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Cloning, expression and biological function of the bovine CD40 homologue: role in B-lymphocyte growth and differentiation in cattle

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SUMMARY

Members of the tumour necrosis factor receptor superfamily play a key role in B-lymphocyte survival, proliferation, differentiation and programmed cell death. A member of this superfamily, the CD40 molecule, plays an important role in the differentiation of B lymphocytes into effector cells and in early activation through cognate interaction with T lymphocytes. In this report, we describe a cDNA and its protein product identified in cattle with approximately 70% sequence conservation at the nucleic acid level with the human CD40 gene. Transcripts for the boCD40 molecule were identified in resting and activated B lymphocytes, some but not all $CD4^+$ and $CD8^+$ T lymphocyte clones, and peripheral blood-derived T lymphocytes. Coculture of resting B cells with simian virus 40 (SV40)-transformed NIH3T3 (MOP8) cells stably transfected with the ligand for CD40 (bovine CD40 ligand, boCD40L), resulted in proliferation which was enhanced by addition of rbo interleukin-4 (IL-4). Cross-linkage of CD40 on bovine B lymphocytes upon coculture with CD40L-transfected cells resulted in the increased production of secretory IgM and, to a lesser extent, of IgG. Addition of rboIL-4 to these cultures increased levels of IgM and IgG secretion approximately twofold over those induced by CD40L alone. Our results indicate that many of the functions described for human and mouse CD40 are also conserved in the bovine but that differences in subset distribution of expression of the CD40 molecule in lymphoid cell types in cattle may impact on regulation of the early activation steps in the acquired immune response.

INTRODUCTION

Initial interactions involving cognate signalling between helper T cells and B cells are the key to the development of T-dependent antibody responses. Binding of CD40 and trimerization by its counter-receptor CD40 ligand (CD40L) as well as the interactions of CD28–CD80/CD86 play an important role in early contact-dependent B cell–T cell signalling events.[1,2] It is evident from a variety of studies that the CD40 molecule is a key player in the development of antigen-specific immunity. In mammalian species where it has been described, the CD40 molecule is found on a variety of cell types including B lymphocytes, dendritic cells, haematopoietic progenitor cells, epithelial cells and some types of carcinomas.[3] Its counter-receptor, CD40L has been identified on both activated $CD4^+$ and $CD8^+$ T cells.[4–6] The nucleotide sequence of the bovine homologue for CD40L has recently been described by our laboratory and others (Genbank accession #z48469).

Cross-linking of the CD40 molecule with anti-CD40 monoclonal antibodies or cells transfected with CD40L stimulates proliferation of human B lymphocytes *in vitro*.[7,8] In combination with various cytokines such as interleukin (IL)-4 or IL-13, CD40 cross-linkage can allow relatively long-term propagation of non-transformed Epstein–Barr virus (EBV)-negative human peripheral blood B lymphocytes.[7,9–11] Addition of cytokines such as IL-4, IL-13, IL-10, transforming growth factor-β (TGF-β), or non-cytokine factors such as vasoactive intestinal peptide (VIP) results in isotype switching from non-committed B-lymphocyte progenitors to IgG4/IgE, IgA/IgG3/IgG1, or IgA2 respectively.[7,9–13] The CD40–CD40L interaction has also been implicated *in vivo* as a key step in the maturation of B cells into non-IgM-producing cells within germinal centres. This was demonstrated directly by identification of the defect in hyper-IgM syndrome as a mutation in CD40L, which resulted in the diminished production or absence of non-IgM isotypes.[14–17]

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Abbreviations: AC, accessory cells; boCD40L, bovine CD40 ligand; Con A, concanavalin A; IFN, interferon; PBMC, peripheral blood polynuclear cells; PCR, polymerase chain reaction; PMA, phorbol myristate ester; PWM, pokeweed mitogen; rboIL-4, recombinant bovine interleukin-4; TCGF, T-cell growth factor.

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We demonstrate in this study that the critical role of CD40–CD40L interaction in the development of T-dependent antibody responses in humans and rodents is conserved in cattle. Bovine CD40L-transfected cells stimulate B-cell proliferation and antibody production *in vitro* and this contact-mediated event can be enhanced by IL-4 by its ability to stimulate cells to progress through cell cycle, differentiate to high-rate immunoglobulin-producing cells, and undergo isotype switching.

