The Journal of Immunology

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J Immunol 1998; 161:5809-5812; ;
http://www.jimmunol.org/content/161/11/5809

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cutting Edge: Identification of the 2B4 Molecule as a Counter-Receptor for CD48[1]

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The CD48 molecule belongs to a subfamily of the Ig superfamily that also includes the CD2, CD58, 2B4, Signaling lymphocyte activation molecule (SLAM), and Ly-9 molecules. Receptor-ligand interactions are known to occur between several members of this family, and these interactions can strengthen cell to cell adhesion. In mice, the CD48 molecule can bind to CD2. To search for additional ligands of murine CD48, we have generated a chimeric fusion protein consisting of the extracellular domain of murine CD48 and the C region of human IgG1. The results of immunofluorescence and immunoprecipitation experiments in which this reagent was used identify the 2B4 molecule as a novel counter-receptor of CD48. ***The Journal of Immunology,*** **1998, 161: 5809–5812.**

The CD2 subfamily of the Ig superfamily (IgSF)[3] includes the CD2, CD48 (Blast-1, BCM-1, sgp-60, and OX-45), CD58 (LFA-3), 2B4, Ly-9, and SLAM molecules. Membership within this family is defined by structural features and by the chromosomal colocalization and evolutionary relationship of the family members. The Ags of the CD2 subfamily have similar patterns of conserved disulfide bonds and typically comprise one membrane-distal V-set domain and one membrane-proximal C2-set domain, with the exception of Ly-9, which contains a tandem repeat of V-C2-set domains. In mice, the genes encoding the proteins of the CD2 subfamily are situated in two loci: *Cd2* is situated on chromosome 3; *Cd48*, *2b4*, and *Ly-9* are clustered on chromosome 1 (1).

CD2, the prototype of the family, is a transmembrane protein expressed on the surface of T lymphocytes, thymocytes, and NK cells; in mice, it is also found on B cells (1). CD2 has two known ligands. Its major ligand in humans is CD58 (LFA-3) (2), a molecule that is widely expressed on hemopoietic and many nonhemopoietic tissues. No CD58 homologue has been identified in rodents. Instead, another member of the CD2 subfamily, CD48 (3–6) has been identified as a CD2 ligand in mice and rats (1). The expression of CD48 is regulated more tightly than that of CD58 and is restricted to lymphocytes, dendritic cells, and macrophages (1).

The functional significance of murine CD2 and CD48 is still uncertain. Mice carrying a targeted mutation in the *Cd2* gene are almost normal phenotypically (1). In contrast, the phenotype of CD48-deficient mice that we have recently generated is more pronounced. Specifically, T lymphocytes from CD48-deficient mice are impaired in activation (Cabrero et al., manuscript in preparation). This discrepancy in phenotypes led us to ask whether CD48 binds to another counter-receptor in addition to CD2. In this article we provide evidence that CD48 binds the 2B4 molecule.

Materials and Methods

Abs and chemicals

The following mAbs and second-step reagents were obtained from PharMingen (San Diego, CA): anti-mouse CD2 (clone RM2-5), anti-mouse CD48 (clone HM48.1), anti-mouse 2B4 (clone 2B4), anti-Thy-1 mAb (clone 53-2.1), and FITC-avidin. Anti-glycoprotein 200 (gp200)-MR6 (clone MR6 (7)) and anti-H-2K^b (clone Y3 (8)) mAbs were gifts of Drs. Mary Ritter and Hans Stauss (Hammersmith Hospital), respectively. The human CTLA4-Ig fusion protein has been described previously (9). All other Abs and chemicals were obtained from Sigma (Dorset, U.K.) unless otherwise indicated.

Construction of CD48-Ig fusion protein

CD48 was amplified by PCR from a B cell cDNA library (6). The sense primer (5′-TGGGAAAGCTTTTTCAAGGTCATTCAATACCAG-3′) corresponds to base pairs 67–88 of CD48 and to a restriction site for *Hin*dIII. The antisense primer (5′-GAAGGGATCCCCTAGATCACAAG GTAGAGTGAAGTACAC-3′) corresponded to nucleotides 616–643 of CD48 and to a restriction site for *Bam*HI. PCR conditions were 94°C for 1 min, primer annealing at 55°C for 1 min, and an extension at 72°C for 1 min, for 35 cycles. The 598-bp product was digested with *Hin*dIII and *Bam*HI and ligated into the signal pIg plus vector that contains the signal peptide of CD33 and the human IgG1 Fc fragment (Ingenius, Abingdon, U.K.). The sequence of the resulting plasmid was confirmed by MWG-Biotech (Milton Keynes, U.K.).

