The T cell receptor-dependent activation of T cells is tightly regulated by a discreet number of cell surface receptors, including members of the tumor necrosis factor receptor (TNFR) family. 

The T cell activation antigen 4–1BB (CDw137) is a distinctly related member of the tumor necrosis factor receptor family of cell surface receptors. We previously reported that murine 4–1BB (m4–1BB) bound to extracellular matrix (ECM) proteins. Recently, a tumor necrosis factor-like ligand of m4–1BB, m4–1BBL, as well as the human counterparts of 4–1BB (ILA) and 4–1BBL (h4–1BB and h4–1BBL, respectively) have been cloned. No information is currently available on how binding of m4–1BB to ECM proteins affects its binding to m4–1BBL and vice versa and if the ability of m4–1BB to bind ECM proteins is conserved across species. We report that binding of m4–1BBL to m4–1BB blocked its ability to bind laminin (LN), while binding of m4–1BB to LN did not block its ability to bind m4–1BBL. Furthermore, binding of m4–1BBL to the m4–1BB-LN complex did not displace LN. These findings suggest the two ligands bind to proximal but distinct sites on m4–1BB. This is supported by the observation that six of eight anti-m4–1BB monoclonal antibodies blocked the interaction between 4–1BB and 4–1BBL, while seven blocked LN binding. Ligand and monoclonal antibody binding studies with a truncated protein lacking the amino-terminal LN-homologous domain of m4–1BB demonstrated that regions downstream of the LN-homologous domain participate in LN binding and that the intact protein is required for m4–1BBL binding. Studies with h4–1BB showed that h4–1BB only bound h4–1BBL, indicating that the ECM binding activity of 4–1BB is not conserved across species. This finding allowed the construction of murine/human 4–1BB chimeras, which permitted further dissection of the regions of 4–1BB involved in LN and 4–1BBL binding and suggests that sequence differences in the LN-homologous domain of h4–1BB in part account for the inability of h4–1BB to bind ECM proteins.

Analysis of 4–1BBL and Laminin Binding to Murine 4–1BB, a Member of the Tumor Necrosis Factor Receptor Superfamily, and Comparison with Human 4–1BB*

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The T cell receptor-dependent activation of T cells is tightly regulated by a discreet number of cell surface receptors, including members of the tumor necrosis factor receptor (TNFR) family. 4–1BB (CDw137) is a type I membrane protein whose extracellular domain is related to the members of the TNFR family (1, 2) and has low level homology to the extracellular matrix protein laminin (LN) (3, 4). Early studies with an immunoglobulin chimera of murine 4–1BB (m4–1BB-Ig) and with COS and L cell transfectants expressing full-length m4–1BB showed that this protein was able to bind extracellular matrix proteins including LN and vitronectin (3). Later, similar m4–1BB-Ig and human 4–1BB-Ig (h4–1BB-Ig) fusion proteins were used to isolate cDNAs encoding the high affinity TNF-like ligands m4–1BB and h4–1BBL, respectively (5, 6).

M4–1BB was initially identified as a protein whose expression was up-regulated following T cell activation (1). M4–1BB is predominantly expressed by activated thymocytes and T cells but not by resting or lipopolysaccharide-activated B cells (7). This contrasts with the expression of mRNA encoding h4–1BB in human peripheral blood mononuclear cells. Analogous to what has been observed in murine T cells, h4–1BB is expressed by activated T cells; however, unlike m4–1BB, transcripts encoding h4–1BB can be detected in activated human peripheral blood B cells (8). RNA encoding h4–1BB is also detected in interleukin-1β (IL-1β)-activated or phorbol-12-myristate-13-acetate-activated peripheral blood monocytes (8). In addition, transcripts encoding h4–1BB can be detected in an activated human T cell line (Jurkat), in resting human B cell lines (Raji and Epstein-Barr virus transformed B cells from normal donors), and in a number of unstimulated and IL-1β-stimulated nonlymphoid cell lines (8). Expression of 4–1BB by activated murine and human T cells increases gradually over time. Studies using anti-CD3 monoclonal antibodies (mAb) or concanavalin A-activated murine T cells showed that following activation the level of m4–1BB detectable on the surface of T cells steadily increases over 3 days (9). Additionally, h4–1BB mRNA expression by anti-CD3 mAb-activated peripheral blood mononuclear cells can be detected at 4 h and remains strongly expressed at 48 h (8).