MATERIALS AND METHODS

Reagents

Rabbit polyclonal antiserum against amino acids 25–45 of the bovine CD40 N-terminal sequence was generated in rabbits by a previously described method.[18] Affinity-purified goat polyclonal antibodies against bovine IgM were obtained from Kirkegaad & Perry Laboratories Inc. (Gaithersburg, MD). Recombinant bovine IL-4 (rboIL-4) was generated in CV-1 cells as previously described, with modifications.[19] The rboIL-4 was partially purified by ultrafiltration (YM-30, Amicon, Beverly, MA, 30 000 molecular weight cutoff; MWCO), reconcentrated (YM-10, Amicon, 10 000 MWCO), and filter sterilized (Millipore, Bedford, MA).

Molecular cloning and gene structure of the bovine CD40 homologue

A plasmid cDNA library was constructed from pokeweed mitogen-activated (PWM) peripheral blood mononuclear cell (PBMC) poly A^+ RNA using a commercial kit (Fastrack, Invitrogen, San Diego, CA). The first strand cDNA was synthesized using oligo dT with a *Not*I restriction site followed by *Bst*XI/*Eco*RI adapter ligation which allowed unidirectional cloning. The synthesized cDNA was cloned into pcDNA3 (Invitrogen). Initial cloning by polymerase chain reaction (PCR) was performed using internal degenerate primers whose sequences were conserved in the human and mouse CD40 molecules (sense primer 5′-CCCTGCCCAGTCGGCTTCTTC-3′, antisense primer 5′-CCTGCACTGAGATGCGACTCT-3′). For PCR, plasmid template from randomly generated pools was denatured at 95° for 1 min, annealed at 60° for 1 min, and extended at 72° for 2 min for 40 cycles using an Amplitron I thermal cycler (Thermolyne Instruments, Iowa City, IA). To obtain 5' and 3' flanking sequences, further rounds of PCR amplification and extension were performed using a T7 promoter primer on the vector and primer extension from a known sequence to obtain the 5′-proximal sequence of boCD40 (5′-TAATACGACTCACTATAGGG-3′, 5′-CTGACACCAGCATAAGGTCTCT-3′, 5′-GGGCCCTCTAGATGCATGCTCG-3′) using the same conditions except the annealing temperature was reduced to 50°. PCR products of correct size were sequenced by the dideoxy method using a Sequenase kit according to manufacturers instructions (USB, Cleveland, OH). Sequence comparisons were made using the FASTA algorithm (National Center for Biotechnology Information, National Library of Medicine, Washington, DC) and by simple homology (DNasis, Hitachi, San Bruno, CA) to predict domain and homology structures. The characterized boCD40 sequence has been submitted to Genbank (Accession #U57745).

The boCD40L gene was also isolated from the PWM-stimulated PBMC cDNA library by PCR using known sequence (Genbank accession #z48469; sense primer 5′-GGAAGCTTAACTCTAACGCAGCATGATCGA-3′, antisense primer 5′-GGATCCCTGCTTTCCTGGATTGTGAAGA-3′). PCR cloning was performed as described above and the product was ligated into the mammalian expression vector pcDNA3 and the sequence confirmed by dideoxy sequence analysis.

Immunoprecipitation analysis of the boCD40 homologue

BL3 cells (a bovine leukaemia virus (BLV)-transformed B-cell line, American Type Culture Collection, Rockville, MD) were metabolically labelled using ^{35}S-labelled cysteine/methionine (New England Nuclear-DuPont, Boston, MA) in serum-free RPMI-1640 at 37° for 3 hr. Labelled cells were solubilized in 1% Triton-X-100 lysis buffer containing 1% bovine serum albumin (BSA, Sigma Chemicals, St Louis, MO), 1 mM dithiothreitol (DTT) (Boehringer Mannheim, Indianapolis, IN), 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma Chemicals) and aprotinin (0·2 U trypsin inhibitor units, Sigma Chemicals). BL3 crude lysates were precleared three times with normal rabbit serum (preimmunization serum) at room temperature for 30 min. After preclearing, samples were incubated with anti-boCD40 peptide rabbit immune serum precoupled to Protein A–Sepharose beads (Sigma Chemicals) at 4° overnight with shaking. Beads were washed three times with lysis buffer and heated to boiling in 1 X sodium dodecyl sulphate (SDS) sample buffer with 2-mercaptoethanol (2-ME) for 5 min.[20] Precipitated protein(s) were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) (10%) under reducing conditions as previously described.[19,21] SDS–PAGE gels were dried and exposed using X-OMAT AR film (Kodak, Rochester, NY) with intensifying screens for approximately 1 week.