COS-7 cells were transfected with the CD48-pIg plasmid using Lipofectin (Life Technologies, Paisley, UK). Transfected cells were selected with 1 mg/ml of G418 and subcloned. The CD48-Ig protein was purified from culture medium on a protein A column and subjected to Western blot analysis. For this purpose, samples (1 μg) of the CD48-Ig fusion protein or of a gp200-MR6 control fusion protein (P.F.M. and M. Ritter, unpublished observations) were fractionated on a 7.5% SDS-PAGE gel under nonreducing conditions. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Following a blocking step, membranes were cut; the pieces were subjected to one of the following incubations as described previously (10): 1) biotinylated goat anti-human IgG followed by avidin peroxidase (extravidin peroxidase); 2) biotinylated hamster anti-mouse CD48 followed by avidin-peroxidase; or 3)

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Received for publication August 26, 1998. Accepted for publication September 29, 1998.

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[1] This research was supported by a project grant from the Wellcome Trust.

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[3] Abbreviations used in this paper: IgSF, Ig superfamily; ECL, enhanced chemiluminescence; gp, glycoprotein; PVDF, polyvinylidene difluoride.

0022-1767/98/$02.00

mouse anti-human gp200-MR6 Ab followed by peroxidase-labeled rabbit anti-mouse Ig. Membranes were washed after each incubation. Proteins were visualized with enhanced chemiluminescence (ECL) substrate (Amersham, Little Chalfont, U.K.) before autoradiography.

Immunofluorescence and flow cytometry

To determine CD2 expression, cells were incubated with biotinylated anti-CD2 mAb (clone RM2-5) followed by incubation with FITC-avidin. To determine 2B4 expression, cells were incubated with anti-2B4 mAb (clone 2B4) followed by incubation with FITC-conjugated goat anti-mouse IgG. For staining with CD48-Ig or CTLA4-Ig, cells were incubated with purified fusion protein followed by incubation with biotin-conjugated goat anti-human IgG and, finally, by incubation with FITC-avidin. Control stainings included stainings with developing reagents (biotin-conjugated goat anti-human IgG followed by FITC-avidin; FITC-conjugated goat anti-mouse Ig) only. For the sake of clarity, the staining profiles of these control stainings are omitted in several of the figures. All incubations were conducted on ice. Following each step, cells were washed three times with PBS/1% BSA before the next incubation. After the final incubation, cells were washed and fixed with 1% paraformaldehyde. Samples were analyzed on a Coulter Epics XL-MCL instrument (Hialeah, FL). A total of 10,000 cells were analyzed per sample.

Cloning and expression of murine 2B4

The complete coding region of 2B4 was amplified by PCR from CTLL-2 cells and cloned into the pEGFP-C1-*Not*I vector (Clontech, Palo Alto, CA). The sense primer was 5′-GTAGGCGGCCGCGTCCTGTGGTGATGTT GGGGCAAGCTG-3′ and corresponded to base pairs 1–16 of 2B4 and a restriction site for *Not*I. The antisense primer was 5′-GCGAATTCCTAG GAGTAGACATCAAAGTTCTCCAGCTCTC-3′ and consisted of nucleotides 1166–1197 of 2B4 and a restriction site for *Eco*RI. PCR conditions were 94°C for 1 min, primer annealing at 65°C for 1 min, and an extension at 72°C for 1 min, for 35 cycles. The 1.23-kb *Not*I/*Eco*RI-digested PCR product was ligated into the pEGFP-C1-*Not*I vector that had been digested with *Not*I and *Eco*RI to remove the green fluorescent protein. The resulting construct, termed 2B4-pYL, was used to transform DH5α cells. COS-7 cells were transfected with 2B4-pYL DNA by electroporation. As a control, COS-7 cells were transfected with the cDNA encoding the gp200-MR6 Ag cloned into the identical expression vector (P.F.M., unpublished observations). Cells were incubated for 48 h to allow transient expression.

