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The Identification and Characterization of a Ligand for Bovine CD5

Karen M. Haas* and D. Mark Estes2*

CD5, a type I glycoprotein expressed by T cells and a subset of B cells, is thought to play a significant role in modulating Ag receptor signaling. Previously, our laboratory has shown that bovine B cells are induced to express this key regulatory molecule upon Ag receptor cross-linking. To date, a ligand has not been described for bovine CD5. Given the importance of CD5 expression, moderately expressed in the function of CD5 on this B cell subset and on T cells, we sought to characterize the ligand for this protein using a bovine CD5-human IgG1 (CD5Ig) fusion protein produced by both mammalian and yeast cells. As determined by CD5Ig binding, expression of this ligand is negative to low on freshly isolated lymphocytes, with low-density expression being limited to activated B cells. Activation with LPS, PMA, and calcium ionophore, or ligation of CD40 alone or in combination with anti-IgM, resulted in B cell-specific expression of this ligand. Interestingly, activation through B cell Ag receptor cross-linking alone, although able to induce CD5 expression, did not result in expression of CD5 ligand (CD5L). In addition, we demonstrate a functional role for CD5L as a costimulatory molecule that augments CD40L-stimulated B cell proliferation. Finally, immunoprecipitation with CD5Ig suggests that the ligand characterized in this study has a molecular mass of ~200 kDa. The data reported herein, as well as future studies aimed at further characterizing this newly identified bovine CD5L, will undoubtedly aid in understanding the role that the CD5-CD5L interaction plays in immune responses. The Journal of Immunology, 2001, 166: 3158–3166.

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3 Abbreviations used in this paper: BCR, B cell Ag receptor; L, ligand; sIgM, surface IgM; TD, T, T cell-dependent; TI, T cell-independent; CD5Ig, bovine CD5-huIgG1; mCD5Ig, MOP-8-expressed bovine CD5Ig; yCD5Ig, yeast-expressed bovine CD5Ig; DTSSP, 3,3’-dithiobis-(sulfosuccinimidylpropionate); bo, bovine; hu, human.
The constitutive B cell marker, CD72, was described by Van de Velde et al. (37) as the first ligand for CD5. However, recent studies have suggested that CD5 may bind to one or more alternative ligand(s). Using a CD5-huIgG1 fusion protein, Biancone et al. (38) described an activation-induced CD5 ligand (CD5L) expressed by activated murine B cells, as well as T cell clones. Similarly, Bikah et al. (39) have described a ligand expressed by murine peritoneal B cells, activated splenic B cells, and B lymphoma cell lines. More recently, Calvo et al. (40) described a CD5L expressed by cell types of lymphoid, myeloid, and epithelial origin. In addition to these studies, which suggest that CD5 may potentially interact with more than one ligand, CD5 has been reported to interact with 

\[ V_{H} \] Ig framework regions in both rabbit and human studies (41, 42). Given the variation in function CD5 appears to serve with respect to cell-specific expression, as well its structural similarity to scavenger receptors (known to bind multiple ligands), it is plausible to hypothesize that CD5 may indeed have multiple ligands that perform unique functions. Further characterization of these proposed ligands and the interactions that they form with CD5 is required to begin to understand the functional significance that the CD5-CD5L interaction may have in vivo.

Although a variety of ligands have been described for human, mouse, and rabbit CD5, a bovine CD5L has not yet been described. In the following study, we have addressed the ability of a recombinant bovine CD5-huIgG1 fusion protein to interact with the putative ligand for CD5 on the surface of bovine PBMCs. The data demonstrate that CD5Ig expressed in either yeast or mammalian cells interacts with an activation-induced ligand (~200 kDa) expressed by B cells, which functions as a costimulatory molecule to enhance B cell proliferation. Furthermore, we demonstrate that unlike CD5 expression, which is up-regulated on B cells after surface IgM (sIgM) cross-linking but not CD40 ligation, its ligand appears to be regulated in a reciprocal manner, in which CD40 ligation, but not Ag receptor cross-linking, results in expression of the ligand for CD5. The potential implications for the counterregulation of CD5 and its ligand with respect to T cell-dependent (TD) and T cell-independent (TI) B cell responses are discussed.

Materials and Methods

Animals

Blood donors used for these studies were primarily male steers between 2 and 12 mo of age maintained in an indoor housing facility.

Purification of bovine PBMCs and B cells

Blood was collected in 10× sodium citrate anticoagulant solution (0.15 M sodium citrate, 80 mM citric acid, 0.16 M dextrose) and centrifuged 20 min at 2000 rpm (900 × g) to obtain a buffy coat. Buffy coats were harvested and residual RBCs were lysed in ACK RBC lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 M EDTA (pH 7.3)) 7 min at room temperature, centrifuged 7 min at 300 × g, and washed two times in HBSS to obtain PBMCs. B cells were selected by passive panning, as previously described (7). Residual CD3⁺, CD5⁺, and CD14⁺ cells were removed by treatment with Abs to bovine CD3 (MMIA; Washington State University Monoclonal Antibody Center, Pullman, WA), CD5 and CD14 (CC17 and CCG33; generously provided by Chris Howard, Institute for Animal Health, Compton, U.K.) followed by magnetic depletion with sheep anti-mouse IgG-coated magnetic beads (Dynal, Lake Success, NY). Cells were routinely >90% IgM⁺ and <5% CD5⁺ as determined by flow cytometric analysis.

