coomassie brilliant blue R-250 (Nippon Bio-Rad Laboratories, Kanagawa, Japan). The target proteins were excised and loaded onto a protein sequencer (Applied Biosystems, Foster City, CA).

cDNA cloning

A cDNA library was prepared previously from a B cell lymphoma (BCL1) (9). Degenerate primers were designed according to the N-terminal aa sequence (double underlined in Fig. 2A). Two primers were prepared for each direction to reduce degeneracy. The sequences of primers were as follows: forward primer 1, GA(CT)CA(CT)GG(ACGT)TC(ACGT)GA(AG)AA(CT)GG(ACGT)TGG; forward primer 2: GA(CT)CA(CT)GG(ACGT)AG(CT)GA(AG)AA(CT)GG(ACGT)TG; reverse primer 1: TG(AG)TA(ACGT)AC(ACGT)AC(CT)TC(ACGT)AG(ACGT)CC(ACGT)CC; and reverse primer 2: TG(AG)TA(ACGT)AC(ACGT)AC(CT)TC(CT)AA(ACGT)CC(ACGT)CC. Purified DNA from the BCL1 cDNA library was used as a template, and amplification was conducted as follows: a cycle of 94°C for 2 min, 55°C for 2 min, and 72°C for 30 s followed by 29 cycles of 94°C for 45 s, 55°C for 2 min, and 72°C for 30 s. The final step at 72°C was extended to 10 min. Amplified products were subjected to PAGE. A fragment of expected size (71 bp) was recovered using the QIAEX II Gel Extraction kit (Qiagen GmbH, Hilden, Germany) and was cloned into a plasmid by T-A cloning. Cloned fragments were sequenced, and those encoding the obtained aa sequence were used as a probe to screen the cDNA library. Colony hybridization was performed as described previously (9). Sequencing was conducted with an ALFexpress DNA sequencer (Pharmacia) and a Thermo Sequenase cycle sequencing kit (Amersham Japan, Tokyo, Japan).

Northern hybridization

Total RNA was extracted from various tissues with Isogen (Nippon Gene, Toyama, Japan) and subjected to agarose electrophoresis (20 μg/lane). After transfer to a nylon membrane (Hybond-N$^+$, Amersham), RNA was hybridized to a probe that had been labeled by the random priming of a cDNA clone encoding mouse MD-1. The hybridization buffer consisted of 10% dextran sulfate (Pharmacia), 1 M NaCl, 1% SDS, and 50 mM Tris/HCl (pH 7.5). Hybridization was conducted at 65°C for 20 h. Washing was conducted in 2 × SSC and 0.1% SDS at 65°C. Radioactive signals were visualized using an image-analyzer BAS2000 (Fuji Film, Tokyo, Japan). The same membrane was reprobed for glyceraldehyde-3-phosphate dehydrogenase (11).

Cell surface biotinylation and immunoprecipitation

Cell surface biotinylation and immunoprecipitation was conducted as described previously (12). Briefly, cells were washed in HBSS and adjusted to 5 × 10^7/ml in saline containing 100 mM HEPES (pH 8.0). Sulfosuccinimidobiotin (Pierce, Rockford, IL) was added to the cell suspension to make a final concentration at 0.5 mg/ml. After a 30-min incubation at room temperature with occasional shaking, cells were washed in HBSS and lysed in lysis buffer (see above). After a 30-min incubation on ice, lysate was centrifuged, and nuclei were removed. We used either anti-RP105-coupled HiTrap beads or anti-flag mAb (M2)-coupled Sepharose 4B (Eastman Kodak); mouse IgG1-coupled HiTrap beads were used as control beads. The beads were added to cell lysate and rotated for 2 h at 4°C. Beads were washed in lysis buffer, and bound proteins were subjected to SDS-PAGE and Western blotting. Biotinylated proteins were detected with streptavidin-peroxidase (Amersham) and SuperSignal chemiluminescent substrate (Pierce).