Lymphocyte purification and culture conditions

B lymphocytes were purified from the peripheral blood of a 6–8-month-old Holstein steer as previously described with modifications.[19,22] Briefly, resting lymphocytes were isolated over a two-step Percoll gradient (density values of 1·060 and 1·080) as previously described with modifications for a two layer gradient.[23] IgM^+ high-density B lymphocytes (density $>1{\cdot}080$) were positively sorted using biotinylated goat anti-bovine IgM oligoclonal antibody followed by avidin-conjugated magnetic beads (Dynal, Inc. Lake Success, NY) treatment and subsequently utilized for analysis. Purity of B-lymphocyte populations was assessed by the absence of proliferation to concanavalin A (Con A) at 5 μg/ml (Sigma Chemicals) and flow cytometry/immunocytochemical staining for surface and cytoplasmic immunoglobulin, respectively. Purified resting B lymphocytes (>95% sIg^+) were cultured in complete RPMI-1640 medium (cRPMI) with additions to final concentrations as follows: RPMI-1640 supplemented with 4 μg/ml gentamycin sulphate, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma Chemicals), 1mM non-essential amino acids (Sigma Chemicals), and 10% γ-globulin-free normal horse serum (GG-free NHS, Gibco-BRL, Gaithersburg, MD). B lymphocytes were cultured at 1×10^5 cells/well in a total culture volume of 200 μl. The boCD40L-transfected cell line (boCD40L-MOP8) (A. Hirano *et al.*, unpublished results) or

control MOP8 cells were irradiated (6000 rads) and plated at cell densities to achieve a 5:1 B lymphocyte to boCD40L-transfected cell ratio. Cultures were maintained in cRPMI for 3–5 days, pulsed with 1 μCi [^{3}H]thymidine ([^{3}H]TdR) (NEN-DuPont), and harvested 8–18 h later onto glass fibre filters using a cell harvester (Skatron, Oslo, Norway). Thymidine incorporation was determined by scintillation counting (Packard Instruments, Camas, WA). Antibody production was assessed under similar conditions but cultures were extended to 6–7 days post-stimulation. Supernatants were evaluated for immunoglobulin production relative to bovine immunoglobulin standards as previously described.[19,22] Enzyme-linked immunosorbent assay (ELISA) results are presented as the mean and standard error for triplicate cultures and are representative of at least three separate experiments.

T-cell clones consisted of a γ/δ T-cell clone (G8.1H11) obtained from a *Fasciola hepatica*-stimulated T-cell line as described previously[24] and CD4$^+$ (G2.2D7, 99.3D1, 99.3C11 and G1.1C9) and CD8$^+$ (G2.1G8, 99.G1.G3, 99.2G7 and G2.3E4) clones that were obtained from Con A-induced T lymphoblastoid cell lines with no known antigen specificity.[25] The γ/δ clone (G8.1H11) was maintained with irradiated PBMC as a source of accessory cells (AC) and antigen, and the Con A-induced clones were maintained with 10% bovine T-cell growth factor (TCGF) in the absence of AC.[25] To obtain total RNA for analysis of CD40 mRNA expression, T cells were harvested 7 days after the last stimulation with antigen and AC or 3 days after subculture with TCGF, washed, and stimulated for 8 or 22 hr at a density of 2×10^6 cells per ml with Con A, (2·5 μg/ml) or Con A plus recombinant human IL-2 (25 U/ml, Boehringer Mannheim).