Immunoprecipitations

Cell surface proteins were biotinylated with sulfo-*N*-hydroxysuccinimide-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Cell lysates were prepared and immunoprecipitations were conducted as described previously (6). Proteins were separated by SDS-PAGE as described previously (6), except that a separating gel with an acrylamide/bisacrylamide ratio of 30:0.8 was used in the fractionation.

Results

Construction of CD48-Ig fusion protein

A plasmid encoding a fusion protein comprising the extracellular domain of CD48 and the C region of human IgG1 was constructed as detailed in *Materials and Methods*. This construct, termed CD48-pIg, was introduced into COS-7 cells by lipofection. G418-resistant cells were selected, and CD48-Ig fusion protein was subsequently purified from culture supernatants on a protein A column. The presence of fusion protein was confirmed by Western blot analysis (Fig. 1). The purified protein was recognized by anti-human IgG and anti-mouse CD48 Abs (Fig. 1, *left* and *middle panels*) but not by an irrelevant control (anti-gp200-MR6) Ab (Fig. 1, *right panel*).

CD48-Ig fusion protein recognizes an Ag distinct from CD2 on CTLL-2 cells

To assess the usefulness of the CD48-Ig fusion protein, we initially analyzed stable CD2 transfectants by immunofluorescence and flow cytometry. As shown in Fig. 2*A*, CD48-Ig recognizes CD2. This staining was specific, because no reactivity was observed with control transfectants (data not shown). To search for a CD48 counter-receptor distinct from CD2, we subsequently screened a

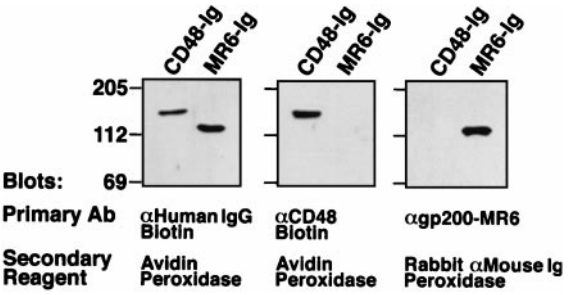

FIGURE 1. Western blot analysis of the CD48-Ig fusion protein. Samples of the CD48-Ig fusion protein or of a truncated MR6 control fusion protein (P.F.M. and M. Ritter, unpublished observations) were loaded onto an SDS-PAGE gel under nonreducing conditions. Following electrophoresis, proteins were transferred onto a PVDF membrane and subjected to Western blot analysis as detailed in *Materials and Methods*. Membranes were cut, and the pieces were subjected to primary and secondary incubations as indicated. After the second-step incubation, each membrane was incubated with ECL substrate before autoradiography. Molecular masses are indicated in kilodaltons.

panel of cell lines for reactivity with anti-CD2 mAb and CD48-Ig. As shown in Fig. 2*B*, CTLL-2 cells do not express CD2 on the cell surface. In contrast, these cells can be stained with the CD48-Ig fusion protein. Staining with this reagent was specific, because a control fusion protein, CTLA4-Ig, did not react with CTLL-2 cells (Fig. 2*B*). Additional staining analyses revealed that CTLL cells express the 2B4 Ag on the cell surface (Fig. 2*B*). Because 2B4 and CD2 belong to the same subfamily of the IgSF and are both composed of a single V-set domain and a single C2-set domain, 2B4 was a good candidate to be a second counter-receptor of CD48.

CD48-Ig fusion protein recognizes the 2B4 Ag on CTLL-2 cells

To investigate whether 2B4 was a counter-receptor for CD48, we initially determined the apparent molecular mass of the Ag recognized by CD48-Ig on CTLL-2 cells. The apparent molecular mass of the 2B4 Ag has been studied previously and was determined to be 66 kDa (11). To determine the apparent molecular mass of the Ag recognized by CD48-Ig fusion protein, we conducted immunoprecipitations on detergent lysates derived from surface-biotinylated CTLL-2 cells. As shown in Fig. 2*C*, the CD48-Ig fusion protein specifically immunoprecipitated a major band of ~66 kDa from CTLL-2 cells. The band comigrates with the protein immunoprecipitated by anti-2B4 mAb (Fig. 2*C*). This result strongly suggested that 2B4 was a counter-receptor for CD48. To test this hypothesis directly, we employed sequential immunoprecipitation studies on lysates from CTLL-2 cells. The results of these experiments revealed that the preclearing immunoprecipitations with anti-2B4 mAb eliminated the band immunoprecipitated by CD48-Ig (Fig. 2*D*).