Transient transfection

The human kidney cell line 293T was plated onto a 24-well plate at 1.5 × 10^5/well on the day before transfection. The calcium phosphate/DNA coprecipitate was prepared as follows. DNA (1 μg) was diluted in 100 μl of deionized water, and 2 M of calcium chloride (14 μl) was added to DNA. An equal amount (114 μl) of 2 × HEPES-buffered saline (280 mM NaCl,
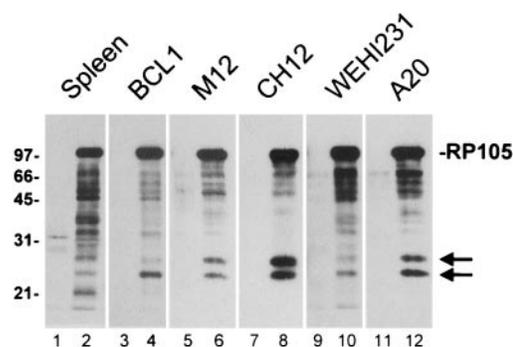

FIGURE 1. Coprecipitation of 22/25-kDa molecules (gp22/25) with RP105. Five B cell lines and normal spleen cells were used for cell surface biotinylation and subsequent immunoprecipitation with the anti-RP105 mAb RP/14 (*lanes 2, 4, 6, 8, 10,* and *12*) or with control IgG (*lanes 1, 3, 5, 7, 9,* and *12*). Precipitates were subjected to SDS-PAGE (10% acrylamide) under reduced conditions, blotted to a polyvinylidene difluoride membrane, and visualized with streptavidin-horseradish peroxidase. RP105 and gp22/25 (arrows) are indicated.

50 mM HEPES, and 1.5 mM sodium phosphate (pH 7.05)) was added slowly in a dropwise manner. After 30-min incubation at room temperature, coprecipitates were added to 293T cells. Medium was changed on the following day and then cultured for 1 to 2 days. Cells were harvested with PBS containing 1 mM EDTA and were used for further analysis.

Results

Molecules of 22/25 kDa are precipitated with RP105

In an attempt to search for RP105-associated molecules, we conducted immunoprecipitation studies. Because the extracellular domain of RP105 has LRRs and the intracellular domain is very short, we expected that the associated molecule would be a cell surface molecule. Therefore, cell surface biotinylation was used for labeling proteins. We used several B cell lymphomas that express RP105. After extracting membrane proteins with buffer containing 1% Triton X-100, RP105 was precipitated with an anti-RP105 Ab. Two extra signals of ~22 and 25 kDa (glycoprotein (gp)22/25) were apparent in precipitates from all five B cell lines and normal spleen cells (Fig. 1, arrows). Interestingly, the amount of gp22/25 was variable with each B cell line, and gp22 was dominant over gp25 in BCL1, WEHI231, and A20. Both signals migrated faster under nonreduced conditions, suggesting an intramolecular disulfide bond (data not shown).

cDNA cloning of 22/25-kDa molecules and identification as mouse MD-1

We subsequently proceeded to purify the 22/25-kDa molecules and determine their N-terminal aa sequence (see *Materials and Methods*). A total of 24 residues were successfully determined from the 25-kDa molecule (Fig. 2A, double underline). The same sequence was obtained from the 22-kDa molecule. Because the sequence did not show any identity to previously identified sequences, we moved on to cDNA cloning (see *Materials and Methods*). A DNA fragment encoding the N-terminal aa sequence was obtained by PCR using degenerate primers. A cDNA library was screened using the DNA fragment as a probe. A total of 18 clones were obtained from 2 × 10^5 colonies. After the size determination and restriction mapping of cDNA inserts, we focused on a 1-kb cDNA clone. An entire sequence of the clone was determined and is shown in Figure 2A. The longest open reading frame begins with a codon at position 95–97 and ends with a stop codon at 578–580. This sequence encodes 162 aa, and the experimentally determined

A

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  1 GAAACTGAACATACTACTCCTTCAGGGGGAGGACGAGCTTCGGAGGTCTTTTTATTGGGC
 61 TGGCTTACCATTGGGCACACACAACCCCCTCGCCATGAATGGTGTCGCAGCTGCCCTCCT
-19                                         M  N  G  V  A  A  A  L  L

