CD48 Is a Counter-Receptor for Mouse CD2 and Is Involved in T Cell Activation

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Summary

CD2 is an intercellular adhesion molecule that has been implicated in T cell activation and differentiation both in humans and mice. Although the ligand for human CD2 has been defined as LFA-3, that for murine CD2 has not been identified yet. To identify the ligand for mouse CD2, we generated a chimeric molecule consisting of the extracellular domain of mouse CD2 and human immunoglobulin (Ig)G1 Fc (mCD2Rg). A hamster monoclonal antibody (mAb), HM48-1, was established by screening mAbs that could block the binding of mCD2Rg to T cell lines at the ligand site. The putative mouse CD2 ligand recognized by this mAb was a glycosyl phosphatidylinositol–anchored glycoprotein with an apparent molecular mass of 45 kD, which were shared characteristics with human LFA-3. However, its expression was predominantly restricted to hematopoietic cells, unlike human LFA-3. Protein microsequencing analysis for the NH_2-terminal 18 amino acid residues of the affinity-purified HM48-1 antigen revealed that it is almost identical with mouse CD48. This identity was further confirmed by the reactivity of HM48-1 with a soluble recombinant CD48 (sCD48) protein and the molecule recognized by a rat mAb raised against sCD48. A rat anti-CD48 mAb blocked the mCD2Rg binding as well as HM48-1. Moreover, sCD48 also inhibited the mCD2Rg binding to the cellular ligand. Finally, like anti-CD2 mAb, HM48-1 inhibited the phytohemagglutinin response and, when crosslinked, augmented the anti-CD3 response of splenic T cells. These results indicate that CD48 is a ligand for mouse CD2 and is involved in regulating T cell activation.

CD2 has been implicated in T cell adhesion and activation (1, 2). A natural ligand for CD2 has been identified as LFA-3, which is expressed on a variety of cells, including nonhematopoietic cells in humans (3–5). CD2 and LFA-3 molecules have a structural similarity (6) and a close genetic linkage (7), suggesting that these molecules have evolved from a common precursor, which has a homotypic adhesion function–like neural cell adhesion molecule, by a gene duplication (8). It has been proposed that the intercellular interaction between CD2 and LFA-3 leads to bidirectional signaling via each molecule, resulting in activation of both T cells and APC (9–11). However, it has been demonstrated that the binding of LFA-3 was not sufficient for triggering T cell activation via CD2 since the addition of anti-CD2R mAbs was required (12–14). This suggests the existence of an additional ligand corresponding to anti-CD2R mAb, but it has not been identified yet.

CD48, which is known as Blast-1 in humans (15), OX-45 in rats (16), and BCM1 in mice (17), is a glycosyl phosphatidylinositol (GPI)[1]-anchored glycoprotein expressed on almost all T and B cells, whose function remains unknown. Molecular cloning studies revealed a high structural homology of human CD48 molecule to LFA-3 (18), suggesting that it would also function as an adhesion molecule. We describe here that CD48 is a ligand for mouse CD2 and is involved in regulating T cell activation.

Materials and Methods

Cell Lines. Mouse T cell leukemia MBL-2 was maintained in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated FCS (JR Scientific Inc.,

[1] *Abbreviations used in this paper:* GPI, glycosyl phosphatidylinositol; ICAM-1, intercellular adhesion molecule 1; PI-PLC, phosphatidylinositol phospholipase C; SF, superfamily.

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Woodland, CA), 100 IU/ml penicillin G, 1 mM sodium pyruvate, 2 mM L-glutamine, 2.3 mg/ml Hepes, and 2.0 mg/ml $NaHCO_3$. Mouse cytotoxic T cell line CTLL-2 was cultured in the same medium supplemented with 50 U/ml of rIL-2 (Shionogi Pharmaceutical Co., Osaka, Japan).

Antibodies. Rat mAbs reactive with mouse CD2 (RM2-1) (19), mouse CD48 (MRC OX78) (A. F. Williams, manuscript in preparation), heat-stable antigen (J11d) (20), LFA-1 (KBA) (21), intercellular adhesion molecule 1 (ICAM-1) (KAT-1) (K. Kato, manuscript in preparation), and hamster mAbs reactive with mouse CD2 (HM2-31), which was raised against affinity-purified mouse CD2 molecule and reacts with a CD2R epitope (H. Yagita, details will be described elsewhere), and mouse CD3 (145-2C11) (22) were purified from ascites by using mAb Trap G (Pharmacia, Uppsala, Sweden) affinity chromatography. FITC-conjugated anti-human IgG and FITC-conjugated anti-hamster IgG antibodies were purchased from Caltag Laboratories (San Francisco, CA).

mCD2Rg Construction. The mouse CD2 cDNA (pMCD2-2) (23) was digested with HpaI, which cleaves the cDNA near the start of the transmembrane region, and a double-stranded synthetic oligonucleotide BamHI adaptor:

AACTGTCCAGAGAAG
TTGACAGGTCTCTTCCTAG

regenerating the codons for Asn-Cys-Pro-Glu-Lys was ligated to the blunt HpaI ends. After EcoRI digestion, the 650-bp EcoRI-BamHI fragment containing the extracellular region of mouse CD2 cDNA and the 1.3-kb BamHI-XbaI fragment containing human IgG1 constant region genomic sequences, which was prepared from an IgG1 expression plasmid provided by Dr. Brian Seed (Massachusetts General Hospital, Boston, MA) (24), were subcloned into EcoRI- and XbaI-digested pBluescript II SK(+) vector (Stratagene, La Jolla, CA). This results in the in-frame fusion of mouse CD2 extracellular region to the human IgG1 hinge region as shown in Fig. 1 *A*. After digestion with XhoI and NotI, a 1.95-kb insert containing the fusion construct was transferred into the XhoI and NotI sites of the BCMGSneo expression vector (25), kindly provided by Dr. H. Karasuyama (Basel Institute, Basel, Switzerland).