Southern blot and reverse transcriptase PCR analysis of boCD40 expression

Total RNA were obtained from resting or stimulated T and B lymphocytes, CD4$^+$ T-cell clones (G2.2D7, 99.3D1, 99.3C11 and G1.1C9), CD8$^+$ T-cell clones (G2.1G8, 99.G1.G3, 99.2G7 and G2.3E4) and one γ/δ T-cell clone (G8.1H11),[24,25] and BL3 cells. Total RNA was reverse-transcribed using random-hexamer priming and Abelson murine leukaemia virus reverse transcriptase and the resulting cDNA were amplified by PCR using boCD40 and actin specific primers respectively. PCR products were transferred onto a nylon membrane (GeneScreen Plus, New England Nuclear-DuPont) after denaturation. The ^{32}P-labelled DNA probes were generated by random priming according to manufacturers instructions (Random Priming Kit, Boehringer Mannheim). Hybridization was performed at 42° overnight with 2×10^5 c.p.m./ml probe as previously described.[26] Membranes were washed three times with 2 × SSC for 30 minutes at room temperature and twice with 0·2 × SSC at 50° for 1 h. Membranes were scanned by phosphoimager (AMBIS Inc. San Diego, CA) and exposed on Kodak X-OMAT-AR film (Kodak) at −70° overnight with intensifying screens.

RESULTS

Cloning and characterization of the boCD40 homologue

The cDNA encoding the boCD40 homologue was isolated from an expression library generated from mitogen-activated bovine PBMC using degenerate primers. Table 1 shows the isolated boCD40 cDNA sequence which contains 807 base pairs (bp) of open reading frame and a predicted polypeptide containing 269 amino acids. Based on sequence homology with the human CD40 gene, approximately 30 bp of coding sequence remains to be identified in order to reach a translational termination codon. Immunoprecipitation and SDS–PAGE analysis using peptide-specific antiserum directed against amino acid residues 25–45 of the predicted amino acid sequence demonstrated that a reactive protein of approximately 33 000 MW was present in the bovine B lymphoblastoid cell line BL3 cell lysates (Fig. 1). The molecular size of the immunoprecipitated native protein from BL3 is consistent with the predicted primary sequence for the boCD40 molecule, suggesting that few residues remain to be identified at the C-terminus. The size of the mature bovine CD40 protein is similar to that of non-glycosylated human CD40.[27–29] Although there are two predicted glycosylation sites which are conserved with human CD40, extensive glycosylation was not observed by immunoprecipitation analysis and molecular weight determinations from unstimulated BL3 cells. Attempts to clone the extreme 3' end from the plasmid cDNA library using vector primers and PCR analysis have not been successful. The boCD40 molecule as presently characterized has approximately 73% homology at the nucleic acid level and 69% homology at the amino acid level with the human CD40 molecule. Chou and Fasman analysis of the predicted structure of the molecule indicates that the boCD40 cDNA encodes a type I transmembrane protein which contains 16 amino acids of leader sequence followed by 174 residues constituting the extracellular domain of the molecule. Similar to the human CD40 molecule, the boCD40 extracellular domain contains 21 cysteine residues (12% of the total amino acid content). Secondary structural analysis suggests that the boCD40 extracellular domain contains a subdomain structure and the cysteine residues in these subdomains are conserved between the human and bovine homologues consistent with other members of the tumour necrosis receptor superfamily.

Expression and distribution of the boCD40 molecule

The CD40 molecule is expressed on a variety of cell types in humans and the mouse. We assessed the expression of the boCD40 molecule by reverse transcription, PCR analysis and Southern blotting from unstimulated T and B cells, phorbol myristate ester (PMA) and calcium ionophore-stimulated B and Con A-stimulated peripheral T cells, and either Con A- and TCGF- or Con A- and IL-2-stimulated CD4$^+$, CD8$^+$ and γ/δ T cell clones (Fig. 2). Our studies indicate that high density IgM$^+$ B lymphocytes (> 1·08) do not express detectable transcripts for CD40, whereas expression is strongly up-regulated upon activation with PMA and ionomycin. All CD8$^+$ T-cell clones (G2.1G8, 99.G1.G3, 99.2G7 and G2.3E4) and one γ/δ T-cell clone (G8.1H11) did express CD40 mRNA. Four CD4$^+$ T-cell clones (G2.2D7, 99.3D1, 99.3C11 and G1.1C9) were tested for expression of CD40 transcript after stimulation. Two of the three Con A–TCGF-stimulated CD4$^+$ T-cell clones (G2.2D7 and 99.3D1) did not express CD40 mRNA (Fig. 2). Interestingly, in a separate experiment, one clone (99.3C11) strongly expressed CD40 transcripts while a Con A–IL-2-stimulated CD4$^+$ T-cell clone (G1.1C9) weakly expressed CD40 mRNA (data not shown). CD40 transcripts were not