121 TGTGTGGATTCTGACTTCTCCGAGCAGCAGTGACCATGGCAGCGAAATGGTTGGCCCAA
-10  V  W  I  L  T  S  P  S  S  S  D  H  G  S  E  N  G  W  P  K

181 GCACACGGCCTGCAACAGTGGGGGCTTGGAAGTAGTCTACCAGAGCTGTGATCCCTTACA
 11  H  T  A  C  N  S  G  G  L  E  V  V  Y  Q  S  C  D  P  L  Q

241 GGATTTTGGCCTTTCCATTGACCAGTGTTCCAAGCAGATCCAATCAAATCTCAACATTAG
 31  D  F  G  L  S  I  D  Q  C  S  K  Q  I  Q  S  N  L  N  I  R

301 ATTTGGCATCATTCTGAGACAGGATATCAGAAAGCTGTTTCTGGACATAACTCTGATGGC
 51  F  G  I  I  L  R  Q  D  I  R  K  L  F  L  D  I  T  L  M  A

361 AAAAGGCTCTTCTATTCTGAACTACTCCTATCCCCTTTGTGAGGAGGACCAGCCCAAGTT
 71  K  G  S  S  I  L  N  Y  S  Y  P  L  C  E  E  D  Q  P  K  F
                                           *
421 CTCATTCTGTGGAAGAAGAAAAGGAGAACAGATATACTATGCCGGCCCTGTCAATAACCC
 91  S  F  C  G  R  R  K  G  E  Q  I  Y  Y  A  G  P  V  N  N  P

481 TGGACTTGATGTTCCACAGGGAGAATATCAGCTCTTGCTGGAACTGTACAATGAAAACCG
111  G  L  D  V  P  Q  G  E  Y  Q  L  L  L  E  L  Y  N  E  N  R

541 TGCTACTGTGGCTTGTGCCAATGCCACTGTCACCTCCTCCTGAGCATGGTCTGCAAGGAA
131  A  T  V  A  C  A  N  A  T  V  T  S  S  .
                          *
601 ATGCACAGTAAACTCAATCTCAGGGGACCCCAAGGTCCCTGGACTCACCTAGCTGCAAGA
661 ACCACTGATAACCAAGAGAGGCTTTACAAAGAAATTTCTTGTGGGTCACTCTTCCGATCT
721 TAGCTCCAGGGACAGATGTTCCCAGACCCAACAGATGTAATAAACCCTCAAAAACTATCT
781 ATTTCTGAGGACCCTGAGTAGTCTTGAAGCCCTATTGTAGTACCTCTCCTTATGTAATTA
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FIGURE 2. Nucleotide and aa sequence of mouse MD-1 and its homology to chicken MD-1. *A*, The first aa in the mature peptide is positioned at 1. The signal sequence is underlined. The sequence obtained from purified MD-1 is double underlined. Canonical *N*-glycosylation sites are indicated by an asterisk. *B*, The aa sequence of chicken and mouse MD-1 is aligned. Identical residues are indicated by an asterisk. Five gaps have been introduced in chicken MD-1 to obtain maximal homology. These sequence data are available from EMBL/GenBank/DDBJ under accession number AB007599.

B

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Mouse  MNGVAAALLV WILTSPSSSD HGSENGWPKH TACNSGGLEV VYQSCDPLQD  50
       *       **    *      *         ** * * *    **    * **** **

chick  MKTLNVLALV LVLLCINAS- ----TEWPTH TVCKEENLEI YYKSCDPQQD  45

mouse  FGLSIDQCSK QIQSNLNIRF GIILRQDIRK LFLDITLMAK GSSILNYSYP 100
       *   *** **         **   *** *   *      *    *   * **

chick  FAFSIDRCSD VTTHTFDIRA AMVLRQSIKE LYAKVDLIIN GKTVLSYSET  95

mouse  LCEEDQPKFS FCGRRKGEQI YYAGPVNNPG LDVPQGEYQL LLELYNENRA 150
       **       *  *** ***   ** **      *** *    * ** **