Preparation of mCD2Rg Chimeric Protein. Mouse myeloma cell line P3U1 was transfected with mCD2Rg cDNA in the expression vector BCMGSneo by using a Cell-Porator (Bethesda Research Laboratories, Gaithersburg, MD). Transfected cells were selected using 1 mg/ml of G418 (Geneticin; Wako Pure Chemical Industries, Osaka, Japan) and analyzed for cytoplasmic expression of the mCD2Rg protein by immunofluorescence assay using FITC-conjugated anti-human IgG. After cloning by limiting dilution, secretion of the mCD2Rg chimeric protein into the medium was detected by immunoprecipitation, and the reactivity of mCD2Rg against mouse cell lines was tested by immunofluorescence assay as follows.

Immunofluorescence Assay. To examine the reactivity of mCD2Rg against mouse cell lines, the pellets of 10^6 cells were incubated with 1 ml of culture supernatant containing mCD2Rg for 1 h at 4°C followed by FITC-conjugated anti-human IgG. After gently washing, the cells were analyzed on a FACScan® (Becton Dickinson & Co., San Jose, CA) and data were processed by using the Consort 30 program.

Immunoprecipitation. The mCD2Rg transfectants (10^6) were cultured with 100 μCi/ml [^{35}S]-Met and [^{35}S]-Cys (TransLabel; ICN Biomedicals Inc., Costa Mesa, CA) at 37°C for 16 h. The culture supernatant was immunoprecipitated with protein G–conjugated Sepharose (Pharmacia) or anti-mouse CD2 mAb (RM2-1)-coupled Sepharose for 1 h at 4°C. After washing with 1% NP-40 in PBS, the bound materials were eluted and subjected to SDS-PAGE analysis under nonreducing or reducing conditions. For fluorography, gels were incubated with Amplify (Amersham Japan, Tokyo, Japan) for 30 min, dried, and exposed to x-ray film (Kodak XAR-5) at −80°C for 24 h.

To identify the HM48-1-recognized antigen on mouse cell lines, cell pellets (10^7) were suspended in saline containing 0.1 M Hepes, and then labeled with 100 μg/ml of sulfo-*N*-hydroxysuccinimide-biotin (Pierce Chemical Co., Rockford, IL) for 40 min at room temperature. Biotin-labeled cells were extracted with a lysis buffer (1% NP-40, 20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 μM A-PMSF, and 50 μg/ml trypsin inhibitor). After preclearing with normal hamster Ig–coupled Sepharose, the lysate was immunoprecipitated with HM48-1-coupled Sepharose for 1 h at 4°C. After washing with the lysis buffer, the eluates were subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane filters (Nihon Millipore Kogyo K.K., Japan). The blots were subsequently incubated with VECTASTAIN ABC solution (Vector Laboratories Inc., Burlingame, CA). After washing, the blots were further incubated with ECL Western blotting detection reagents (Amersham Japan) and exposed to x-ray films for a few minutes.

Generation of mAb. Male Armenian hamsters (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) were immunized by intraperitoneal injection of mouse T lymphoma MBL-2 (10^7) for several times at 10-d intervals. 3 d after final immunization, the splenocytes were prepared and fused with PA-I mouse myeloma cells as described previously (19). After HAT selection, the antibodies that inhibit the binding of mCD2Rg to MBL-2 were screened. HM48-1 was identified by its strong inhibitory effect and cloned by limiting dilution.

Inbred AO rats (MRC Cellular Immunology Unit, Oxford, UK) were immunized twice subcutaneously with 20–100 μg sCD48 (17) in CFA within a 2-wk interval. The rats were boosted with 20 μg sCD48 intravenously 4 d before fusion. The splenocytes were prepared by teasing the spleen in RPMI 1640 and were fused with parental Y3 myeloma cells at a ratio of 2:1 in 1.2 ml 50% polyethylene glycol 4000 in HBSS. The cell mixture was resuspended in 25 ml Isocove's medium with 20% FCS and HAT, and dispensed into a 96-well plate. The culture supernatants were screened by binding assay with BALB/c thymocytes and ^{125}I-labeled rabbit anti-rat Fab, and the positive clones were further cloned by limiting dilution analysis. The antibody isotype of the clones was determined with an isotyping kit (Serotec Ltd., Kidlington, Oxford, UK).

Affinity Purification of the HM48-1 Antigen and Protein Microsequencing. The lysate prepared from 10^{10} MBL-2 cells in the same lysis buffer as used in immunoprecipitation was subjected to 1 ml of HM48-1-conjugated Sepharose. After washing with the lysis buffer, the bound material was eluted with 50 mM glycine buffer (pH 3.0) containing 0.1% NP-40. 900-μl fractions were collected and immediately neutralized with 1 M Tris buffer (pH 7.8). 10-μl aliquots of each fraction were subjected to SDS-PAGE and silver staining. The fractions containing the antigen were pooled, dialyzed against distilled water, and lyophilized. Approximately 10 μg of the affinity-purified antigen was subjected to a 4–20% gradient SDS-PAGE, electroblotted onto a polyvinylidine difluoride membrane (Applied Biosystems, Inc., Foster City, CA), and stained with coomassie blue. The band was cut out and subjected to microsequencing on a sequencer equipped with on-line PTH analysis (477A; Applied Biosystems, Inc.).

Phosphatidylinositol Phospholipase C (PI-PLC) Treatment. Spleen

cells (10^6/ml) were incubated in serum-free RPMI 1640 with or without 50 mU/ml of PI-PLC (Boehringer Mannheim, Mannheim, Germany) for 60 min at 37°C. After washing three times with the medium, the reactivity with HM48-1 and J11d was analyzed by immunofluorescence.