B cell culture conditions

B cells were cultured in cRPMI containing 10% FCS at a concentration of 2 × 10⁵ cells/ml. Cross-linking of bovine IgM was conducted using F(ab′)₂ of goat anti-bovine IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD) generated using peptic cleavage (Immobilized Pepsin, Pierce, Rockford, IL) according to manufacturer’s instructions. Culture of B cells with a murine fibroblast cell line, DAP3, stably transfected with bovine CD40L (boCD40L-DAP3), was conducted at a ratio of 1 CD40L-DAP3 cell to every four B cells, as previously described (7). Before culture, transfected cells and nontransfected DAP3 cells were treated with 50 ng/ml mitomycin C for 30 min at 37°C, washed three times in HBSS, and allowed to adhere to culture dishes for 1 h and residual unbound fibroblasts were removed. PMA and calcium ionophore A23187 (Sigma, St. Louis, MO) stimulation of B cells was performed at final concentrations of 10 ng/ml and 1 μg/ml, respectively. Endotoxin (from Escherichia coli O55: B5; Sigma) and pokeweed mitogen (Phytolacca americana lectin; Sigma) were used at concentrations of 20 EU/ml and 10 μg/ml, respectively.

Proliferation assay

B cell proliferation assays were conducted on B cells cultured in triplicate at a concentration of 2 × 10⁶ B cells per well in a 96-well plate. Twenty-four hours after culture, CD5Ig or isotype control (huIgG1) was added to cultures at concentrations of 25, 12.5, or 2.5 μg/ml. B cells were pulsed with 1 μCi [³H]thymidine (DuPont/NEN, Boston, MA) after 72 h culture and harvested 18 h later onto Skatron filter mats (Skatron Instruments, Lier, Norway) using a cell harvester (Skatron Instruments). Thymidine incorporation was determined by scintillation counting (Beckman Coulter, Fullerton, CA).

Flow cytometry

The following Abs specific for bovine Abs were used for staining: CC17 recognizing CD5 (provided by Chris Howard, Institute for Animal Health), MM1A (anti-CD3), and GB25A (anti-CD21) (WSU mAb Center); goat anti-bovine IgM-FITC (Kirkegaard & Perry Laboratories) and mouse anti-bovine IgM (BM23; Sigma). Secondary detection reagents used include rat anti-mouse IgG1-PE (BD Becton Dickinson, San Jose, CA), streptavidin-PE, and streptavidin-APC (BD PharMingen, San Diego, CA). Abs used as negative staining controls include the secondary detection Abs listed above used in the absence of primary Abs and in conjunction with isotype-matched mouse IgG1 and huIgG1, and goat Ig-FITC (Sigma). Cells were stained and washed in cold PBS containing 1% BSA and 0.1% sodium azide and were fixed in 2% buffered paraformaldehyde. Cells were analyzed using a FACSVantage flow cytometer and CellQuest acquisition and analysis programs (BD Becton Dickinson). To characterize CD5Ig binding, cells were allowed to bind CD5Ig in the presence of PBS alone or PBS containing 25 mM EDTA. 1 M glucose, 1 M fructose, or 1 M mannose. Protease pretreatment was conducted by incubating cells in 0.05% trypsin-EDTA (Sigma) or 1 mg/ml proteinase K (Sigma) in PBS for 20 min before CD5Ig staining.

Production and purification of chimeric fusion proteins

Yeast-expressed bovine CD5Ig (yCD5Ig). The cDNA encoding the extracellular domain of bovine CD5 lacking its native signal sequence was fused to the cdna encoding the Fc portion of huIgG1 (CH2 and CH3 domains minus hinge region) and cloned into the yeast expression vector, picZαA (Invitrogen, Carlsbad, CA). Pichia pastoris was transformed with CD5Ig-picZαA via electroporation, according to manufacturer’s instructions (Gene Pulser; Bio-Rad, Richmond, CA). Transformed yeast was selected for growth on yeast extract peptone dextrose plates containing (100 μg/ml) Zeocin. Individual yeast clones transformed with CD5Ig-picZαA were further identified by PCR screening. PCR-positive clones were grown in methanol-inducing medium and supernatants were analyzed at various time points for the presence of secreted CD5Ig by Western blot analysis. Large-scale production of yCD5Ig followed. yCD5Ig was purified by Amicon filtration (XM50; Amicon, Beverly, MA) followed by protein A purification (Pierce) in the presence of 0.05% Tween 20.