Early evidence for a role of m4–1BB in the regulation of the immune response came from mAb cross-linking studies, which showed that co-immobilized suboptimal concentrations of an anti-CD3 mAb with the anti-m4–1BB mAb 53A2 could efficiently drive resting T cells to proliferate (7). These studies were extended by Goodwin et al. (5), who showed that CV-1 cells expressing m4–1BB could significantly enhance the proliferation of IL-2- and concanavalin A-stimulated thymocytes. M4–1BB-expressing CV-1 cells could also significantly enhance the proliferation of phytohemagglutinin-stimulated

VCAM-1, vascular cell adhesion molecule-1; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate.
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splenic T cells (5). Independent evidence for a role of 4–1BB in T cell activation came from studies which sought to identify the molecules responsible for the T cell costimulatory activity of the B cell lymphoma K46J. These studies showed that the costimulatory activity of K46J B cells could be blocked with a soluble alkaline phosphatase chimeric of m4–1BB (m4–1BB-AP) but not with a soluble immunoglobulin chimera of CTLA4 (CTLA4-Ig) or mAbs against B7–1, B7–2, intercellular adhesion molecule-1, or heat-stable antigen (10). This is consistent with the results of experiments showing that stimulation of purified resting T cells by anti-CD3 mAb and fixed antigen presenting cells could be partially blocked with an immunoglobulin chimera of m4–1BB (m4–1BB/Fc) (11). In addition, m4–1BB/Fc was capable of blocking IL-2 production by purified T cells coactivated with anti-CD3 mAb and accessory cells (11). Similar studies with an anti-h4–1BB mAb (M14), or CV-1 cells expressing the h4–1BB, showed that h4–1BB can function as a costimulatory molecule for resting human T cells and enhance the anti-CD3 mAb-driven apoptosis of chronically activated T cells (6). Consistent with a role of 4–1BB in T cell signaling, the cytoplasmic domain of m4–1BB contains a CXCP motif recognized by protein-tyrosine kinases of the Src family. Indeed, p56

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—COS cells were grown and maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin. Generation of the rat anti-m4–1BB monoclonal antibodies is described elsewhere.2 The anti-Lyt2a mAb 53–6, directed against CDS, was a gift from Dr. J. Ledbetter (Bristol-Myers Squibb, Seattle, WA).

Construction of Full-length 4–1BB cDNA—Complementary DNA (cDNA) encoding the full-length m4–1BB and CD40 molecules was constructed and prepared as described previously (3) using the following primers: 5’-CCG CGG GTA CCA AGA AAA TAC AAT CCA GTC TCC TGG-3’ and 5’-CCG TCT AGA CCA TGA AGG ATG GAG TAG TGG-3’. The m4–1BB domain (residues 1–45) was generated using the following primers: 5’-CCG AAG CTT GCT TTG CAC GGG AGG AGT GTC CTG GCT CTC TCG C-3 and 5’-GCA AAT CCT CTT ATT CCT ATT ATC ACA GAA GAT TGG-3’. The two PCR products were joined together using the flanking primers 5’-AAC AAG CAC ACC TCT CGT GAA CCA CAC GGG AGG GAT TGG-3 and 5’-GCA GAT CTG GTC CTT TGC CAC TGG-3’. The binding of purified m4–1BB-Ig to rat laminin (Life Technologies, Inc.) was tested using an ELISA assay as described (3). Blocking by m4–1BB-Ig was tested by adding neat m4–1BB supernatant to the wells for 1 h at 22°C. Wells were washed, and purified 4–1BB-Ig or CD40-Ig was added at 2.5 μg/ml in PBS and incubated 1 h at 22°C. Wells were washed, and peroxidase-conjugated goat anti-human IgG (1:10,000; Jackson Immunoresearch, West Grove, PA) was added and incubated 1 h at 22°C. Wells were washed and treated with the EIA chromogen reagent (Genetics Systems, Seattle, WA) for 15 min at 22°C. The colorimetric reaction was quenched with 1 N H2SO4, and the optical density was measured on an ELISA plate reader at dual wavelengths of 450 and 630 nm. Blocking by rat laminin was tested by preincubating 4–1BB-Ig with rat laminin at 10 μg/ml.