Table 1. Nucleotide and predicted primary amino acid sequence of the bovine CD40 homologue. PCR products isolated from cDNA library were sequenced and homology determined relative to the human CD40 homologue using FASTA algorithm. The predicted open reading frame of the protein is indicted resulting in a polypeptide of approximately 33 000 MW

1	ATGGTTCGTTTGCCACTGCAGTGTCTCTTCTGGGGCTTCTTT	42
1	M V R L P L Q C L F W G F F	14
43	CTGACCGCCGTCCACTCAGAACCAGCCACTGCTTGTGGAGAG	84
15	L T A V H S E P A T A C G E	28
85	AAGCAATACCCAGTGAACAGTCTTTGCTGTGATTTGTGCCCG	126
29	K Q Y P V N S L C C D L C P	42
127	CCGGGACAGAAACTGGTGAACGACTGCACAGAGGTCAGCAAA	168
43	P G Q K L V N D C T E V S K	56
169	ACAGAATGCCAGTCCTGCGGTAAAGGCGAATTCTTGTCCACC	210
57	T E C Q S C G K G E F L S T	70
211	TGGAACAGAGAGAAATACTGTCACGAGCACAGATACTGCAAC	252
71	W N R E K Y C H E H R Y C N	84
253	CCCAACCTAGGGCTCCGGATCCAGAGCGAGGGTACCTTGAAT	294
85	P N L G L R I Q S E G T L N	98
295	ACAGACACCATTTGTGTATGTGTCGAAGGCCAACACTGTACC	336
99	T D T I C V C V E G Q H C T	112
337	AGTCACACCTGCGAAAGTTGCACGCCCCACAGCTTGTGTCTC	378
113	S H T C E S C T P H S L C L	126
379	CCTGGCTTCGGGGTCAAGCAGATCGCTACAGGGCTTTTGGAT	420
127	P G F G V K Q I A T G L L D	140
421	ACCGTCTGTGAACCCTGCCCGCTCGGCTTCTTCTCCAACGTG	462
141	T V C E P C P L G F F S N V	154
463	TCATCTGCTTTTGAAAAGTGTCACCGTTGGACAAGCTGCGAG	504
155	S S A F E K C H R W T S C E	168
505	AGAAAAGGCCTGGTGGAACAACACGTGGGGACGAACAAGACA	546
169	R K G L V E Q H V G T N K T	182
547	GATGTTGTCTGCGGTTTCCAGAGTCGGATGAGGACCCTGGTG	588
183	D V V C G F Q S R M R T L V	196
589	GTGATCCCCGTCACGATGGGAGTCTTGTTTGCTGTCCTGTTG	630
197	V I P V T M G V L F A V L L	210
631	GTATCTGCCTGTATCAGGAACATAACCAAGAAGCGGCAGCTA	672
211	V S A C I R N I T K K R Q L	224
673	AGGCCCTGCACCCTATGGCTGAAAGGCAGGATCCCGTGGAGA	714
225	R P C T L W L K G R I P W R	238
715	CGATTGATCCGGAGGATTTTCCCGGCCCCCACCCGCCTCTCC	756
239	R L I R R I F P A P T R L S	252
757	GGTGCAAGAGACCTTATGCTGGTGTCAGCCGGTCGCCCAGGA	798
253	G A R D L M L V S A G R P G	266
799	GGACGGCAAAG	809
267	G R Q	270

expressed by unstimulated or Con A-stimulated peripheral blood T cells. The α/β T-cell clones that expressed CD40 mRNA were propagated continually in the absence of AC, ruling out the potential contribution of CD40 transcripts by B cells or other AC present in irradiated feeder cells.