Proliferation Assay. Spleen cells were cultured with 1 μg/ml of Con A (Sigma Chemical Co., St. Louis, MO) or 10 μg/ml of PHA (Difco Laboratories, Detroit, MI) at 37°C in the presence or absence of various mAbs (10 μg/ml) for 48 h. The cultures were pulsed with 1 μCi [^{3}H]TdR for the last 4 h. Cells were collected by using a cell harvester (Skatron, Lier, Norway), and the radioactivity of each well was measured by a standard liquid scintillation counting.

To examine a stimulatory effect of mAbs, nylon wool–nonadherent spleen cells were cultured with 1 μg/ml of anti-CD3 mAb (145-2C11) in the presence or absence of HM2-31 or HM48-1 mAbs on anti–hamster IgG-coated plates for 48 h, and the incorporation of [^{3}H]TdR was measured as above.

Results

Construction and Characterization of mCD2Rg Chimeric Protein. The construction of the mCD2Rg chimeric protein was depicted in Fig. 1 *A*. The mCD2Rg construct was subcloned into the mammalian expression vector BCMGSneo and introduced into the myeloma cell line P3U1 by electroporation. Transfected cells were selected by G418 and cloned by limiting dilution. To determine the secretion of mCD2Rg protein into the culture supernatant of the transfectants, we performed immunoprecipitation with RM2-1 (anti–mouse CD2 mAb)-conjugated Sepharose and protein G–conjugated Sepharose. As shown in Fig. 1 *B*, a single polypeptide with an apparent molecular mass of 180 kD was immunoprecipitated by both RM2-1 and protein G from the supernatant of mCD2Rg transfectants under nonreducing conditions. After reduction, a single band of 80 kD was detected in the immunoprecipitates, indicating that the mCD2Rg chimeric protein was expressed as a disulfide-linked homodimeric molecule.

Generation of HM48-1 mAb, Which Inhibits mCD2Rg Binding to Mouse T Cells. We first estimated the binding of mCD2Rg to various mouse cell lines by immunofluorescent staining with FITC-conjugated anti–human IgG and flow cytometric analysis. The mCD2Rg bound to several T cell lines, including CTLL-2, MBL-2, and FBL-3. The binding of mCD2Rg to CTLL-2 was completely abrogated by preincubation with RM2-1 (Fig. 2 *A*) but not with anti-LFA-1 or anti-ICAM-1 mAbs of the same isotype (data not shown), indicating that the mCD2Rg binding was CD2 dependent. To generate the mAbs that inhibit the mCD2Rg binding at the ligand site on the cells, we immunized Armenian hamsters with MBL-2, and the resulting immune splenocytes were fused with PA-I myeloma. After screening the ability to inhibit the mCD2Rg binding and cloning, we established a mAb HM48-1 (hamster IgG) that could completely block the binding of mCD2Rg to MBL-2 and CTLL-2 (Fig. 2 *B*). HM48-1 exhibited an inhibitory effect even when the cells were preincubated with it and washed before estimating the mCD2Rg binding, suggesting that this mAb recognizes a ligand for mouse CD2. A similar pretreatment of the cells with anti-CD2, anti-LFA-1, or anti-ICAM-1 mAbs did not affect the mCD2Rg binding (data not shown).

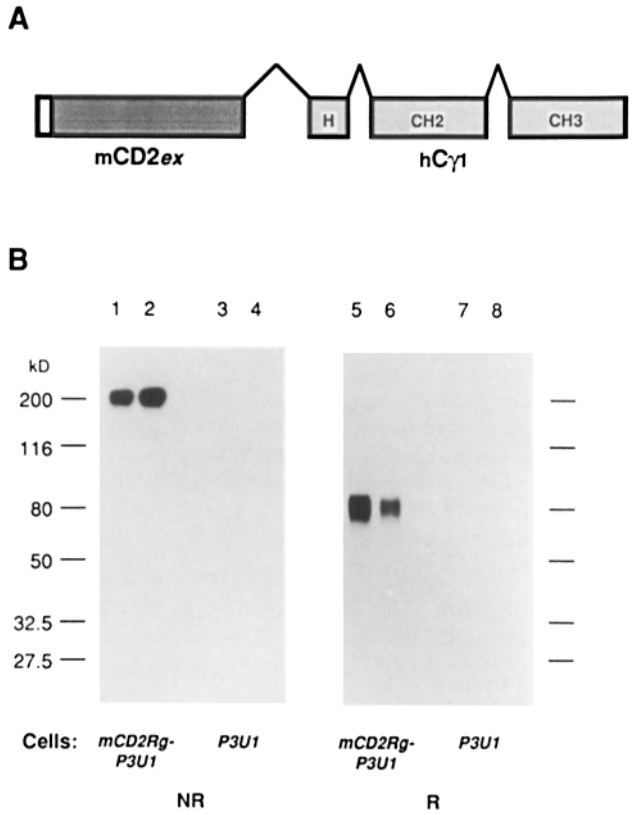

Figure 1. Construction and characterization of mCD2Rg chimeric protein. (*A*) Schematic representation of mCD2Rg construction. cDNA encoding the extracellular region of mouse CD2 (mCD2ex) was fused with genomic human Cγ1 (hCγ1) gene–containing exons for hinge (H), CH2, and CH3 regions. (*B*) Immunoprecipitation of radiolabeled mCD2Rg. Expression plasmid containing the chimeric construct was transfected into P3U1 myeloma cells. mCD2Rg transfectants (mCD2Rg-P3U1; lanes *1*, *2*, *5*, and *6*) and parent cells (P3U1; lanes *3*, *4*, *7*, and *8*) were labeled with [^{35}S]Met and [^{35}S]Cys, and the labeled proteins secreted into culture supernatants were immunoprecipitated with protein G–Sepharose (lanes *1*, *3*, *5*, and *7*) or anti–mouse CD2 mAb–conjugated Sepharose (lanes *2*, *4*, *6*, and *8*). The precipitates were subjected to SDS-PAGE (4–20%) under nonreducing (*NR*; lanes *1–4*) or reducing (*R*; lanes *5–8*) conditions and then to fluorography. Position of the molecular mass markers are indicated on the left.