As an alternate format, purified 4–1BB-Ig (2 μg/ml) was captured on goat anti-human IgG Fc-coated wells as described previously (3). Following washing, serial dilutions of 4–1BB supernatants or purified 4–1BB-Ig were added to the wells and incubated 1 h at 22°C. Wells were washed and biotinylated anti-Ly-2 (CD8) mAb (Dr. D. T. Loo, laboratories, South San Francisco, CA) diluted 1:2000 in 1 × specimen diluent (SD, Genetics Systems, Seattle, WA) was added and incubated 1 h at 22°C. Wells were washed and horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) diluted 1:2000 in 1 × SD was added to the wells and incubated 1 h at 22°C. Wells were washed and treated with the EIA chromogen reagent as described

2 W. W. Shuford, K. Klussman, D. D. Tritcher, D. T. Loo, J. Chalupny, A. W. Sidak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, T. C. Pearson, J. A. Ledbetter, A. Aruffo, and Robert S. Mittler, manuscript in preparation.

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Blocking by rat laminin was tested by incubating the wells with LN (10 μg/ml) for 1 h at 22 °C, followed by washing, then the addition of 4–1BB.

Binding of Rat Anti-m4–1BB Monoclonal Antibodies to 4–1BB-Ig Fusion Proteins and Blocking of Ligand Binding—The binding of rat anti-m4–1BB mAbs to the various 4–1BB-Ig fusion proteins was tested using an ELISA assay. Purified 4–1BB-Ig (2 μg/ml) was captured on goat anti-human IgG Fc-coated wells as described previously (3). The wells were then blocked for 1 h at 22 °C. The wells were washed, and purified rat anti-m4–1BB mAbs were added at 10 μg/ml in 1 × SD and incubated for 2 h at 22 °C. After washing, horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:3000 in 1 × SD was added and incubated 1 h at 22 °C. Wells were washed and treated with the EIA chromogen reagent as described above. To examine the ability of the rat anti-m4–1BB mAbs to block laminin and m4–1BBL binding to m4–1BB-Ig fusion protein, an analogous ELISA assay was performed. Following incubation of the rat anti-m4–1BB mAbs (10 μg/ml in 1 × SD) on m4–1BB-Ig fusion protein-coated wells for 1 h at 22 °C, biotinylated rat laminin (5 μg/ml) or purified m4–1BBL (0.05 μg/ml) was added to antibody-containing wells and incubated 1 h at 22 °C. Wells were washed, and m4–1BBL was detected with biotinylated anti-Ly-2 (CD8α) mAb, followed by horseradish peroxidase-conjugated streptavidin. Biotinylated laminin was detected with horseradish peroxidase-conjugated streptavidin directly. Wells were washed and treated with the EIA chromogen reagent as described above.

Binding Assays in COS Cells—The binding of m4–1BBL, h4–1BB, sgp39, and LN to m4–1BB, h4–1BB, and CD40 was tested by staining transfected COS cells. COS cells were either mock-transfected or transfected with full-length m4–1BB, h4–1BB, or CD40 cDNAs using DEAE-dextran (15). One day after transfection, cells were trypsinized and replated. Two days later, cells were washed with PBS then recovered following incubation with Ca²⁺/Mg²⁺-free PBS containing 0.5 mM EDTA. Cells were stained with m4–1BBL, h4–1BB, or sgp39 supernatants (1:10 in DMEM, 2% FBS) for 1 h on ice, followed by incubation with biotinylated anti-Ly-2 (CD8α) mAb (1:200 in DMEM, 2% FBS) for 45 min on ice, then avidin-FITC (1:500 in DMEM, 2% FBS; Molecular Probes, Eugene, OR) for 45 min on ice. Cells were stained with biotinylated LN (10 μg/ml in DMEM, 2% FBS) for 2 h on ice followed by avidin-FITC (1:500 in DMEM, 2% FBS) for 45 min on ice. Flow cytometry was performed with a FACSscan unit (Becton Dickinson, San Jose, CA). At least 5000 viable cells were analyzed in each sample.