Bovine CD40 ligation induces B-cell proliferation and immunoglobulin production

We investigated whether cross-linkage of boCD40 by boCD40L-transfected MOP8 cells stimulated proliferation of dense sIgM-positive B cells in the presence or absence of exogenous cytokine (rboIL-4). As shown in Fig. 3, neither isolated resting B cells nor irradiated boCD40L-transfected cells alone demonstrated significant thymidine uptake in the presence or absence of IL-4. However, when B cells were cocultured with irradiated, boCD40L-transfected MOP8 cells, a fourfold increase in proliferative responses was observed. Importantly, B cells cultured with the parental MOP8 cell line or Con A did not proliferate above background values obtained with B cells alone (data not shown). Addition of rboIL-4 to cultures of B cells and CD40L-transfected MOP8 cells further enhanced B-cell proliferation by approximately 1·6-fold.

As CD40 cross-linkage on B cells not only enhances cell proliferation and survival but also affects immunoglobulin production, the effects of coculture of IgM^+ resting B cells and boCD40L-transfected cells on secretory IgM and IgG production *in vitro* was determined. Immunoglobulin production was normalized against culture medium of CD40L-transfected cells alone. As shown in Fig. 4, B cells cultured with CD40L–MOP8 increased secretory IgM production by approximately 1·7-fold. The rboIL-4 itself can synergize with the goat anti-bovine IgM-coated magnetic beads which remain

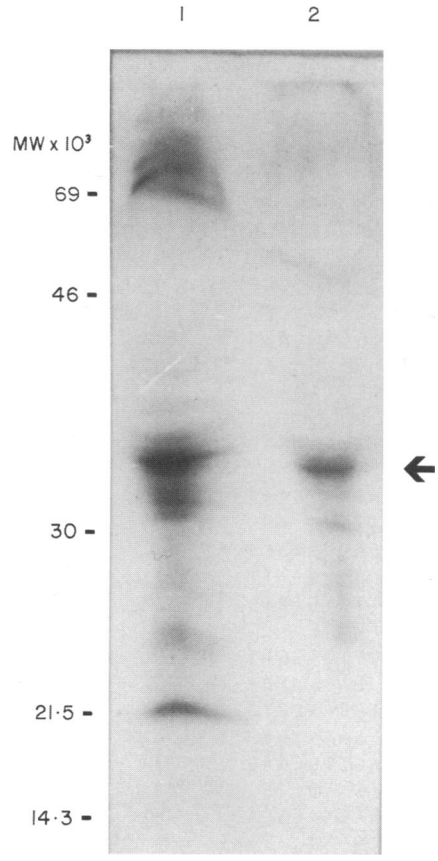

Figure 1. Immunoprecipitation of boCD40 from BL3 cell lysates using anti-boCD40 peptide serum. Lysates from BL3 cells pulsed for 3 hr with ^{35}S-labelled methionine/cysteine were incubated with rabbit anti-boCD40 peptide antiserum. immunoprecipitates were analysed under reducing conditions on 10% SDS–PAGE gels. Lane 1 represents crude lysates from BL3 cells. Lane 2 represents immunoprecipitates from BL3 cells using boCD40 peptide-specific antiserum following preclearing with normal rabbit serum. A protein of approximately 33 000 MW (arrow) was identified from BL3 lysates.