chick  LCGPGLSKLI FCGKKKGEHL YYEGPITLGI KEIPQGDYTI TARLTNEDRA 145

mouse  TVACANATVT SS                                         162
       ***** **

chick  TVACADFTVK NYLDY                                      160
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sequence starts at the aa-1 (double underlined in Fig. 2*A*). Therefore, the hydrophobic stretch from the aa-19 to the aa-1 is a signal sequence (underlined in Fig. 2*A*). Poly(A) signals locate in a 3′ untranslated region. Mature peptide consists of 143 aa and a deduced molecular mass of 17.8 kDa. Asparagine residues at positions 77 and 137 are canonical *N*-glycosylation sites (represented by an asterisk in Fig. 2*A*). The databases were searched again using the entire aa sequence, and chicken MD-1 (10), a *v-myb*-regulated gene, showed significant homology (Fig. 2*B*). Chicken MD-1 cDNA is ~1 kb and codes for 160 aa. With 5 gaps between the aa 19 and aa 20 in chicken MD-1, 64 aa are identical (40% identity). We conclude that the cDNA encodes a mouse homologue of chicken MD-1. Hereafter, it is referred to as mouse MD-1. Northern hybridization was conducted to visualize tissue distribution (Fig. 3). The MD-1 transcript is ~1 kb in size and is most abundant in the spleen. It was also detectable in other tissues such as the liver, brain, thymus, and kidney. MD-1 may have an additional role in nonimmune systems. Most B cell lines expressed the MD-1

transcript, but an IL-3-dependent line, Ba/F3, did not (data not shown).

MD-1 is a secreted protein but is present on cell surface in association with RP105

The cDNA clone does not have the second hydrophobic stretch, suggesting that MD-1 is a secretory molecule. We created a construct that tagged MD-1 with the flag epitope at the C terminus (MD-1flag). This construct was transfected into 293T cells. As expected, MD-1flag was not expressed on 293T cells but was demonstrable in the supernatant (Fig. 4, *A*1 and *B*). A broad signal was apparent that was 22 to 35 kDa. However, the flag epitope appeared on the cell surface when RP105 was coexpressed (Fig. 4*A*2). The expression of MD-1flag showed a linear correlation with that of RP105 (Fig. 4*C*). An irrelevant cDNA did not confer MD-1 expression on the cell surface (data not shown). Consequently, MD-1 is a secreted protein but can be present on the cell surface in the presence of RP105.
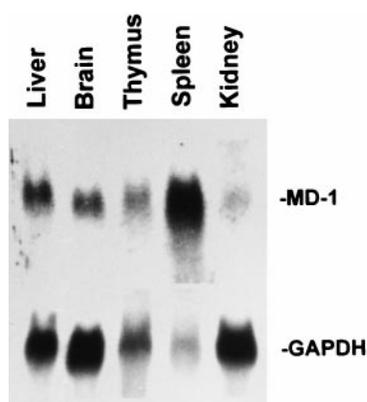
FIGURE 3. Tissue distribution of the MD-1 transcript. Total RNA was extracted from several tissues and subjected to electrophoresis (20 μg/lane) and subsequent transfer to a nylon membrane. The MD-1 cDNA was used as a probe, and signals were visualized with an image analyzer. The same membrane was reprobed for the glyceraldehyde-3-phosphate dehydrogenase transcript as an internal control.