Sequence Alignments—Initially, segments of the 4–1BB extracellular region sequence were aligned manually against sequences of members of the TNFR family to identify TNFR repeat domains in 4–1BB. Due to the low sequence similarities, these alignments were guided by structural consensus residues of TNFR (18). 4–1BB sequence segments were aligned using ClustalW (19) to identify regions that have conservation that are distinct from other TNFR family members.

Fig. 1. A, binding of m4–1BBL to m4–1BB-Ig and blocking by laminin. Serial dilutions of m4–1BBL supernatant were immobilized on plastic coated with anti-Lyt2A mAb 53–6 and its ability to bind m4–1BBL was measured by an ELISA. The ability of laminin to block binding of m4–1BBL to m4–1BB-Ig was tested by preincubating m4–1BBL-Ig with rat laminin (10 μg/ml). As a control, the binding CD40-Ig to m4–1BBL was monitored. B, binding of laminin to m4–1BB-Ig and blocking by m4–1BBL. m4–1BB-Ig was immobilized on plastic coated with affinity-purified goat anti-human IgG antibodies, and its ability to bind to increasing amounts of biotinylated rat laminin was assessed by ELISA. The ability of m4–1BBL to block laminin binding to m4–1BB was tested by incubating the wells with m4–1BBL supernatant prior to addition of biotinylated rat laminin. As a control, the binding CD40-Ig to m4–1BBL was monitored. C, the displacement of laminin by m4–1BBL was examined by ELISA. m4–1BBL was immobilized on plastic coated with affinity-purified goat anti-human IgG antibodies. The ability of m4–1BBL to displace laminin was tested by first incubating the wells with laminin (10 μg/ml), followed by the addition of m4–1BBL supernatant, then staining the wells for laminin (LN/m4–1BBL (LN)). Blocking of m4–1BBL binding to m4–1BB-Ig by laminin was assessed by first incubating the wells with laminin (10 μg/ml), followed by the addition of m4–1BBL supernatant, then staining the wells for m4–1BBL (LN/m4–1BBL (m4–1BBL)). Blocking of laminin by m4–1BBL was assessed as in B (m4–1BBL/LN (LN)). For positive controls, the ability of laminin (LN) or m4–1BBL (m4–1BBL) alone to bind to m4–1BB-Ig was tested. m4–1BBL was immobilized on plastic coated with affinity-purified goat anti-human IgG antibodies, and its ability to bind m4–1BBL supernatant or biotinylated rat laminin (10 μg/ml) was assessed by ELISA as above. Data points represent the mean ± S.E. value (n = 3 for each data point).
outside identified TNFR-like domains were then searched against the SwissProt and GenPept data bases using GCG programs. Obtained alignments for these regions were manually modified to adjust gaps.

RESULTS

Binding and Cross-blocking Studies with m4–1BBL and LN—Our initial attempts to identify a ligand for m4–1BB showed that a m4–1BB-Ig fusion protein and COS and L cell transfectants expressing m4–1BB were able to bind to extracellular matrix proteins. Subsequently, using a similar m4–1BB-Ig fusion protein, another group identified and cloned a second m4–1BB ligand, which they named m4–1BBL (5). This molecule, which is expressed by activated T cells, is homologous to TNF. Although other lymphocyte cell surface receptors such as CD2, CD28, and integrins (for example lymphocyte function-associated molecule-1 and very late antigen-4 (VLA-4)) have more than one ligand, 4–1BB is the first member of the TNFR family known to bind both a TNF-like molecule and extracellular matrix proteins.

To gain a better understanding of the interaction of m4–1BB with its ligands, we carried out a series of binding and cross-blocking studies using m4–1BB-Ig, LN, and a soluble recombinant form of m4–1BBL, _m_4–1BBL. As shown in Fig. 1A, _m_4–1BBL bound to m4–1BB-Ig but not to the immunoglobulin fusion of the related receptor CD40, CD40-Ig. Ligand cross-blocking studies showed that the interaction between m4–1BB and _m_4–1BBL was not blocked by preincubating m4–1BB with a high concentration of LN (10 μg/ml). Furthermore, the binding of _m_4–1BBL to the LN-m4–1BB complex did not result in the displacement of LN, indicating that m4–1BB was capable of simultaneously binding to both ligands (Fig. 1C). However, preincubating m4–1BB with a high concentration of _m_4–1BBL prevented LN from binding m4–1BB-Ig (Fig. 1B). In these studies, CD40-Ig binding to _m_4–1BBL and LN was carried out to evaluate nonspecific binding. As reported previously, CD40-Ig was unable to bind to either m4–1BBL or LN (Fig. 1, A and B). These studies suggest that the interaction of m4–1BB with m4–1BBL and LN is specific, and that m4–1BBL and LN bind to distinct but proximal sites on m4–1BB.