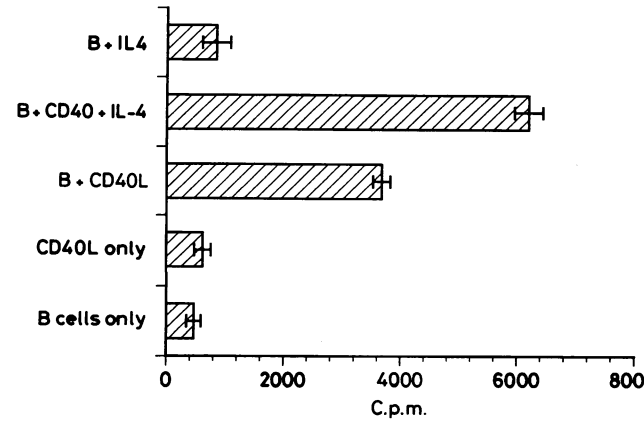

Figure 3. Induction of B-cell proliferation by IL-4 and CD40 cross-linkage by MOP8 cells expressing boCD40L. High-density enriched bovine B cells isolated over Percoll and positively selected for sIgM expression were cocultured with $1–2 \times 10^4$ irradiated (6000 rads) MOP8 cells transfected with boCD40L. The rboIL-4 was added at approximately 60 ng/ml final concentration. The cultures were harvested 5 days later after addition of [^{3}H]thymidine for the last 8 hr of culture. The values represent means $\pm$ SE of triplicate cultures.

from the purification procedure. This effect of the residual anti-IgM-coated beads is reflected in the net increase in IgM production with B cells cultured with IL-4 alone which is similar if not marginally greater than that observed for cells cocultured with boCD40L–MOP8. CD40 cross-linkage also resulted in increased production of IgG from an enriched population of sIgM-bearing cells. While coculture of B cells with rboIL-4 and the remaining anti-IgM-coated beads can increase production of IgG (215 ± 7 ng/ml versus 20 ± 1 ng/ml in control cultures with B cell only), immunoglobulin secretion was further enhanced by approximately 1·6-fold upon culture with both boCD40L–MOP8 and rboIL-4 (338 ± 20 ng/ml). Similar results have been reported by others using related assay systems with human B lymphocytes.[30]

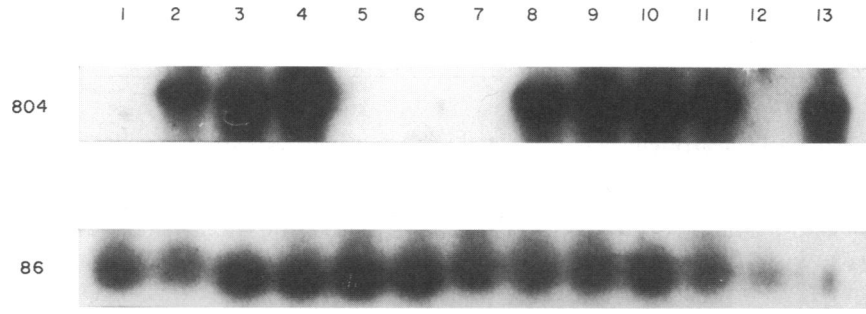

Figure 2. Bovine CD40 mRNA expression by resting and activated B and T cells. Peripheral blood-derived resting B cells and T cells were isolated as described in the Materials and Methods. B cells, T cells, or cells from the bovine leukaemic B-cell line BL3 were incubated with PMA (10 ng/ml) and ionomycin (0·5 μM) for 18 hr or left unstimulated. T-cell clones were stimulated with Con A and Il-2 for 8–22 hr. RT-PCR analysis of 1 μg of total RNA was performed using a bovine CD40 cDNA probe and specific primers (top). As a control, bovine actin primers and a bovine actin cDNA probe were used to illustrate the relative amounts of template analysed for each sample (bottom). Samples from left to right are: (lane 1) unstimulated B cells; (lane 2) activated B cells; (lane 3) unstimulated BL3 cells; (lane 4) activated BL3 cells; (lane 5) unstimulated T cells; (lane 6) Con A-stimulated T cells; (lane 7) CD4$^+$ T-cell clone (G2.2D7); (lane 8) CD8$^+$ T-cell clone (G2.1G8); (lane 9) CD8$^+$ T-cell clone (99.G1.G3); (lane 10) CD8$^+$ T-cell clone (G2.3E4); (lane 11) CD8$^+$ T-cell clone (99.2G7); (lane 12) CD4$^+$ T-cell clone (99.3D1); (lane 13) γ/δ T-cell clone (G8.1H11).