MOP-S-expressed bovine CD5Ig (mCD5Ig) and CD40Ig. The extracellular domain of bovine CD5 including its native signal sequence and an added Kozak’s consensus sequence was fused to cdna encoding the human Fc portion of CD40 (CH2 and CH3 domains lacking the hinge region) and cloned into the mammalian expression vector, pcDNA3.1/neo (Invitrogen). CD5Ig-pcDNA3.1(+) was introduced into MOP8 NIH3T3 cells (CRL-1709; American Type Culture Collection, Manassas, VA) using Li-pofectamine (Life Technologies, Gaithersburg, MD) according to manufacturer’s instructions. Stable transfectants were selected by limited dilution cloning and selection in complete DMEM-10 containing 200 μg/ml G418 (Life Technologies). Supernatants were analyzed for mCD5Ig via dot blot and Western blot analysis. CD5Ig was purified from supernatants by dialysis against PBS (pH 8) using 50000 MWCO cellulose dialysis tubing (SpectraPor; Spectrum Laboratories, Los Angeles, CA). Similarly, a CD40Ig fusion protein containing the extracellular portion of bovine CD40 and the CH2 and CH3 domains of huIgG1 was purified from stably transfected MOP8 cells. CD40Ig was purified from conditioned medium using protein A-Sepharose (Amersham, Arlington Heights, IL). Purified human CTLA-4g, derived from American Type Culture Collection CRL-10762 (generously supplied by David R. Lee) did not demonstrate appreciable binding to bovine PBMCs and thus, was used in addition
to huIgG1 as a negative control for nonspecific fusion protein binding attributable to human Fc interactions. Fusion proteins were biotinylated for use in flow cytometric analysis.

**Immunoprecipitation**

In experiments which the thiol-cleavable cross-linking agent, 3,3′-dithiobis-(sulfosuccinimidylpropionate) (DTSSP; Pierce) was used, cells were allowed to bind CD5Ig or isotype control (huIgG1 or CTLA4Ig) for 30 min, washed three times in cold PBS (pH 8), and simultaneously surface biotinylated and cross-linked with equal amounts of EZ-Link Sulfo-NHS-LC biotin (Pierce) and DTSSP in PBS (pH 8) for 1 h on ice. Cells were then washed three times in PBS and lysed in CHAPS lysis buffer (10 mM CHAPS, 150 mM NaCl, 20 mM Tris-Cl (pH 8), 0.2 mM PMSF, 10 mM iodoacetamide, 1 mM EDTA, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) for 1 h on ice. Cell lysate was then centrifuged for 10 min at 10,000 × g at 4°C. Lysate supernatant was then incubated with protein A-Sepharose. Precleared lysate was then incubated with CD5Ig or biotinylated as described above, lysed, and precleared overnight with protein A-Sepharose. Precleared lysates were then incubated with CD5Ig or huIgG1 for 2 h at 4°C and then incubated overnight with protein A-Sepharose. Samples were then treated and analyzed as described above.

**Results**

**CD5L expression, as assessed by CD5Ig binding, is activation-state dependent**

To begin to characterize and identify the ligand for bovine CD5, we constructed a chimeric fusion protein consisting of the extracellular bovine CD5 domain fused to the Fc portion of huIgG1 (CD5Ig) and expressed this protein in both yeast (yCD5lIg) and mammalian cells (mCD5lIg). Similar to CD5, which exists as a monomer, the CD5Ig fusion protein was designed to be secreted as a monomer, the CD5Ig fusion protein was designed to be secreted as a monomer, which allows for dimerization. CD5Ig was verified to be produced in monomeric form by nonreducing PAGE analysis. Using this fusion protein, we sought to characterize the cellular expression of the ligand for CD5 by FACS analysis. PBMCs freshly isolated from five healthy donors demonstrated low to undetectable levels of mCD5Ig binding (Fig. 1A, d0, and data not shown) relative to the negative staining controls, huIgG1 and huCTLA4Ig. However, activation of PBMCs with PMA and ionophore resulted in the gradual increase in CD5Ig binding over a 3-day period, followed by a decrease in binding by day 4 (Fig. 1A). In some cases, culture of cells in medium alone over a 3-day period resulted in increased CD5Ig binding, albeit at lower levels than PMA plus ionophore-activated cells. In the interest of determining whether PMA or calcium ionophore could act independently to induce CD5L expression, we treated cells for 3 days with calcium ionophore, PMA, or the combination of PMA and ionophore, and compared the level of CD5Ig binding between treatments. As shown in Fig. 1B, PMA alone is unable to enhance CD5L expression over that observed for cells cultured in medium alone. However, activation of cells with ionophore alone results in a CD5Ig binding pattern that is nearly identical with that observed for cells activated with both PMA and ionophore. Thus, activation of cells with ionophore alone is sufficient to induce CD5L expression, as assessed by CD5Ig binding. Moreover, PMA does not appear to enhance this effect.