Ligand Blocking Studies with Anti-m4–1BB mAbs—To further characterize the LN and m4–1BBL binding sites on m4–1BB, eight anti-m4–1BB mAbs were examined for their ability to block the binding of m4–1BB to either m4–1BBL or LN. As shown in Fig. 2A, six of the eight anti-m4–1BB mAbs effectively blocked the binding of m4–1BB to _m_4–1BBL. On the other hand, seven of the mAbs were able to effectively block the interaction between m4–1BB and LN (Fig. 2B). As expected the interaction of m4–1BB with either m4–1BBL or LN could not be blocked with a mAb directed to gp39, the TNF-like ligand of CD40 (14) (Fig. 2). These findings provide further evidence that the m4–1BBL and LN binding sites of m4–1BB are distinct.

4–1BB Extracellular Domain Organization and Sequence Alignment—A preliminary comparison of the amino acid sequence of the extracellular domain of m4–1BB with that of other proteins revealed the presence of regions homologous to both members of the TNFR family and LN. This observation led us to reexamine in more detail the extracellular domain organization of m4–1BB and its homology to other proteins. Consistent with an earlier proposal (2), only two TNFR domains could be identified with certainty in the extracellular region of m4–1BB (Fig. 3). The two TNFR-like domains in m4–1BB are best aligned with TNFR domains 1 and 3, respectively. These two TNFR-like domains are not adjacent, but separated by a short cysteine-rich segment. Data base searches with the sequence of this short segment revealed significant matches of its cysteine pattern to that found in metallothionein (MT; Fig. 3). MT is a small cysteine-rich cytoplasmic protein, which binds divalent cations using clusters of cysteine residues (16) and is not related to TNFR molecules. However, on the cell surface, a motif with cysteine spacing similar to MT may form a small and highly disulfide bond constrained domain. The region of m4–1BB/MT homology overlaps with the COOH-terminal region of the first TNFR-like domain of m4–1BB (Fig. 3). Although the structural significance of this overlap remains unclear, the finding that the two TNFR-like domains in m4–1BB may be connected by a constrained structural motif suggests that this domain organization may result in a “fusion” of the two TNFR domains. In contrast, the NH2-terminal region in m4–1BB displays sequence similarities to LN (Fig. 3). The
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X-ray structure of three consecutive LN-type epidermal growth factor-like modules was reported recently (17). These modules were arranged in a linear "stacked" fashion, reminiscent of the gross domain arrangement of TNFR (18). Based on this structure and the amino acid sequence alignment (Fig. 3), it appears that the LN-homologous domain of m4–1BB includes about half of a LN repeat module, corresponding to the disulfide stabilized "loops" d and c (17). Taken together, these observations suggest the possibility of a combined arrangement of a LN-type and two TNFR-type domains.

**Ligand and mAb Binding to a Panel of Truncated m4–1BB Proteins**—To begin investigating if the different ligand binding properties of m4–1BB resided within discrete domains or required multiple domains of the extracellular region of m4–1BB, we prepared a series of DNA constructs encoding truncated forms of m4–1BB (a total of nine constructs) lacking various NH₂- and COOH-terminal extracellular domains and domain fragments. The initial design of these constructs was based on early sequence alignment studies, which predicted that 4–1BB contained one LN-homologous domain and three TNFR-like domains. Subsequent sequence alignment of 4–1BB, as described herein, predicted that most of these truncated proteins contained incomplete domains. Most of the proteins encoded by these constructs were unable to bind to our panel of anti-m4–1BB mAbs, m4–1BBL, and LN and thus were not further studied (data not shown). However one truncated protein, m4–1BBdLN (Fig. 4A), which lacked only the amino-terminal LN-homologous domain of m4–1BB was recognized by the anti-m4–1BB mAb 1D8, the mAb that blocked LN binding but not m4–1BBL binding (Fig. 4B). This protein was capable of weakly binding to LN but was no longer able to bind m4–1BBL (Fig. 4, C and D). These findings suggest that some of the binding determinants responsible for m4–1BB-LN interactions reside downstream from the LN homologous domain of m4–1BB and that the presence of an intact LN-homologous domain is required for m4–1BBL binding.