Cell Surface Expression of the Antigen Recognized by HM48-1. We next examined the distribution of the HM48-1-defined Ag on various mouse cells. HM48-1 reacted not only with CTLL-2 and MBL-2 but also with all spleen cells and thymocytes (Fig. 3 *A*). The Ag defined by HM48-1 was widely distributed in hematopoietic cell lines, including helper T cell lines, cytotoxic T cell lines, T and B lymphomas, and macrophages, but not in nonhematopoietic cell lines, including fibroblasts, carcinomas, and melanomas (data not shown).

We next examined the biochemical nature of the HM48-1-defined Ag on lymphocytes by using the enzyme PI-PLC, which cleaves the GPI-anchored membrane proteins, including human LFA-3 (26) and mouse heat-stable antigen (27). The

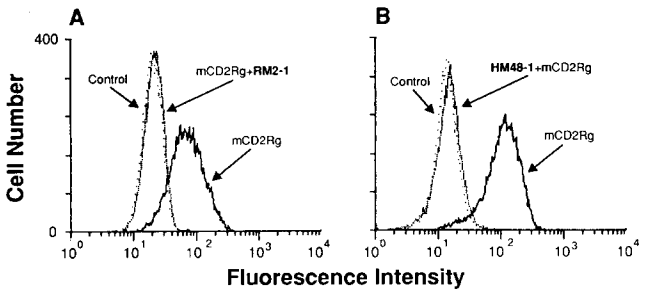

Figure 2. Binding ability of mCD2Rg chimeric protein. (*A*) Inhibitory effect of RM2-1 on mCD2Rg binding. Mouse T cell line CTLL-2 was stained with mCD2Rg chimeric protein in the presence or absence of RM2-1 at 10 μg/ml, followed by FITC-conjugated anti-human IgG antibody. (*B*) Inhibitory effect of HM48-1 on mCD2Rg binding. CTLL-2 was first pretreated with HM48-1 at 10 μg/ml. After washing with PBS, the cells were stained with mCD2Rg and FITC-conjugated anti-human IgG. A total of 10,000 cells each was analyzed on FACScan®.

treatment of spleen cells with PI-PLC resulted in a marked decrease in binding of HM48-1 and J11d as determined by indirect immunofluorescence (Fig. 3 *B*). This decrease was specific for the HM48-1 Ag and was not observed with CD2, which possesses a cytoplasmic tail (data not shown). These results indicate that the HM48-1-defined Ag is a GPI-anchored membrane molecule, which is a shared characteristic with human LFA-3.

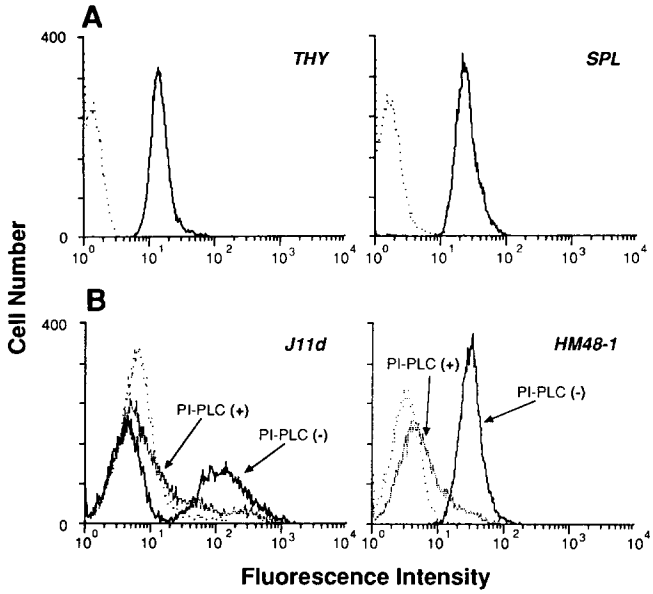

Figure 3. Expression and PI-PLC sensitivity of the HM48-1-defined antigen on mouse thymocytes and splenocytes. (*A*) Reactivity of HM48-1 with mouse thymocytes (*THY*) and splenocytes (*SPL*). Cells were stained with HM48-1 at 10 μg/ml, followed by FITC-conjugated anti-hamster IgG. (*B*) Effect of PI-PLC treatment on HM48-1 reactivity. Splenocytes were treated with PI-PLC (*thin lines*) and then stained with J11d (*left*) or HM48-1 (*right*). FITC-conjugated anti-rat IgG or FITC-conjugated anti-hamster IgG were used as second-step reagents for J11d and HM48-1, respectively. Solid lines indicate splenocytes treated with the medium without PI-PLC. Dotted lines indicate the staining with second-step reagents alone.

Molecular Characterization of the HM48-1-defined Ag. Biochemical natures of the HM48-1-defined Ag were further examined by immunoprecipitation and SDS-PAGE analysis. Lysates of surface biotinylated MBL-2, CTLL-2, thymocytes, and splenocytes were subjected to immunoprecipitation with HM48-1-conjugated Sepharose, and SDS-PAGE analysis was performed under nonreducing or reducing conditions. As shown in Fig. 4 *A*, the Ag defined by HM48-1 was a single polypeptide with apparent molecular masses of 45 or 50 kD under nonreducing or reducing conditions, respectively. A similar molecule was also identified from CTLL-2, thymocytes, and spleen cells (Fig. 4 *B*).