**Characteristics of the CD5Ig-CD5L binding interaction**

In an attempt to further characterize the CD5Ig-CD5L interaction, various binding conditions and cell pretreatments were examined for their ability to inhibit CD5Ig binding. To determine the requirement for divalent cations for CD5Ig binding to CD5L, cells activated for 3 days with PMA and ionophore were stained with mCD5Ig by flow cytometry. B, PBMCs were activated for 3 days in the presence of medium (dashed line), PMA (gray shaded area), Ca2+ ionophore (bold solid line), or Ca2+ ionophore and PMA (solid line) and assayed for mCD5Ig binding. HuIgG1 and huCTLA4Ig are shown as negative staining controls. Results are representative of three experiments.

**FIGURE 1.** Binding of CD5Ig by bovine PBMCs. A, PBMCs isolated from various donors were cultured in medium alone or in the presence of PMA and Ca2+ ionophore for the indicated period and assayed for binding to mCD5Ig by flow cytometry. B, PBMCs were activated for 3 days in the presence of medium (dashed line), PMA (gray shaded area), Ca2+ ionophore (bold solid line), or Ca2+ ionophore and PMA (solid line) and assayed for mCD5Ig binding. HuIgG1 and huCTLA4Ig are shown as negative staining controls. Results are representative of three experiments.

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<th>Treatment</th>
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<td>EDTA</td>
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* aPBMCs were activated for 3 days with PMA+ ionophore and analyzed for CD5Ig binding under the indicated conditions (see Materials and Methods).

b Relative to untreated (+ indicates no change in binding; – indicates complete abrogation of binding).
of several monosaccharides (D-glucose, D-fructose, and D-mannose). Concentrations of 1 M D-glucose, D-fructose, and D-mannose had no inhibitory effect on PBMC CD5Ig binding (Table I). Thus, these carbohydrates are not likely to contribute significantly to the CD5-CD5L interaction. Finally, pretreatment of activated cells with either trypsin or proteinase K totally abrogated CD5Ig binding (Table I).}

**CD5L expression is detected on activated B cells**

Independent studies aimed at characterizing the ligand for CD5 have demonstrated the ligand to be expressed on a variety of cell types (37–42). Analysis of freshly isolated bovine PBMCs indicated that cells expressing low levels of CD5L in vivo were of an activated phenotype based on forward angle light scatter and were CD3+CD5−IgM+ (data not shown). To determine which cell population(s) could be induced to express the CD5L, small resting (Percoll-fractionated) PBMCs were activated for 3 days with PMA and Ca2+ ionophore and analyzed by flow cytometry using Abs against various bovine surface markers. Concordant with the results observed for freshly isolated PBMCs, mCD5Ig binding was primarily confined to B cells, according to CD21 (Fig. 2A) and IgM (data not shown) dual staining results. However, PMA and ionophore-activated CD3+ T cells did not appear to express the ligand that binds the CD5Ig fusion protein. Importantly, preincubation of cells with huIgG1 or CTLA4Ig before staining did not block CD5Ig binding. Similar to the results obtained for resting lymphocytes activated in vitro, bulk (nonfractionated) PBMCs activated to express CD5L were primarily IgM+ cells (Fig. 2B). In some experiments, a small fraction of non-IgM+ cells was observed to bind CD5Ig. Although the exact identity of this cell population is presently unknown, these cells could potentially be non-IgM-expressing B cells that have undergone isotype switching. Alternatively, these CD5L-expressing cells could belong to a non-B, non-T cell type. Importantly, as shown in Fig. 2B, yCD5Ig bound to activated cells in a pattern analogous to mCD5Ig. In addition, preincubation of activated cells with unlabeled yCD5Ig was found to partially inhibit mCD5Ig binding, thereby demonstrating that yCD5Ig and mCD5Ig compete for binding to a similar site (23% vs 11%; Fig. 2C). This result suggests that any differences in protein modifications occurring between yeast and mammalian-cell expression of CD5L do not substantially affect binding of CD5L to its ligand.

Additional cell populations analyzed did not demonstrate CD5L binding. CD14+ adherent bovine macrophages were not observed to bind the CD5L fusion protein under proper blocking conditions (Fig. 2D). Interestingly, a CD5+ bovine B lymphoblastoid cell line (BL3) was unable to bind to CD5L. Finally, K562 cells, shown by Calvo et al. (40) to bind huCD5Ig, were not observed to bind CD5Ig (Fig. 2D), nor were murine fibroblast cell line DAP3, liver cell line BNLSV A.8, or monkey kidney cell line COS-7 (data not shown). These results indicate the ligand for bovine CD5, as measured by both mCD5Ig and yCD5Ig binding, is primarily expressed by activated B cells. This finding is similar to recent descriptions of the alternative murine CD5L as characterized by huCD5Ig binding (38, 39).