**Binding of h4–1BB to h4–1BBL and LN**—Recently, cDNA clones encoding the putative human homologues of m4–1BB (19) and m4–1BBL (6) were isolated and characterized. To determine if the interaction between m4–1BB and extracellular matrix ligands was conserved in different species, we isolated cDNA clones encoding h4–1BB and h4–1BBL. COS cell transfectants expressing h4–1BB bound to h4–1BBL but were unable to bind LN (Fig. 5A). These findings contrast with those using COS cell transfectants expressing m4–1BB, which were capable of binding efficiently to sm4–1BBL and LN (Fig. 5B). COS cell transfectants expressing either h4–1BB or m4–1BB were unable to bind sgp39. COS cell transfectants expressing CD40 bound to gp39, but were unable to bind to m4–1BBL, h4–1BBL, or LN (Fig. 5C). To ensure that the inability of h4–1BB to bind extracellular matrix proteins was not due to our assay format, we prepared a chimeric gene encoding an h4–1BB immunoglobulin fusion protein, h4–1BB-Ig. As shown in Fig. 6, h4–1BB-Ig bound to h4–1BB but not LN. These findings suggest that the extracellular matrix binding activity of 4–1BB is not conserved in different species.
Comparison of the amino acid sequence of h4–1BB with m4–1BB, other TNFR molecules, LN and MT suggested equivalent domain organization in m4–1BB and h4–1BB. Like m4–1BB, the amino-terminal region of h4–1BB is homologous to LN. However, the c loop of LN is more closely mimicked by murine than by human 4–1BB sequences (Fig. 3). Presently it is unclear if these gross sequence differences are sufficient to account for the differences in the ability of human and murine

**Fig. 4.** Binding of rat anti-m4–1BB monoclonal antibodies, rat laminin, and m4–1BB to m4–1BB-Ig and m4–1BBdLN-Ig. A, schematic representation of m4–1BB-Ig and m4–1BBdLN-Ig immunoglobulin fusion proteins. The laminin-homologous domain (LN), TNF-like domains (TNFr1 and TNFr3), and the metallothionein domain (MT) are shown in the open boxes. The human immunoglobulin Fc region, containing the hinge and second (CH2) and third (CH3) constant regions of the human IgG1 are shown in the shaded box. m4–1BB-Ig or m4–1BBdLN-Ig was immobilized on plastic coated with affinity-purified goat anti-human IgG antibodies and their ability to bind rat anti-m4–1BB mAbs (B), increasing concentrations of biotinylated rat laminin (C), or increasing concentrations of m4–1BBL (D) was measured using an ELISA. For antibody binding, the anti-gp39 antibody, 39.5, represents a negative control. For ligand binding, CD40-Ig represents a negative control. Data points represent the mean ± S.E. value (n = 3 for each data point).

**Fig. 5.** Binding of 4–1BBL and laminin to 4–1BB-transfected COS cells. Flow cytometry profiles of the binding of the indicated ligands to h4–1BB-transfected COS cells (A), m4–1BB-transfected COS cells (B), and CD40-transfected COS cells (C) (dark profiles). The empty profiles represent mock-transfected COS cells.
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4–1BB to bind LN.

Preparation and Binding Studies with Chimeric 4–1BB Proteins—The isolation of cDNA clones encoding h4–1BB opened a different avenue to study the structural organization and function of 4–1BB. The finding that h4–1BB was unable to bind to LN permitted further investigation as to which regions of m4–1BB are involved in m4–1BB and LN binding through the generation of human/mouse 4–1BB chimeric proteins. In one construct, termed m/h4–1BB, the LN-homologous domain of m4–1BB replaced the equivalent domain of h4–1BB (Fig. 7A). In the converse construct, termed h/m4–1BB, the LN-homologous domain of h4–1BB replaced the equivalent domain of m4–1BB (Fig. 7A). Ligand binding studies showed that replacement of the h4–1BB LN-homologous domain with the equivalent m4–1BB domain conferred weak binding to LN (Fig. 7B). The m/h4–1BB chimeric protein displayed reduced binding to h4–1BB (Fig. 7C) and was unable to bind m4–1BB (data not shown). Conversely, replacement of the m4–1BB LN-homologous domain with the equivalent h4–1BB domain abolished LN binding, while it did not alter binding to m4–1BB (Fig. 7, D and E). The h/m4–1BB chimera was unable to bind to h4–1BB (data not shown). CD40-Ig was used as a control in these experiments and, as shown previously, was unable to bind LN, m4–1BB, or h4–1BB (Fig. 7). These findings suggest that a portion of the LN binding determinants of m4–1BB are located in the LN-homologous region of m4–1BB and that some of the binding determinants required by 4–1BB ligand reside in the TNFR homologous region of 4–1BB. In addition, these findings indicate that the inability of h4–1BB to bind LN is in part due to amino acid differences in the LN-homologous domain of m4–1BB and h4–1BB.