We next performed the affinity purification of the HM48-1-defined Ag. Detergent lysate of MBL-2 T lymphoma cells (10^{10}) was subjected to the HM48-1-coupled Sepharose column, and the bound proteins were eluted and fractionated. As shown in Fig. 5 *A*, three fractions containing the 45-kD molecule were pooled and subjected to protein microsequencing. The NH_2-terminal 18–amino acid residues were determined, except for one residue, which was obscure and indicated with an *X* in Fig. 5 *B*. We performed a computer search for the protein data base and unexpectedly found an extremely high homology to mouse CD48 rather than human LFA-3. In Fig. 5 *B*, the NH_2-terminal sequences of the HM48-1 Ag, mouse, rat, or human CD48 (16–18), and human LFA-3 (6) are aligned. 16 of 18 NH_2-terminal residues of the HM48-1-defined Ag were identical with that of mouse CD48. The obscurity of residue 16 of the HM48-1 Ag might result

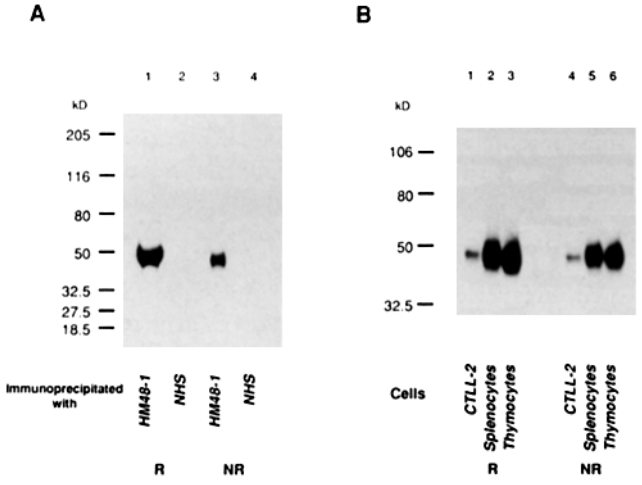

Figure 4. Immunoprecipitation of the antigen recognized by HM48-1. (*A*) MBL-2 mouse T lymphoma cells (10^7) were surface biotinylated and lysed in lysis buffer containing 1% NP-40. Immunoprecipitation was performed by using normal hamster serum (*NHS*; lanes *2* and *4*) or HM48-1 (lanes *1* and *3*). Eluates were subjected to SDS-PAGE (4–20%) under reducing (*R*; lanes *1* and *2*) or nonreducing (*NR*; lanes *3* and *4*) conditions and then blotted onto nitrocellulose membranes. Biotinylated proteins were detected by ABC complex and chemiluminescence. (*B*) CTLL-2 mouse T cell line (10^7), mouse splenocytes (2×10^8), and thymocytes (2×10^8) were surface biotinylated and lysed. Immunoprecipitates with HM48-1 were subjected to SDS-PAGE under reducing (*R*; lanes *1–3*) or nonreducing (*NR*; lanes *4–6*) conditions and then to blotting. Biotinylated proteins were detected as above. Positions of the molecular mass markers are indicated on the left.

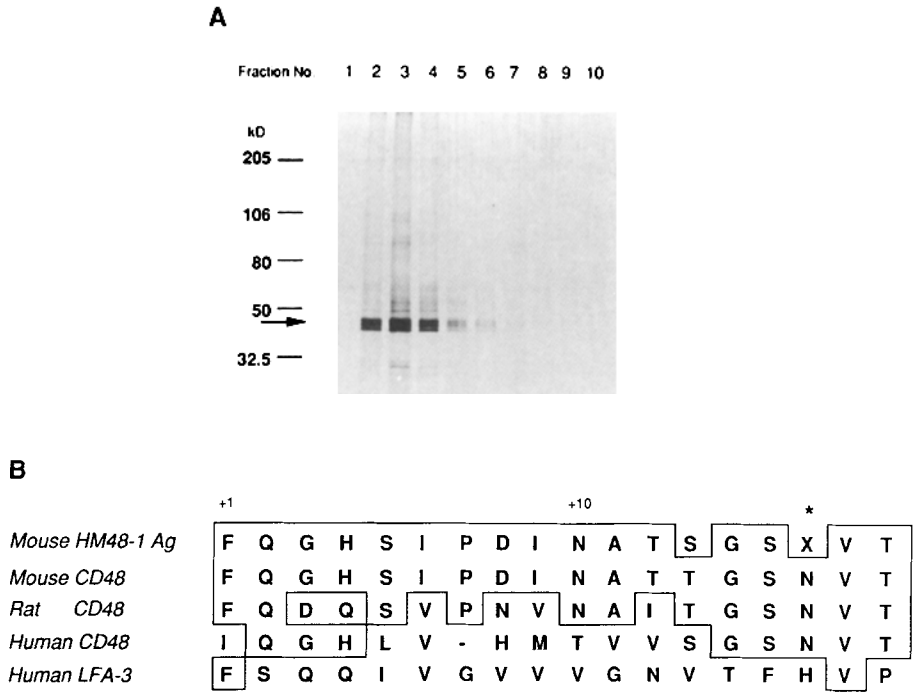

Figure 5. Affinity purification of the HM48-1 Ag and comparison of its NH_2-terminal sequence with CD48 and LFA-3. (*A*) The lysate of MBL-2 (10^{10}) was subjected to HM48-1 conjugated Sepharose. After washing with lysis buffer, the bound materials were eluted with glycine buffer. 10-μl aliquots of each fraction were subjected to SDS-PAGE and silver staining. The arrow indicates the HM48-1 antigen. The fractions containing the antigen were pooled and subjected to protein microsequencing. (*B*) NH_2-terminal amino acid sequence of the HM48-1 antigen, those of mouse, rat, or human CD48, and that of human LFA-3 are aligned. Identical residues with the HM48-1 antigen are boxed. The asterisk above residue 16 indicates the potential N-glycosylation site of mouse CD48.

from its potential N-glycosylation. In contrast, no similarity was observed between the HM48-1-defined Ag and human LFA-3. These results strongly suggested that the HM48-1 Ag is not a mouse homologue of human LFA-3 but of mouse CD48.