In addition to PMA and ionophore, other cellular activators were examined for their ability to induce CD5L expression on B cells. As shown in Fig. 3, stimulation of PBMCs with endotoxin also resulted in increased CD5L expression on IgM+ cells compared with cells cultured in medium alone. Whereas only 20% of B cells were found to express CD5L after 3 days of culture in medium alone (as assessed by mCD5Ig and yCD5Ig binding), 40% of B cells expressed the ligand after activation with endotoxin (Fig. 3). This result is similar to the observed increase in CD5Ig binding by B cells activated with PMA and ionophore (data not shown). Unlike endotoxin activation, PWM stimulation did not result in an appreciable increase in CD5L binding. Although the percentages of B cells induced to express CD5L were observed to vary from experiment to experiment and from donor to donor, ligand expression on B cells was typically increased twofold or greater after activation with ionophore or endotoxin.

**Ligation of CD40, but not B cell receptor cross-linking, results in CD5Ig binding**

Recently, we have demonstrated that CD5 expression can be induced on bovine B cells after slgM cross-linking, but not CD40 ligation, and furthermore, that activation of B cells through CD40 functions to inhibit slgM-mediated CD5 expression (7). Given the differences in the regulation of CD5 expression with regard to CD40 and slgM signaling, we were interested in determining whether the expression of CD5L was affected by these distinct
activation pathways. To determine the effect of CD40 ligation or sIgM cross-linking on CD5L expression, highly purified nonfractionated CD5^+ IgM^+ B cells were cultured for 2 days in the presence of nontransfected DAP3 cells, CD40L transfectants, goat anti-bovine IgM F(ab')_2 (25 μg/ml) and DAP3 cells, or a combination of CD40L-DAP3 cells and goat anti-bovine IgM F(ab')_2. These cells were subsequently analyzed for CD5L binding. The result of CD5Lg binding by IgM^+ gated cells is shown in Fig. 4A. One-third of the B cell population was observed to express CD5L in the absence of activation (35%, culture with DAP3 cells alone). However, activation of B cells via BCR cross-linking did not result in an increase in the percentage of B cells expressing CD5L (38%). Interestingly, CD40 ligation did cause an increase in the number of B cells that bound CD5L (48%). Finally, CD40 ligation and sIgM activation synergistically resulted in a higher percentage of B cells expressing CD5L (57%). However, activation of B cells with Ca^{2+} ionophore was the most efficient stimulus, resulting in induction of B cell expression of CD5L in 70% of B cells.

One potential explanation for the relatively high level of CD5L binding by B cells not artificially activated in vitro (35%; Fig. 4A; DAP3) was that there was a pre-existing population of naturally activated B cells at the onset of culture. To determine whether this was indeed the case, small resting CD5^+ IgM^+ B cells were isolated by percoll fractionation and stimulated as described above for 2 days and IgM^+ gated cells were analyzed for CD5L binding. As predicted for a population of unstimulated resting B cells, only 3% of the IgM^+ B cell population cultured with control DAP3 cells expressed the ligand for CD5 (Fig. 4B). Similar to the observation reported for nonfractionated cells stimulated with goat anti-bovine IgM F(ab')_2, no significant increase was observed in the percentage of B cells expressing CD5L after slgM cross-linking (5%). However, CD40 ligation dramatically increased the percentage of IgM^+ cells demonstrating CD5L binding (42%). Furthermore, dual activation of B cells with both CD40L transfectants and anti-IgM treatment appeared to synergize to result in an even greater percentage of B cells expressing CD5L compared with cells activated through CD40 alone (57% vs 42%). Importantly, higher concentrations of goat anti-bovine IgM F(ab')_2 (50, 75, and 100 μg/ml) were analyzed for the ability to increase CD5L binding, yet while observed to result in increased CD5 expression, were ineffective at causing expression of its ligand to be enhanced (data not shown). Notably, B cells which were observed to express CD5 after BCR cross-linking were not found to coexpress CD5L, although in cultures of nonPercoll-fractionated B cells,
CD5<sup>−</sup>CD5L<sup>+</sup> B cells were indeed present. Given the finding that stimulation of B cells via both Ag receptor cross-linking and CD40 ligation display enhanced CD5Ig binding, we sought to determine whether B cells stimulated to express CD5 in these cultures were also induced to coexpress CD5L. To determine this, purified B cells were stimulated in the presence of CD40L transfectants alone or in combination with anti-IgM. As shown in Fig. 4C, the percentage of B cells (IgM<sup>+</sup>-gated cells) coexpressing CD5 and CD5L after 2 days stimulation with CD40L transfectants is only 2%. A greater percentage of B cells that are activated via BCR cross-linking and CD40 ligation are induced to express CD5 compared with those cultured in the presence of CD40L transfectants alone, as expected. Although both CD5 and its ligand are induced on B cells after CD40 ligation and anti-IgM activation, CD5 and CD5L appear to be dual-expressed on only a small fraction of costimulated B cells (4%; Fig. 4C). Although a minor fraction of CD5<sup>+</sup> B cells may be capable of coexpressing CD5L, the majority of B cells expressing CD5L after coculture with CD40L transfectants and BCR cross-linking Ab do not express CD5 (26%), despite the presence of a significant number of CD5<sup>+</sup> B cells (25%). Thus, most CD5<sup>+</sup> B cells in cultures stimulated by anti-IgM and CD40L are not observed to express CD5L. This finding is further supported by the observation that freshly isolated PBMCs (which include CD5<sup>+</sup> B cells) expressing low levels of CD5L are CD5-negative. In addition to this, the CD5<sup>+</sup> bovine B lymphoblastoid cell line, BL3, was not observed to bind CD5Ig (Fig. 2D). Thus, most B cells expressing CD5L do not appear to coexpress CD5. Overall, these results indicate that CD5L is indeed an activation Ag, whose expression is induced through ligation of CD40, or the combination of CD40 ligation and slgM cross-linking. Importantly, slgM cross-linking solely, even at high concentrations, was unable to modulate expression of CD5L. Interestingly, these observations are the reciprocal of what we have reported for the regulation of CD5 expression by CD40 and B cell receptor signaling.