CD48-Ig stains 2B4 transfectants

To independently demonstrate the CD48–2B4 interaction, we examined whether CD48-Ig could recognize the 2B4 expressed on specific transfectants. For this purpose, we cloned the 2B4 cDNA into an expression vector for use in gene transfection experiments. COS-7 cells were transfected with this plasmid by electroporation as described in *Materials and Methods*. Cells were incubated for 48 h to allow transient expression. After the incubation period, the cell surface expression of Ags was studied by indirect immunofluorescence and flow cytometry. Fig. 3 shows a representative experiment. As shown in Fig. 3*A*, the CD48-Ig fusion protein reacts with the product

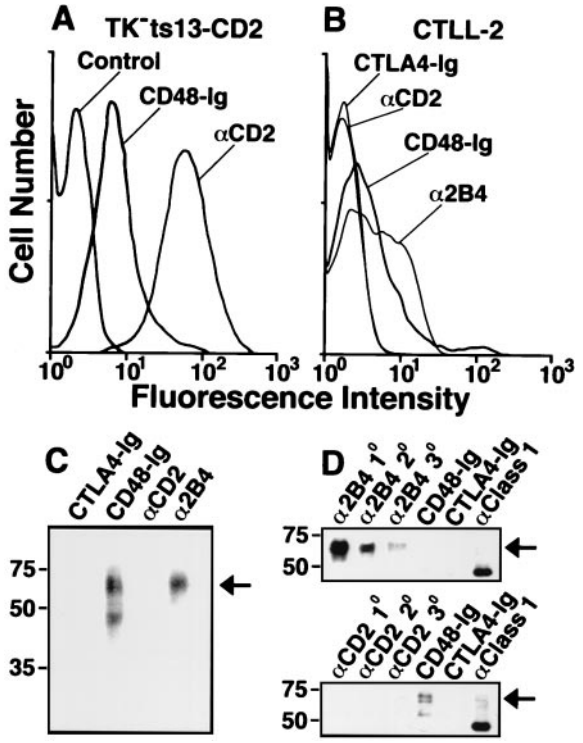

FIGURE 2. CD48-Ig fusion protein recognizes an Ag on CTLL-2 cells that is distinct from CD2. *A*, Flow cytometry profile of CD2-transfected tk$^-$ ts13 cells. CD2-transfected tk$^-$ ts13 cells (16) were stained with anti-CD2 mAb (αCD2) or with CD48-Ig fusion protein (CD48-Ig) as detailed in *Materials and Methods*. Negative control cells were stained with FITC-avidin only (control). Neither anti-CD2 mAb nor CD48-Ig stained untransfected tk$^-$ ts13 cells (data not shown). *B*, Flow cytometry profile of CTLL-2 cells. CTLL-2 cells were stained with anti-2B4 mAb (α2B4), anti-CD2 mAb (αCD2), CD48-Ig fusion protein (CD48-Ig), or CTLA4-Ig fusion protein (CTLA4-Ig) as detailed in *Materials and Methods*. Control stainings with developing reagents (biotin-conjugated goat anti-human IgG followed by FITC-avidin; FITC-conjugated goat anti-mouse Ig) were negative and were undistinguishable from the stainings with CTLA4-Ig and anti-CD2 mAb displayed in the graph. *C*, Western blot analysis of CTLL cells. Immunoprecipitations were performed on lysates of biotinylated CTLL-2 cells using either fusion proteins (CTLA4-Ig, CD48-Ig) or mAbs (anti-CD2, anti-2B4) as indicated. Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and transferred onto a PVDF membrane. The membrane was incubated with Vectastain avidin-biotin complex (ABC) (Vector Laboratories, Burlingame, CA) and developed with the ECL substrate. The position of the 2B4 protein is indicated by an arrow. The band of smaller apparent molecular mass that was immunoprecipitated by CD48-Ig was not consistently observed in all experiments. *D*, Sequential preclearing experiment. Three sequential immunoprecipitations were conducted using either anti-2B4 mAb (*upper panel*, *lanes 1–3*) or anti-CD2 control mAb (*lower panel*, *lanes 1–3*). Subsequently, each of the lysates was split, and immunoprecipitations were conducted using either CD48-Ig (*lane 4*) or CTLA-4 (*lane 5*, negative control). Following immunoprecipitation with CD48-Ig, immunoprecipitations were conducted with anti-class I mAb (*lane 6*, positive control). Gel electrophoresis and Western blot analysis were conducted as described in *C*. The position of 2B4 is indicated by an arrow. In *C* and *D*, molecular masses are indicated in kilodaltons.