Mouse MD-1 binds to RP105

To prove that MD-1 binds to RP105, coprecipitation studies were conducted using the BaRPMD cell line expressing RP105 and MD-1flag (see below). After cell surface biotinylation, immunoprecipitation was conducted with either anti-RP105 or anti-flag mAb (Fig. 5). Precipitated proteins were probed with either avidin (for cell surface molecules) or anti-flag Ab (for MD-1flag). The anti-RP105 mAb precipitated RP105 and additional molecules that were 25 to 30 kDa (*lane 2*). These molecules are MD-1flag, since similar signals of 28 and 25 kDa were apparent by probing with anti-flag mAb (*lane 5*). Signals in *lane 2* are stronger than those in *lane 5*, since *lanes 1* through *3* were exposed to film longer than *lanes 4* through *6* to show MD-1 signals in *lane 3*. When immunoprecipitation was conducted with anti-flag mAb, signals were weaker than with anti-RP105 mAb. Anti-flag mAb may have difficulty recognizing the flag epitope on MD-1 when it is associated with RP105. Although weaker, MD-1 signals were apparent in *lane 3* as well as *lane 6*. RP105 was also visible when probed with avidin (*lane 3*). In addition, we noticed another signal just below the upper MD-1 signal in *lane 3*; it was not detected by probing with anti-flag mAb (*lane 6*). A similar signal was observed in *lane 2* with shorter exposure. Further study is underway to identify this signal. Immunoprecipitation with control IgG (mouse IgG1) had no signal (*lanes 1* and *4*). From these results, we conclude that MD-1 is associated with RP105.

A role of MD-1 in RP105 expression

With an aim to understand a role of MD-1 in RP105 expression and function, we attempted to differentiate the RP105/MD-1 complex from the RP105 monomer. The cell line 293T was transiently transfected either with RP105 alone or with RP105 and MD-1 and then stained with the anti-RP105 mAb. Three independent experiments were conducted, and the percentages of RP105-positive cells are shown in Table I. In a control transfection in which RP105 was expressed with an irrelevant molecule, Ly-6A/E, the mean percentage of RP105-positive cells was less than half of the transfection of RP105 alone. On the other hand, the transfection of RP105 and MD-1 resulted in more than twice as many RP105-positive cells when compared with the transfection of RP105 alone. Moreover, we established and used stable transfectants. The BaRP30 cell line was previously established by transfecting an expression vector encoding RP105 into the IL-3-dependent line
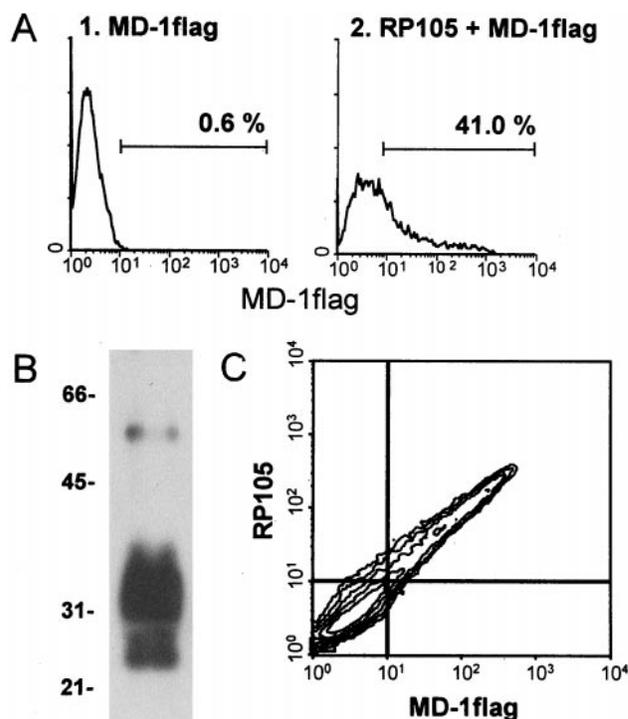

FIGURE 4. MD-1 is secreted in culture fluid but tethered to a cell surface in the presence of RP105. *A*, MD-1 cDNA was tagged with the flag epitope at the C terminus (MD-1flag) and expressed in 293T cells either alone (A1) or in combination with RP105 (A2) by transient transfection. Cells were then stained with an anti-flag mAb followed by goat anti-mouse IgG-FITC. The percentages of flag-positive cells are also shown. *B*, Supernatant from 293T cells expressing MD-1flag was collected and subjected to immunoprecipitation with the anti-flag mAb. Precipitated proteins were resolved with SDS-PAGE, blotted, and detected with the anti-flag mAb followed by goat anti-mouse IgG-peroxidase. A signal of ∼60 kDa is not MD-1flag, since this signal was detected without the anti-flag mAb (data not shown). *C*, The dual staining of 293T cells that had been transiently transfected with RP105 and MD-1flag is shown. The anti-flag mAb was followed with an FITC-rat-anti-mouse κ mAb that does not react with a rat mAb. Biotinylated anti-RP105 mAb and avidin-phycoerythrin were used for counterstaining.