**CD5Ig augments B cell proliferation mediated by CD40 ligation**

Upon determining that CD5L was induced by ligation of CD40, we sought to determine whether CD5Ig had an effect on B cell proliferation mediated by stimulation of CD40. To determine the effect of CD5Ig on proliferation, panned B cells were cultured in the presence of CD40L-DAP3 cells and various concentrations of CD5Ig or isotype control (huIgG1) for 4 days and assayed for proliferation in the final 18 h of culture via [3H]thyminidine uptake. As shown in Fig. 5A, proliferation of CD40L-activated B cells was enhanced nearly 2.5-fold in the presence of 25 μg/ml CD5Ig compared with that observed for B cells activated by CD40L-DAP3 cells alone or CD40L transfectants and isotype control. This enhancement of proliferation by CD5L stimulation appeared to be dose dependent, because decreasing concentrations of CD5Ig resulted in reduced enhancement of proliferation. In addition to investigating the effect of CD5Ig on proliferation elicited by ligation of CD40, we were interested in determining whether CD5Ig would also have an effect on CD40L- and anti-IgM-stimulated proliferation. As demonstrated in Fig. 5B, in the presence of CD5Ig, B cell proliferation was slightly enhanced over that of B cells activated with CD40L and anti-IgM, in the absence or presence of huIgG1 control. In addition to investigating the effect of CD5Ig on B cell proliferation, the effect of yCD5Ig on B cell proliferation was examined. Not surprisingly, yCD5Ig enhanced CD40L-mediated B cell proliferation to a level similar to that observed with mCD5Ig (Fig. 5C). Notably, in the absence of additional stimuli, CD5Ig alone was not observed to have a noticeable effect on background B cell proliferation (Fig. 5D).

**FIGURE 5.** CD5Ig augments CD40L-stimulated B cell proliferation. B cells purified by passive panning were cultured with CD40L transfectants alone (A) or CD40L-DAP3 cells combined with goat anti-bovine IgM F(ab')<sub>2</sub> (25 μg/ml) (B), along with varying concentrations of MOP8-CD5Ig or huIgG1 for 4 days and assayed for proliferation, measured via [3H]thyminidine uptake. C, Proliferation of panned B cells cultured with CD40L-DAP3 cells alone (–), or 25 μg/ml huIgG1, MOP8-CD5Ig, or yCD5Ig was assayed 4 days poststimulation. D, Proliferation of B cells cultured with yCD5Ig (25 μg/ml) or mCD5Ig (12.5 μg/ml) in the presence or absence of CD40L transfectants. A–D, Data are shown as means of triplicate cultures. Error bars represent the SD of the mean. Results are representative of two to three separate experiments.

**CD5Ig immunoprecipitates a 200-kDa protein**

To further characterize the putative CD5L bound by CD5Ig, an attempt was made to immunoprecipitate the ligand from activated
B cells. B cells activated with PMA and Ca\textsuperscript{2+} ionophore for 3 days were allowed to bind CD5Ig or huIgG1, washed, and simultaneously biotinylated and cross-linked using DTSSP. Cross-linking of CD5Ig to CD5L was performed in initial experiments in an attempt to stabilize an interaction that potentially would not be maintained under conditions of lysis. A similar procedure was used to successfully identify the ligand for CD6, ALCAM (43). The CD5Ig-CD5L complexes were isolated by protein A-Sepharose immunoprecipitation and after denaturation (and hydrolysis of cross-linking agent), resolved by denaturing SDS-PAGE. The putative ligand was detected by Western blotting with avidin-peroxidase. The immunoblots in Fig. 6A show that a protein of \(200 \text{ kDa}\) is immunoprecipitated by mCD5Ig and yCD5Ig. However, immunoprecipitation of B cells using huIgG1 as an isotype control did not result in the detection of a band of this size, nor did huCTLA4Ig (data not shown). Upon demonstrating that CD5Ig immunoprecipitated a 200-kDa protein, we next determined whether CD5Ig, in the absence of cross-linking, could immunoprecipitate its ligand from cell surface biotinylated B cell lysates.