DISCUSSION

There are multiple examples of leukocyte cell surface receptors with more than one ligand; however, to date 4–1BB is the only member of the TNFR supergene family that has been shown to bind to both a TNF-like protein and a protein that is not related to TNF. The finding that m4–1BB is capable of binding to both LN and m4–1BB suggests that m4–1BB has a dual function as a cell adhesion and activation molecule. A number of studies have shown that the interaction between m4–1BB and m4–1BB results in m4–1BB-mediated intracellular signaling. By analogy to other members of the TNFR/TNF family of receptor/ligands, this likely occurs via a m4–1BB-driven receptor oligomerization, which is mediated by the trimeric nature of the TNF-like ligand. The observation that m4–1BB is capable of binding to the m4–1BB-LN complex suggests that m4–1BB-m4–1BB signaling is likely to take place even if m4–1BB is binding extracellular matrix proteins. This is significant since extracellular matrix proteins such as LN are very abundant and widely distributed and are likely to bind m4–1BB shortly after it is expressed on the surface of activated T cells. Presently there is no information on the signaling effects that might result from the formation of molecular complexes between m4–1BB and extracellular matrix or on the effect that extracellular matrix protein binding might have on m4–1BB-mediated signaling.

The finding that m4–1BB was capable of simultaneously binding to m4–1BB and LN provides evidence that the binding sites for these two ligand are distinct. Additional evidence for this is provided by the identification of an anti-m4–1BB mAb, which is capable of blocking LN binding to m4–1BB but was unable to effectively block the interaction between m4–1BB and m4–1BB. The dual ligand binding activity of m4–1BB is not unique. The leukocyte antigen VLA-4 is capable of binding to vascular cell adhesion molecule-1 (VCAM-1) (20), a type I cell surface protein of the Ig supergene family, and fibronectin (21). Similar to our observations with m4–1BB, VLA-4 is able to bind VCAM-1 and fibronectin via distinct sites. In this system it appears that the function of binding to the different ligands is overlapping, with both interactions being involved in VLA-4-mediated signaling and cell adhesion. The availability of the anti-m4–1BB mAb 1D8, which is capable of blocking extracellular matrix protein binding without affecting m4–1BB binding, as well as other anti-m4–1BB mAbs, which are capable of blocking the interaction between m4–1BB and its two ligands, provides an avenue for the investigation of the role of extracellular matrix protein binding on m4–1BB signaling.

Unexpectedly, we found that h4–1BB was unable to bind the extracellular matrix protein LN. Although we cannot rule out the possibility that expression of h4–1BB in a heterologous mammalian expression system (COS cells instead of T cells) selectively affected the ability of h4–1BB to bind extracellular matrix proteins.
matrix proteins, this is unlikely. It is more likely that this function of 4–1BB is not conserved. This is not unique to 4–1BB and has been observed with the T cell antigen CD2, which has been reported to bind both CD48 (22) and CD58 (23) in the murine system. However, human CD2 is only able to bind CD58 with high affinity (24). Based on our data, the function of 4–1BB in the regulation of the immune system in humans and mice may be different. This suggests that data obtained from in vitro and in vivo experiments investigating the function of 4–1BB in murine systems may not be fully predictive of the function of 4–1BB in the regulation of the human immune system.