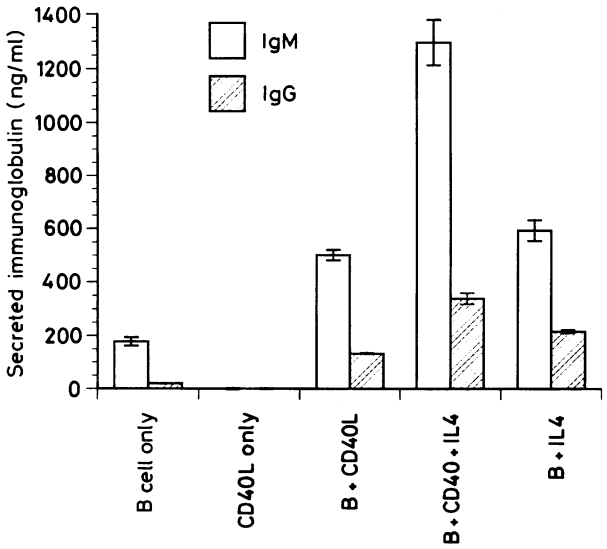

Figure 4. Induction of immunoglobulin synthesis by coculture of bovine B cells with IL-4 and MOP8 cells expressing bovine CD40L. Enriched $sIgM^+$ resting peripheral blood-derived B cells were cultured alone or with MOP8 cells (10^4 cells/well) transfected with boCD40L. The rboIL-4 (60 ng/ml) was added as indicated. Culture supernatant were harvested at 6–7 days of culture and analysed by capture ELISA for IgM and IgG levels relative to bovine immunoglobulin standards (Sigma). Results are presented as the mean ± SE of triplicate culture. Immunoglobulin profiles are representative of three separate experiments.

DISCUSSION

In the present study we demonstrate that the bovine CD40 homologue is relatively conserved by comparison with its human counterpart in both sequence and function (73% at nucleic acid level, 69% at amino acid level). The extracellular domain of the bovine CD40 molecule contains 12% cysteine residues suggesting that the four cysteine-rich domains found in the human CD40 molecule are conserved in ruminants. Based on the conservation of structure in the intracellular and extracellular domains of the molecule, we predicted that the function of boCD40 would be similar to that described in humans.

B-cell proliferation induced by CD40L–MOP8 was enhanced by IL-4. This is similar to previous findings with T-independent activation systems, where rboIL-4 has been shown to have B-cell growth-promoting activity.[19] Coculture of a CD40L–MOP8 with high-density surface IgM-expressing bovine B cells in the presence of IL-4-enhanced IgM and IgG production. This is consistent with numerous reports in human systems in which CD40 cross-linkage combined with exogenous cytokines known to promote B-cell growth and differentiation, induced enhanced expression of secreted immunoglobulin *in vitro* (for review see refs. 1 and 3). Cognate T-cell help provided via CD40L is therefore an important component of B-cell activation, proliferation and differentiation in cattle.

Of considerable interest is the potential for expression of CD40 on $CD4^+$ T cells, $CD8^+$ T cells and γ/δ T cells. The expression of CD40 on T cells in cattle may have an impact on the development of physical interaction of antigen-specific cells by enhancement of cell contact via the formation of both heterotypic and homotypic aggregates. Receptor regulatory mechanisms which facilitate the formation of ligand–receptor interactions across relatively large membrane surface areas may be advantageous to reaching a signalling threshold necessary to up-regulate the expression of downstream cytokine receptors and other molecules such as CD28–CD80/CD86 which serve important roles in costimulation. CD40 ligation as an early signalling event has been shown to improve accessibility to switch regions and their associated heavy-chain gene segments in mice and therefore serves as a potent initiator of B-cell differentiation to the effector cell stage.[31] Importantly, the binding of additional molecules which may play accessory roles in early T–B interactions were not considered here as we investigated signalling mediated via CD40 alone. We are presently investigating the role of cytokines such as interferon-γ (IFN-γ) and TGF-β in immunoglobulin isotype switching using CD40 cross-linkage for costimulation. Previous studies in our laboratory demonstrated that cytokines such as IFN-γ can up-regulate isotype expression by bovine B cells stimulated with T-cell-independent activators such as *Staphylococcus aureus* Cowan strain I (SAC).[22] Together, these results extend our present understanding of T-cell-dependent versus T-cell-independent activation pathways and cytokine control of B-cell differentiation in cattle.

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