**FIGURE 6.** CD5Ig immunoprecipitates a protein of \(200 \text{ kDa}\). A, Immunoprecipitation of lysates from cell surface biotinylated B cells activated with PMA + Ca\textsuperscript{2+} ionophore for 3 days (A and B) or BL3 cells (B) by CD5Ig and huIgG1. In A, cell surface protein cross-linking (using DTSSP) and cell surface biotinylation was performed simultaneously before immunoprecipitation. Immunoprecipitated proteins were reduced, resolved on a 6% (A) or 5–20% gradient (B) SDS-PAGE gel, and detected by enhanced chemiluminescent immunoblotting. The putative ligand is indicated by the arrow.

BL3 cells were used as a negative control cell population, because these cells do not demonstrate CD5Ig binding (Fig. 2D). As shown in Fig. 6B, a similar 200-kDa band was immunoprecipitated by mCD5Ig from activated PBLs, but not from BL3 cells. However, the isotype control did not immunoprecipitate proteins of this size. A similar result was obtained using activated bovine spleen cells, in which a 200-kDa protein was immunoprecipitated by CD5Ig, but not huIgG1 (data not shown). Although attempts have been made to isolate sufficient quantities of the 200-kDa protein for sequence analysis, sequence information for this protein has not yet been attainable due to the poor efficiency of immunoprecipitation by the CD5Ig fusion protein in the absence of cross-linking. However, these preliminary results indicate that the CD5L may indeed be a protein of 200 kDa, although further investigation of this putative protein is required to confirm that it is, in fact, the ligand for CD5.

**Discussion**

In this report, we describe the cellular distribution, as well as the induction, of bovine CD5L based on the binding characteristics of a chimeric bovine CD5Ig fusion protein. As determined by CD5Ig binding, expression of this ligand was typically negative to low on freshly isolated PBMC from various donors, with low-density expression being limited to B cells. We demonstrate that CD5L is transiently up-regulated 1–4 days after stimulation with PMA and Ca\textsuperscript{2+} ionophore, with maximal expression occurring 2–3 days poststimulation. Notably, Ca\textsuperscript{2+} ionophore alone was capable of inducing CD5L, independent of PMA stimulation. Importantly, this finding indicates that a [Ca\textsuperscript{2+}]\textsuperscript{1} increase stimulated by Ca\textsuperscript{2+} ionophore, but not activation of pathways targeted by PMA (PKC, etc.), is sufficient to up-regulate CD5L expression.

Similar to murine CD5Ls described recently (38, 39), we demonstrate that the ligand for CD5 is not constitutively expressed, but inducible on B cells after various modes of activation (i.e., Ca\textsuperscript{2+} ionophore, LPS, or CD40 ligation). Although Bikah et al. (39) have described a ligand on murine spleen cells induced by IgM cross-linking, our data demonstrate that BCR cross-linking alone on resting peripheral blood B cells is not sufficient to induce ligand expression. This observed difference can perhaps be attributed to differences in cellular activation state or the source of cells (peripheral blood vs spleen) or even differences in species. Our results regarding CD5L induction are similar to those reported by Biancone et al. (38), which demonstrated that the induction on murine splenic B cells requires the presence of T cells activated by anti-CD3 and anti-CD28, although no role for CD40 ligation was shown. Our data indicate that whereas CD40 ligation alone results in the up-regulation of CD5L, activation of B cells by both CD40 ligation and sIgM cross-linking results in even greater cell surface expression. Interestingly, activation through BCR cross-linking alone, although able to induce CD5 expression, did not result in expression of CD5L. The apparent discrepancy in CD5L up-regulation between B cells activated by BCR cross-linking, known to result in Ca\textsuperscript{2+} mobilization (44), and B cells activated by Ca\textsuperscript{2+} ionophore, is perhaps explained by differences in the signaling pathways that become activated as a result of ionophore-stimulation vs BCR ligation, as has previously been reported (45). Moreover, the level of intracellular Ca\textsuperscript{2+} increase is likely greater in response to Ca\textsuperscript{2+} ionophore than to BCR cross-linking via soluble anti-IgM, and this could potentially account for the differences observed in CD5L up-regulation.

The lack of CD5L induction by BCR cross-linking is an interesting finding, because it indicates that B cell-specific expression of CD5 and its ligand are reciprocally regulated by CD40 and BCR signaling (Fig. 7). The implications for the counterregulation of
or responses to TI-2 Ags. Thus, the CD5-CD5L interaction could regulate BCR signaling, and thereby inhibit further B cell activation in the absence of other signals. CD5L up-regulation is inhibited by signals generated upon ligation of CD40. However, CD40 ligation, as reported here, is able to instead, stimulate B cell expression of the ligand for CD5. Similarly, additional signals received by B cells, such as LPS, would likely result in expression of CD5L. Although BCR cross-linking alone appears to be unable to result in the expression of CD5L, it synergizes with signals generated by ligation of CD40 to enhance CD5L up-regulation. Interaction between a CD5⁺ B cell and a CD5L⁺ B cell may result in inhibition of the negative regulatory effect CD5 may otherwise have on BCR signaling through physical sequestration of CD5.