HM48-1 Reacts with Mouse CD48. To conclude whether HM48-1 is directed to mouse CD48, sequential immunoprecipitation was carried out by using HM48-1 and a rat anti-mouse CD48 mAb, MRC OX78, which was prepared against a soluble recombinant mouse CD48 protein (A. F. Williams, manuscript in preparation). HM48-1 immunoprecipitated a molecule the same size as that precipitated by OX78 from the surface-biotinylated MBL-2 lysate (Fig. 6). After preclearing with OX78 mAb, the 45-kD HM48-1 Ag disappeared. This result indicates that HM48-1 and OX78 do react with the same molecule. Moreover, HM48-1 reacted with the soluble recombinant mouse CD48 (sCD48) protein (17) in ELISA and Western blotting (data not shown). These results definitively indicated that HM48-1 is directed to mouse CD48.

sCD48 Protein and Anti-CD48 mAbs Block the mCD2Rg Binding. To confirm that mouse CD48 recognized by HM48-1 is a ligand for mouse CD2, we examined the inhibitory effects of sCD48 protein (17) and the rat anti-mouse CD48 mAb (OX78) on the mCD2Rg binding to MBL-2. As shown in Fig. 7 *A*, pretreatment of MBL-2 with OX78 as well as HM48-1 could have completely inhibited the mCD2Rg binding. Furthermore, preincubation of mCD2Rg with sCD48 perfectly abrogated its binding ability to MBL-2 (Fig. 7 *B*). These results clearly indicated that mouse CD48 acts as the ligand for mouse CD2.

Mouse CD48 Is Involved in T Cell Activation. We finally performed preliminary studies on the biological functions of mouse CD48 by using HM48-1 mAb. We previously observed that PHA, but not Con A, response of mouse spleen cells was highly susceptible to anti-CD2 mAb (28). HM48-1 exhibited a similar inhibitory effect to that of RM2-1 on PHA and Con A responses (Fig. 8). The addition of both RM2-1 and HM48-1 exhibited a similar extent of inhibition as those observed with each mAb alone (data not shown), suggesting that CD2 and CD48 act as a receptor ligand pair involved in PHA response.

It was of interest to study whether mouse CD48 itself would

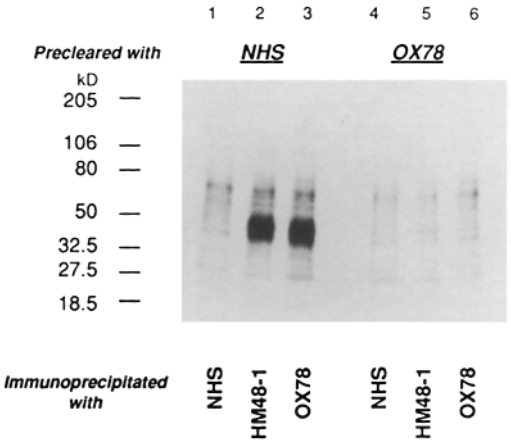

Figure 6. Sequential immunoprecipitation of the antigen recognized by HM48-1 and MRC OX78. NP-40 lysate of surface biotinylated CTLL-2 (10^7) was precleared with normal hamster serum (*NHS*; lanes *1–3*) or mAb reactive with murine CD48 (*OX78*; lanes *4–6*). The precleared lysates were then subjected to immunoprecipitation with NHS (lanes *1* and *4*), HM48-1 (lanes *2* and *5*), or OX78 (lanes *3* and *6*) as described in Fig. 4. SDS-PAGE was performed under nonreducing conditions.

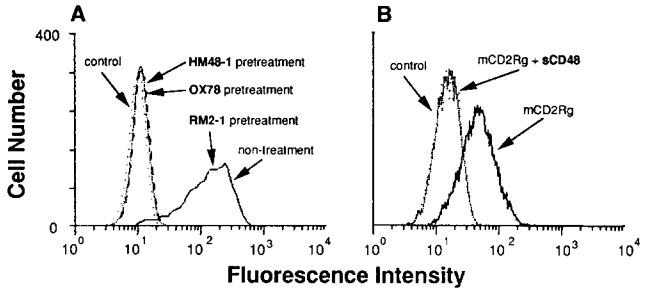

Figure 7. Inhibitory effects of a rat anti-mouse CD48 mAb and sCD48 on mCD2Rg binding. (*A*) CTLL-2 was first pretreated with HM48-1, OX78, or RM2-1, as indicated. After washing with PBS, the cells were stained with mCD2Rg and FITC-conjugated anti-human IgG antibody. (*B*) CTLL-2 was stained with mCD2Rg in the presence or absence of sCD48 (50 μg/ml), followed by FITC-conjugated anti-human IgG. A total of 10,000 cells each was analyzed on FACScan®.

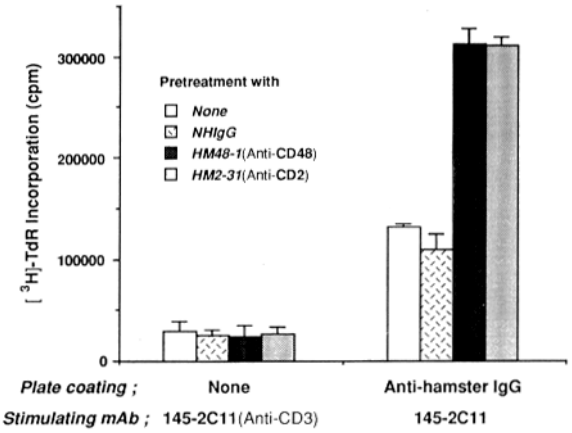

Figure 9. Costimulatory effect of HM48-1 on anti-CD3 response. Nylon wool–nonadherent spleen cells were cultured with anti-CD3 mAb (*145-2C11*; 1 μg/ml) in the presence or absence of normal hamster IgG (*NHIgG*), anti-CD48 mAb (HM48-1), or anti-CD2 mAb (*HM2–31*) at 10 μg/ml on anti-hamster IgG-coated plates for 48 h. The incorporation of [3H]TdR was measured as described in Fig. 8. Data represent means ± SD or triplicate wells.