Ba/F3 (9). BaRP30 was further transfected with another expression vector encoding MD-1. Therefore, the newly established line, BaRPMD, differs from BaRP30 in MD-1 expression only. BaRP30 expresses RP105 alone, while BaRPMD expresses RP105 and MD-1. Indeed, MD-1 is present on BaRPMD but not on BaRP30

Table I. *Cotransfection of MD-1 up-regulates cell surface expression of RP105[a]*

Transfected with Vectors Encoding:	Percentages of RP105-Positive Cells			
	Expt. 1	Expt. 2	Expt. 3	Mean ± SD
Ly-6A/E	0.1	0.6	0.1	0.3 ± 0.3
MD-1 flag	0.1	0.1	0.1	0.1 ± 0
RP105	21.6	25.1	7.4	18.0 ± 9.4
RP105 + Ly-6A/E	12.8	6.8	2.5	7.4 ± 5.2
RP105 + MD-1 flag	41.7	41.2	43.9	42.2 ± 1.4

[a] The human kidney cell line 293T was transfected with calcium phosphate-mediated transfection (see *Materials and Methods*). After 2 (experiments 1 and 3) or 3 days (experiment 2), cells were harvested and used for cell surface staining with the anti-RP105 mAb RP/14. Percentages of RP105-positive cells and mean values ± SD from three independently conducted experiments are shown.
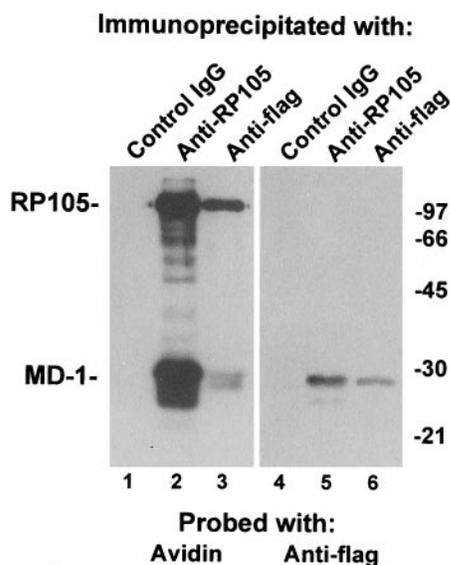
Immunoprecipitated with:

FIGURE 5. Association of MD-1 and RP105. A stable transfectant, BaRPMD, that expresses RP105 and MD-1 was used. Cell surface proteins were biotinylated, extracted, and immunoprecipitated with either control mouse IgG1 (*lanes 1* and *4*), anti-RP105 mAb (*lanes 2* and *5*), or anti-flag mAb (*lanes 3* and *6*). Precipitated proteins were subjected to SDS-PAGE (12% acrylamide) and blotted onto a membrane. Proteins were detected with either streptavidin-peroxidase (*lanes 1*, *2*, and *3*) or the anti-flag mAb followed by goat anti-mouse IgG-peroxidase (*lanes 3*, *4*, and *5*) to detect cell surface molecules and MD-1, respectively. To indicate MD-1 in *lane 3*, *lanes 1* through *3* were exposed to film longer than *lanes 4* through *6* during detection with chemiluminescence.

(Fig. 6, *A* and *C*). The introduction of MD-1 conferred approximately a sevenfold increase in the mean fluorescence intensity of RP105 (Fig. 6*B*: 75.4; Fig. 6*D*: 519.0). The binding of anti-RP105 mAb RP/14 to BaRP30 is as saturable as it is to BaRPMD, and a sufficient amount of anti-RP105 Ab was used in these experiments. Therefore, the difference in fluorescence intensity stems from the increased expression of RP105 molecules. We have established eight other lines by transfecting BaRP30 with MD-1flag. All of these lines showed an increased expression of RP105 (data not shown). Taken together with the results presented in Table I, these results lead to the conclusion that MD-1 positively regulates the cell surface expression of RP105 (see *Discussion*).