of the 2B4 cDNA clone in transfected COS-7 cells. Two findings support the specificity of this result. First, a control fusion protein, CTLA4-Ig, did not react with the 2B4-transfected cell (Fig. 3*A*). This latter reagent recognizes B7-1 transfectants (ref. 9 and data not shown). Second, the CD48-Ig fusion protein did not react with COS-7 cells that, as a control, had been transfected with the cDNA (P.F.M. and M. Ritter, unpublished observations) encoding the human gp200-MR6 Ag (Fig. 3*B*). Taken together, our data demonstrate that the CD48-Ig fusion protein recognizes the 2B4 Ag on the surface of 2B4-transfected COS-7 cells, and that 2B4 is a counter-receptor for CD48.

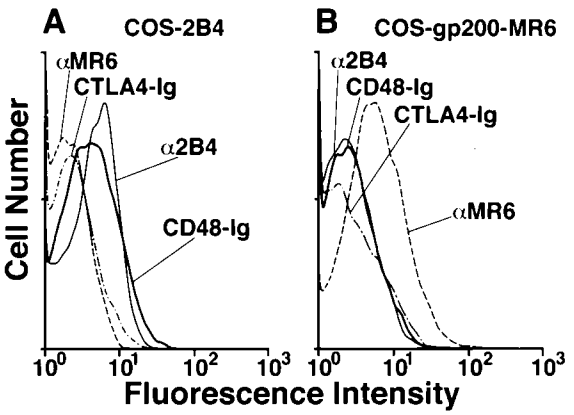

FIGURE 3. Staining of 2B4 transfectants by the CD48-Ig fusion protein. COS-7 cells were transfected with the 2B4-encoding plasmid 2B4-pYL (*A*) or the gp200-MR6-encoding plasmid MR6-pPMK (*B*). After 48 h, cells were harvested and stained with anti-MR6 mAb (αMR6), anti-2B4 mAb (α2B4), CD48-Ig fusion protein (CD48-Ig), or CTLA4-Ig fusion protein (CTLA4-Ig) as detailed in *Materials and Methods*.

Discussion

To search for additional ligands of murine CD48, we have employed a chimeric fusion protein consisting of the extracellular domain of murine CD48 and the C region of human IgG1. This fusion protein recognizes the 2B4 Ag on CTLL-2 cells and reacts with COS-7 cells that have been transfected with the murine 2B4 cDNA cloned into an expression vector. Taken together, these experiments provide clear evidence that murine CD48 binds to the 2B4 molecule.

Like CD48, the 2B4 Ag belongs to the CD2 subfamily of the IgSF. Indeed, 2B4, CD2, and CD48 show significant homology (1, 12), and all comprise a V-set domain as well as a C2-set domain. Therefore, the CD48 molecule as well as both of its known ligands (CD2 and 2B4) have similar primary structure, similar overall structure, and are evolutionarily related. Our finding that CD48 and 2B4 can interact is consistent with the model developed by Davis and van der Merwe (1) who have analyzed the binding characteristics of the CD2-CD48 interaction.

The 2B4 molecule, which is expressed on NK cells, T cells mediating non-MHC-restricted cytotoxicity, and murine epidermal γδ T cells, has been found to transduce activation signals (11, 13). Most importantly, NK cells and γδ T cells express critical effector and regulatory functions. For example, NK cells can interact with and stimulate T and B lymphocytes, and these interactions are known to require cell to cell contact (14, 15). Because CD48 is expressed on T and B cells as well as on dendritic cells, the binding of 2B4 to CD48 may participate in a variety of cell to cell interactions that are important for immune responses. Future studies are needed to explore this possibility.

Acknowledgments

We thank Dr. Mary Ritter for helpful comments on this manuscript.

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