be involved in T cell activation, since some GPI-anchored T cell surface molecules, including Thy-1 and Ly6, have been implicated in T cell activation (29–31). Although HM48-1 alone did not induce the proliferative response of splenic T cells even when crosslinked with anti-hamster IgG antibody (data not shown), it was shown that HM48-1 greatly augmented the proliferative response when cocrosslinked with an anti-CD3 mAb (Fig. 9). A similar costimulatory effect was also observed with an anti-CD2R mAb (HM2-31). These results indicate that CD48 can also act as a signal-transducing molecule regulating the CD3-mediating T cell activation as well as CD2.

Discussion

In the present study, we generated a mAb against the mouse CD2 ligand by screening mAbs that could inhibit the binding of a mouse CD2–human Ig fusion protein. Surprisingly, the Ag recognized by this mAb was not a mouse homologue of human LFA-3 but was mouse CD48. The fact that CD48 is a ligand for mouse CD2 was further confirmed by using an authentic anti-CD48 mAb and sCD48 protein. In addition, we finally indicated that CD48 is involved in mouse T cell activation like CD2.

To identify the mouse CD2 ligand, we first constructed a chimeric protein consisting of the extracellular region of mouse CD2 and the human IgG1 constant region. This mCD2Rg bound to several T and B cell lines but not to nonhematopoietic cell lines. The binding of mCD2Rg was inhibited by anti-CD2 mAb but not by anti-LFA-1 or anti-ICAM-1 mAbs of the same isotype, indicating that it was CD2 dependent. Our findings with mCD2Rg are consistent with the results recently reported by Rutschmann and Karjalainen (32). They also pointed out a preferential expression of the mouse CD2 ligand on lymphoid cells by using a mouse CD2–human Cμ fusion protein, although they did not identify the ligand. These observations are quite different from the distribution of human LFA-3, which is broadly expressed on various hematopoietic and nonhematopoietic cells (33). To identify the mouse CD2 ligand, we established a mAb that could interfere with the mCD2Rg binding at the ligand site. mAb HM48-1 completely inhibited the mCD2Rg binding when preincubated with the target cells. As far as

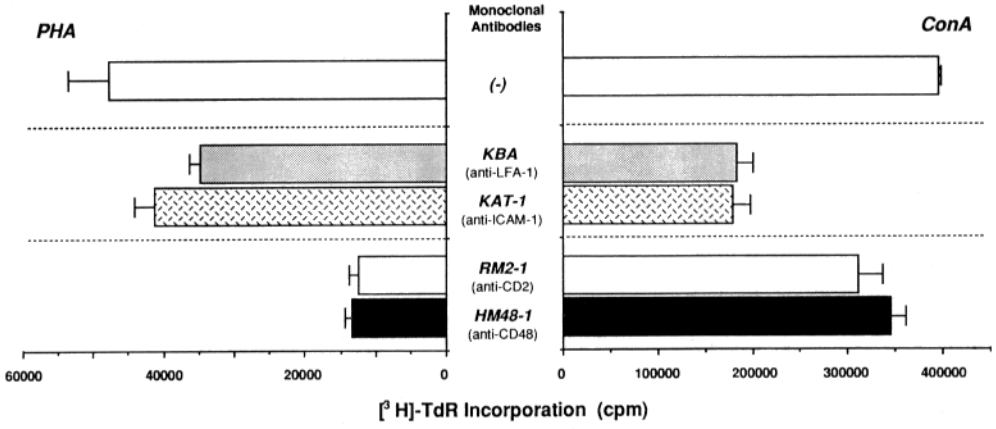

Figure 8. Blocking effect of HM48-1 on PHA and Con A responses. Mouse splenocytes (5 × 10^5) were cultured with 10 μg/ml of PHA (*left*) or 1 μg/ml of Con A (*right*) in the presence or absence of the indicated mAb (10 μg/ml) for 48 h. The cultures were pulsed with [3H]TdR for the last 4 h, and incorporation of [3H]TdR was determined by scintillation counting. Results are shown as means ± SD of triplicate wells.

we tested, all cell lines that bound mCD2Rg expressed the HM48-1-defined Ag, and the binding of mCD2Rg to these cells was always completely blocked by HM48-1. These results indicate that the Ag recognized by HM48-1 is the predominant (or apparently sole) ligand for mouse CD2.

We next performed biochemical characterizations of the HM48-1 Ag to identify whether it is mouse LFA-3 or not. In immunoprecipitation experiments, the Ag's have an apparent molecular mass of 45 and 50 kD under nonreducing or reducing conditions, respectively. The PI-PLC treatment abolished the HM48-1 reactivity, indicating that the Ag is a GPI-anchored membrane protein. These characteristics were shared with human LFA-3. Surprisingly, however, the NH_2-terminal amino acid sequence of the affinity-purified HM48-1 Ag was almost identical to that of mouse CD48, but was totally different from human LFA-3. In addition, the results obtained by using an authentic anti-CD48 mAb and sCD48 protein indicated that HM48-1 does react with mouse CD48. Furthermore, sCD48 protein as well as another anti-CD48 mAb could inhibit the mCD2Rg binding like HM48-1. Based on these results, we concluded that CD48 is the predominant ligand for mouse CD2.