Discussion

The present study reports a molecule that is associated with RP105. An anti-RP105 Ab precipitated gp22/25 as well as RP105 from B cell lines and normal spleen cells (Fig. 1). A cDNA clone for gp22/25 was isolated and found to be a mouse homologue of chicken MD-1 (10). The physical association of MD-1 and RP105 was confirmed by the following results: 1) MD-1 is a secretory molecule but is tethered to the cell surface in the presence of RP105. The expression of MD-1 shows a linear correlation with that of RP105. 2) MD-1 was coprecipitated in combination with RP105 (Fig. 5).

The Ba/F3 cell line does not express the MD-1 transcript (data not shown), but RP105 was able to come out by stable transfection (Ref. 9 and Fig. 6). Therefore, MD-1 is dispensable for RP105 expression. However, MD-1 has an effect on RP105 expression. The transient transfection of RP105 and MD-1 resulted in about twice a many RP105-positive cells compared with the transfection of RP105 alone (Table I). The stable transfection of MD-1 conferred approximately a sevenfold increase in cell surface RP105 on
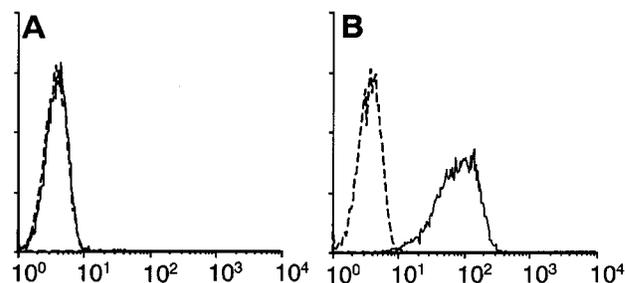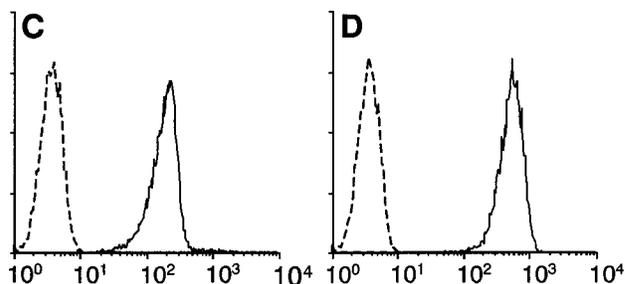

FIGURE 6. Introduction of MD-1 up-regulates the cell surface expression of RP105. BaRP30 is an IL-3-dependent line that was transfected with an expression vector coding for RP105. BaRPMD was established by the further transfection of BaRP30 with MD-1flag. Both were stained with either anti-flag mAb (*A* and *C*) or anti-RP105 mAb (*B* and *D*) followed by FITC-conjugated goat anti-mouse or rat IgG Ab, respectively. The hatched lines depict histograms that were stained with the second reagent alone. The mean fluorescence intensity of each sample was 4.0 (*A*), 75.4 (*B*), 178 (*C*), and 519.0 (*D*).

a cell line that expresses RP105 alone. Moreover, we also found that human MD-1 plays a similar role in the expression of human RP105 (Miura, Y., R. Shimazu, K. Miyake, S. Akashi, H. Ogata, Y. Yamashita, Y. Narisawa, and M. Kimoto. 1998. RP105 is associated with MD-1, and transmits an activation signal in human B cells. *Blood In press*). Considering the physical association, the RP105/MD-1 complex would be favored over the RP105 monomer for cell surface expression. MD-1 seems to be requisite for efficient expression of RP105. There are several other molecules that require an additional, physically associated subunit for proper expression on the cell surface. A heavy chain of the class I MHC complex is associated with β_2-microglobulin (β_2m). β_2m is similar to MD-1 in that it does not have a transmembrane portion; both are either secreted or attached to another molecule on the cell surface. Some class I MHC heavy chains such as H-2D[b] are able to reach the cell surface without β_2m. However, those free heavy chains are not stable on the cell surface and during intracellular transport, most likely due to an inappropriate conformation (13). This may also be the case with RP105. RP105 may take a more stable conformation in the presence of MD-1. We recently obtained an anti-RP105 mAb. The mAb recognized the RP105/MD-1 complex but not the RP105 monomer (our unpublished observations). Therefore, the RP105 monomer would be different from the RP105/MD-1 complex in its conformation. MD-1 is likely to have an important role in the proper conformation and efficient expression of RP105.