Similarly, positive costimulatory signals received through CD5L (expressed on a study that demonstrated that the sequestration of CD5 away from IgM before BCR cross-linking blocks the inhibitory effect of CD5 on BCR signaling (32), engagement of CD5, through interaction with its ligand, may similarly block the negative influence CD5 potentially exerts on signals transmitted through the BCR. Such a B-B cell interaction might be expected to occur during TI-2 Ag responses. Our data suggest that surface IgM cross-linking, mimicking TI-2 Ag activation, although able to result in CD5 expression, is not effective at up-regulating CD5L in the absence of other stimuli (i.e., CD40 ligation). The interaction of a B cell expressing CD5 in response to the cross-linking of its polyreactive Ab receptor (via encounter with a TI-2 Ag) with a B cell expressing CD5L (via activation by LPS, CD40L, or other signal) may allow for further activation of the CD5⁺ B cell. In the absence of the CD5-CD5L interaction, CD5 may continue to negatively regulate BCR signaling, and thereby inhibit further B cell activation or responses to TI-2 Ags. Thus, the CD5-CD5L interaction could potentially serve as a checkpoint for CD5⁺ (or B-1) cell activation, such that in the absence of other activation or danger signals, CD5L is not expressed, and CD5⁺ B cell responsiveness is suppressed. Loss of this regulatory mechanism would presumably contribute to the uncontrolled expansion and transformation, as well as to the production of pathogenic autoantibodies by this B cell subset. The CD5-CD5L interaction might also play a role in the process of Ag presentation that may potentially occur between a CD5⁺ B cell and a CD5⁺ CD5L⁺ (B-2) B cell. Due to their expression of low affinity, polyspecific Ag receptors, CD5⁺ B cells could potentially present TI-2 Ags captured by their Ab receptors to CD5⁺ B cells expressing high affinity receptors, as has been previously proposed (46). During such an encounter, CD5, through its interaction with its ligand, could potentially costimulate activation of a CD5L⁺ B cell. Although much speculation can be made, many questions remain to be answered regarding the role of the CD5-CD5L interaction in immune responses, both protective and pathogenic.

Similar to murine and human studies, which have suggested that CD5 binds to a ligand other than CD72 (38–40), it is unlikely that the ligand we have characterized in this report is bovine CD72. First, the size of the protein immunoprecipitated by CD5lg (180–200 kDa) argues against a CD5Ig-CD72 interaction, because immunoprecipitation of CD72 would have been expected to result in much smaller sized protein (45 kDa). However, it must be noted that immunoprecipitation of the 200-kDa protein by CD5Ig does not confirm that this protein is itself CD5L, because it may be a protein that associates with the true ligand. Sequence information for this protein will assist in designing experiments directed toward confirming whether it is indeed the ligand for CD5. Assuming CD72 follows a similar expression pattern in ruminants, if CD72, as expressed on resting B cells, acts as a ligand for CD5, resting bovine B cells would have been expected to bind CD5lg. However, this was not observed. These findings are in agreement with recent studies that suggest murine and human CD5 bind to an activation-induced B cell-expressed ligand other than CD72 (38, 39). However, to definitely prove that bovine CD5 does not interact with CD72, bovine CD72 must first be cloned in order that experiments that are aimed at examining the interaction CD72 may form with CD5 can be performed.

An important observation reported here is that the CD5Ig fusion proteins produced by MOP8 cells (mammalian) and Pichia pastoris (yeast) demonstrated nearly identical binding patterns and were capable of competing for a similar binding site as determined by blocking inhibition experiments. This suggests that potential differences in processing or modifications in the fusion protein that exist between mCD5Ig and yCD5Ig have no apparent effect on the binding of CD5Ig to the CD5L. Furthermore, as assessed by their ability to augment proliferation, these two fusion proteins appear to demonstrate equal functional activity. Additionally, unlike recent studies in which huCD5ig constructs have been used to characterize the murine CD5L (38, 39), we have used a species-specific construct to avoid potential complications resulting from differences in CD5 species cross-reactivity. Overall, this lends further credence to the data presented within this study, which for the first time, characterize the expression pattern, binding requirements, activation conditions for induction, putative size (~200 kDa), and costimulatory role, for the first ligand to be identified for bovine CD5. The data reported herein, as well as future studies aimed at further characterizing this newly identified bovine CD5L, will undoubtedly aid in understanding the role that the CD5-CD5L interaction plays in immune responses. In addition, the characterization of this newly identified bovine CD5L may assist in elucidating the role that CD5 expression plays on bovine B cells during natural
infections, such as bovine leukemia virus and trypanosomosis in which the CD5+ B cell population becomes expanded.

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References