A detailed comparison of the amino acid sequence of both human and murine 4–1BB with that of other members of the TNFR family and with other published proteins clearly shows that the domain organization of 4–1BB differs from other members of the family. In particular, 4–1BB contains at its amino terminus a LN-homologous domain and has only two extracellular TNFR-like domains. Additionally, in a region upstream of the second TNFR-like domain of 4–1BB, which overlaps the
COOH-terminal portion of the first TNFR-like domain of 4–1BB, we identified a cysteine-rich motif that is homologous to MT. The availability of crystallographic data on the structure of a LN fragment (17) and the TNFR (18) has shown that these proteins are elongated structures built by the “stacking” of either LN-like or TNFR-like domains, respectively. This suggests that LN-like and TNFR-like subdomains of 4–1BB may be combined. These observations, in conjunction with the results of our sequence alignment studies, led us to investigate whether the different ligand binding activities of m4–1BB and LN may be combined. In these experiments, manipulation of the extracellular region of one or more extracellular domains failed to bind m4–1BBL and LN, and were no longer recognized by any of our anti-m4–1BB mAbs. This further suggests an intimate association of these domains. There was one exception, the m4–1BBdLN-Ig protein, which lacked the LN-like domain of m4–1BB. This m4–1BBdLN-Ig protein was recognized by the anti-m4–1BB mAb 1D8 and was able to weakly bind to LN. The latter finding, in conjunction with the observation that mAb 1D8 is capable of blocking the interaction between m4–1BB and LN without affecting m4–1BBL binding, clearly indicates that regions located carboxyl-terminal to the LN-like domain of m4–1BB are involved in LN binding. This finding was surprising since we had hypothesized, based on the observation that LN can form homooaggregates, that the m4–1BB-LN interaction was mediated via interactions between the LN-like domain of m4–1BB and LN. However, the finding that the h/m4–1BB chimera, which contains the LN-like domain from m4–1BB and the TNFR-like regions of h4–1BB, is capable of weakly binding to LN, while the h/m4–1BB chimera does not bind LN, indicates that the LN-like motif of m4–1BB also contributes to LN binding.

The observation that the m4–1BBdLN-Ig protein was unable to bind to m4–1BBL suggests that the interaction between 4–1BB and its TNF-like ligand requires that the extracellular domain of 4–1BB be intact. Interestingly, the m/h4–1BB chimera displayed reduced binding to h4–1BBL, while the h/m4–1BB chimera displayed binding to m4–1BBL equivalent to that of the native m4–1BB protein. This suggests that the 4–1BB binding determinants contributed by the LN-homologous domain of h4–1BB are capable of supporting m4–1BBL binding but not vice versa. This one-sided interaction between ligands and receptors across species has been observed in other systems, including interleukin-2 (25) and interleukin-6 (26). To date there are no reports describing the results of experiments in which the extracellular domains of other members of the TNFR family have been truncated to map the interaction between this receptor family and their respective ligands. However, a subset of human patients with human autoimmune lymphoproliferative syndrome have defects in Fas expression and function (27). These patients were shown to have a mutation that led to aberrant splicing, resulting in the deletion of a portion of the extracellular domain of Fas. T cells from this patient were unable to respond to Fas, suggesting that this truncation was either not expressed or, if expressed, was unable to bind Fas. Our in vitro studies with truncated forms of m4–1BB are consistent with the second explanation. Although studies with truncated forms of m4–1BB and with the m/h4–1BB chimera provide some information of the molecular mechanisms that allow 4–1BB to interact with its ligands, in general we found that manipulation of the extracellular region of this protein was difficult and resulted in structural perturbations that compromised its binding functions.

In summary, this study provides the first in-depth analysis of the molecular basis of the interaction of 4–1BB with its different ligands in different species, and allow the identification of a mAb that is capable of blocking the interaction between m4–1BB and extracellular matrix proteins but not m4–1BBL. Thus, the design of functional experiments to shed light on the role of the interaction between m4–1BB and its different ligands in regulating the immune response may now be possible. Recently, molecules in this family of proteins have been targeted for the development of novel drugs to treat immunological diseases in humans. The development of these drugs has relied upon the use of murine models of human disease. The finding that the extracellular matrix binding activity of m4–1BB is not conserved in humans indicates that in vivo studies in mice designed to evaluate the efficacy of targeting 4–1BB function to treat human disease should be interpreted with caution in light of the different binding functions of this receptor in these two species.

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