Two distinct bands of 22 and 25 kDa were coprecipitated with RP105. These two species behaved similarly during SDS-PAGE

under either reduced or nonreduced conditions and have the same aa sequence at the N termini. They are probably the same protein with a distinct posttranslational modification. Indeed, we obtained two distinct signals of MD-1 by transfecting an MD-1 cDNA (Fig. 5). Glycosylation would be one of the most probable causes of the size difference. Gp25 would have heavier glycosylation than gp22.

RP105 belongs to the LRR superfamily, in which some members are implicated in linking innate and adaptive immunity (14). CD14 is the monocyte/macrophage LPSR and activates these monocytes/macrophages in response to LPS. Activated macrophages secrete cytokines that then act on lymphocytes. The Toll receptor protects Drosophila from fungal infection (5). A human homologue of the Toll receptor was recently isolated (6). It is expressed on lymphocytes as well as on monocytes/macrophages and delivers an activation signal. These LRR molecules are activated in innate immunity and potentiate adaptive immunity (14). RP105 is another LRR molecule that is specifically expressed on B lymphocytes. Given that RP105 has a function that is similar to other LRR proteins, it may help B cells to communicate with and respond to innate immunity. In this regard, a ligand of RP105 could be a product of pathogens, as is the case with tomato genes *cf-2* and *cf-9*. In seeking a ligand, MD-1 must be taken into consideration. It may regulate the ligand binding of RP105 by modulating its conformation or even by providing a ligand-binding site. Our attempts to search for a ligand have been conducted using a fusion protein that contains the extracellular domain of RP105. However, two new probes must be prepared (fusion proteins of MD-1 or RP105/MD-1). The preparation of new fusion proteins is currently underway; such proteins will be used in our continuous effort to search for a ligand of RP105 in lymphocytes, monocytes/macrophages, and pathogens.

Previously, we have proposed two subdomains in the LRRs of RP105 (15). There is a cluster of nonhomologous repeats (from 7–10) in the middle of the 22 LRRs. This cluster would divide the RP105 LRRs into two subdomains, an N-terminal subdomain and a membrane-proximal subdomain. Preliminary studies localized the MD-1-binding site at the N-terminal LRRs (our unpublished observations). RP105 may allow another molecule to bind to membrane-proximal LRRs. The cytoplasmic region of RP105 consists of only 11 aa. An associated signal transducer is expected to transmit an activation signal. Such a signaling molecule might bind to membrane-proximal LRRs. Other LRR molecules in the immune system have given us an idea regarding signaling in RP105. The Toll receptor has a cytoplasmic portion that is similar to the IL-1R and activates the NF-κB pathway in both Drosophila and humans (6, 16, 17). Also, plant resistance genes have LRRs and activate the NF-κB pathway (reviewed in Ref. 4). This connection of LRR molecules and the NF-κB pathway seems to be true of RP105, because B cell activation with RP105 ligation is severely affected in c-*rel* knockout mice (18). Moreover, we confirmed IκBα degradation in response to RP105 cross-linking (our unpublished observations). Thus, a signal through RP105 is likely to activate the NF-κB pathway, and a signal transducer, if any, would link RP105 with the NF-κB pathway.

In conclusion, the present study determined that MD-1 is an RP105-associated molecule. Its association changes the conformation of RP105 and facilitates its cell surface expression. This finding should accelerate our search for a ligand and a signaling molecule of RP105.

Acknowledgments

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