Human CD48 (Blast-1) was first identified as a cell surface molecule highly expressed on EBV-transformed B lymphoblasts (34). Further studies indicated that CD48 is a GPI-anchored membrane protein and is expressed not only on activated B cells but also on T cells, monocytes, and endothelial cells (35). However, little has been known about the function of CD48. Molecular cloning studies revealed a structural and evolutionary relationship of CD48 with LFA-3 and CD2 (16–18). CD48 contains two Ig superfamily (IgSF) domains with the first being V-like without a disulfide bond, and the second being a C2-SET domain. This is a shared property with CD2 and LFA-3. Notably, the similarity between the NH_2-terminal domains of CD48 and LFA-3 scored 63% when considering conservative amino acid substitutions (18). Based on these observations, CD48 has been proposed to constitute a subgroup within the IgSF along with CD2 and LFA-3, which is closely related in evolution and suspected to have evolved from a common precursor (16, 17). This, in turn, implied that CD48 might function as an adhesion molecule like LFA-3 and CD2 (18). Recently, Yokoyama et al. (15) proposed that human CD48 might interact with CD2, but they did not exhibit any evidence for this interaction. Therefore, this is the first study indicating the function of CD48 as a CD2 ligand. In our preliminary studies with CD2-Ig fusion proteins containing the extracellular portion of human or rat CD2, it has been observed that CD48 acts as a CD2 ligand also in humans and in rats (unpublished observations). Therefore, the interaction between CD48 and CD2 appears to be conserved among these three species.

In mice and rats, at present, nothing is known about the human LFA-3 homologue. Our repeated trials for genomic or cDNA cloning of mouse and rat LFA-3 by crosshybridization with the human LFA-3 cDNA probe were so far unsuccessful. Our present observations that HM48-1 always completely blocked the mCD2Rg binding to all cells tested suggest a possibility that LFA-3 may not be expressed in mice. The gene for human CD48 and those for human CD2 and LFA-3 have been mapped in the pericentric regions of human chromosome 1 in the lq21-23 and lp12 regions, respectively (7, 18, 36). In mice, the CD48 gene is located in a region of distal chromosome 1 (17) that is syntenic with human chromosome lq21-32 (37), and the CD2 locus is located in a region of chromosome 3 (38) that is syntenic with the human lp12 region (39, 40). In our previous study (17), it has been suggested that the duplication of a chromosomal region including the precursors of the genes for CD48, CD2, and LFA-3 has occurred and the duplicated regions have stayed together on chromosome 1 in humans with the insertion of a centromere, while, in mice, the genetic regions have become dispersed in the formation of chromosomes 1 and 3. If LFA-3 was not present in mice, it might mean that CD2 and CD48 first evolved from the common precursor before their genetic regions dispersed in mice, and LFA-3 evolved from CD48 later in humans by a further gene duplication in chromosome 1. This speculation is consistent with the fact that CD48 and LFA-3 are more similar in sequence than CD2 and LFA-3. Such a case, that more members constituting an IgSF subgroup have evolved from a common precursor in humans than in mice, has been known for the low affinity Fcγ receptors, for which five genes (hFcγRIIA, hFcγRIIB, hFcγRIIC, hFcγRIIIAα, and hFcγRIIIB) have been found in humans while only two genes (mFcγRII and mFcγRIIIα) have been found in mice (41). Therefore, CD48 might be the primary ligand for CD2 commonly used in all mammalian species. However, it has been demonstrated that the genomic organization of a set of markers defining the region around the CD2 gene locus on human chromosome lp and on mouse chromosome 3 is highly conserved between these two species (17). This suggests that the murine LFA-3 gene might be located near the CD2 gene locus, as is the human LFA-3 gene. Further genetic studies will be needed to clarify this issue.

Alternatively, our failure to find another ligand for CD2 might be due to a lower affinity of mCD2Rg to murine LFA-3 than to CD48. However, it seems unlikely since a human CD2-Ig chimeric protein (hCD2Rg), which was constructed in a similar way to mCD2Rg, exhibited an almost equivalent affinity to CD48 and LFA-3 (our unpublished observation).

Several GPI-anchored membrane molecules, including Thy-1 (30), Ly6 (31), and sgp-60 (42, 43), have been implicated in T cell activation. Therefore, it was of particular interest to investigate the function of CD48 not only as a CD2 ligand but also as a potential T cell–activating molecule. We first examined the effect of HM48-1 on mitogen responses of mouse T cells in order to estimate the involvement of CD48 as a ligand of CD2. As shown in Fig. 8, HM48-1 strongly inhibited the proliferative response to PHA, while the Con A response was not significantly affected. This paralleled the effect of RM2-1, suggesting that CD48 would act as the CD2 ligand in the PHA response.

We next demonstrated that the crosslinking of CD48 would deliver a costimulatory signal augmenting T cell activation. A synergistic proliferative response was observed when CD48 was cocrosslinked with CD3. A similar costimulatory effect

was also observed when CD2 was cocrosslinked with CD3. These results suggest that both the intercellular interaction between CD2 on a T cell and CD48 on an opposite cell, and that between CD48 on a T cell and CD2 on an opposite cell, can be costimulatory for T cell activation and that CD48 acts as a functional counter-receptor regulating T cell activation. The T-T and T-B cell interactions are critically involved in eliciting and regulating cellular and humoral immune responses. CD48 is expressed on both T and B cells in mice (this study) and humans (15), and CD2 is expressed on B cells in mice in addition to T cells (19). Although the involvement of CD48 in B cell activation remains to be determined, the CD2-CD48 interaction revealed in this study might play an important role in regulating immune responses by providing bidirectional costimulatory signals during T-T and T-B cell interactions. To support this notion, a critical role of CD2 in regulating noncognate and cognate T-B interactions (44) and a dramatical immunosuppressive effect of anti-CD2 mAb in vivo (45, 46) have been demonstrated. The physiological role for CD48 in regulating immune responses will be elucidated by such studies in mice using anti-CD48 mAbs described in this study.

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