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Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34

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A ligand was cloned for murine OX40, a member of the TNF receptor family, using a T cell lymphoma cDNA library. The ligand (muOX40L) is a type II membrane protein with significant identity to human gp34 (gp34), a protein whose expression on HTLV-1-infected human leukemic T cells is regulated by the tax gene. The predicted structures of muOX40L and gp34 are similar to, but more compact than, those of other ligands of the TNF family. Mapping of the muOX40L gene revealed tight linkage to *gld*, the FasL gene, on chromosome 1. gp34 maps to a homologous region in the human genome, 1q25. cDNAs for human OX40 receptor were cloned by cross-hybridization with muOX40, and gp34 was found to bind the expressed human receptor. Lymphoid expression of muOX40L was detected on activated T cells, with higher levels found on $CD4^+$ rather than $CD8^+$ cells. The cell-bound recombinant ligands are biologically active, co-stimulating T cell proliferation and cytokine production. Strong induction of IL-4 secretion by muOX40L suggests that this ligand may play a role in regulating immune responses. In addition, the HTLV-1 regulation of gp34 suggests a possible connection between virally induced pathogenesis and the OX40 system.

Key words: HTLV-1/ligand/receptor/T cell (lymphocytes)/TNF

Introduction

The OX40 molecule was originally described as a cell-surface antigen found on activated rat T cells (Paterson *et al.*, 1987). The gene encoding rat OX40 was subsequently cloned and predicted to belong to the TNF receptor family due to sequence similarity with other members (Mallet *et al.*, 1990). These studies of rat OX40 and others with its murine homologue, muOX40 (Calderhead *et al.*, 1993), demonstrated that expression of the molecule appeared to be restricted to activated $CD4^+$ T lymphocytes. Although human OX40 (huOX40) had not been described at the outset of this work, cDNA clones with substantial homology to the rodent receptors have been reported recently (Godfrey *et al.*, 1993; Latza *et al.*, 1994). Little is known about the function of OX40 or its ligand(s).

To define an OX40 ligand (OX40L) and begin an analysis of its biological function, we adopted an expression cloning strategy using as a detection reagent the extracellular domain of muOX40 fused to a modified Fc region from human IgG1. Here we report the cloning of murine OX40L (muOX40L) and its similarity to human gp34 (gp34), a protein whose expression on T cells is regulated by the tax gene of the pathogenic human retrovirus HTLV-1 (Miura *et al.*, 1991). In addition, we report cloning and preliminary characterization of huOX40. The human receptor gene was used in transfection experiments to demonstrate that gp34 is a human OX40L. We also show that the recombinant ligands from both species are biologically active. Both gp34 and muOX40L generate a strong proliferative response by human T cells during co-stimulation, whereas only the murine ligand co-stimulates murine T cells to proliferate. We also have investigated the induction of cytokine secretion in the murine system and found that T cells co-stimulated with muOX40L secrete high levels of IL-2 and IL-4.

Results

Molecular cloning and biochemical characterization of murine OX40 ligand

To identify cells expressing OX40L, muOX40–Fc and muOX40–Fc mutein, soluble forms of muOX40 receptor were prepared (see Materials and methods). The fusion proteins were found to bind specifically to S49.1 cells, a murine lymphoma cell line, and binding was increased by phorbol myristic acetate (PMA) stimulation (Figure 1B and D). A cDNA library was prepared by isolating RNA from stimulated S49.1 cells, and muOX40L was cloned by a slide-binding autoradiographic method (Gearing *et al.*, 1989; Goodwin *et al.*, 1993). The use of muOX40–Fc mutein as a binding reagent was essential for successful cloning of muOX40L with this sensitive technique. The mutationally altered Fc portion of human immunoglobulin γ1 heavy chain in this fusion protein lowered binding to Fc receptors encoded by cDNAs in the library, thereby allowing detection of the expressed muOX40L. The cloning involved transfection of cells on slides with DNA from clone pools and detection of muOX40L expression by a two-step binding method utilizing muOX40–Fc

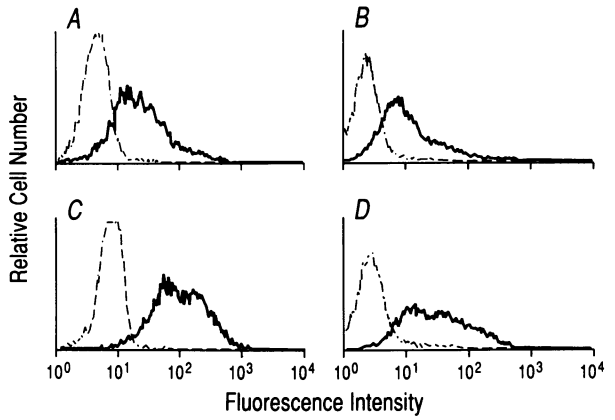

Fig. 1. Cell-surface expression of murine OX40L. Unstimulated S49.1 cells were stained with (**A**) muOX40−Fc mutein (solid line) or a control huIL-4R−Fc (dotted line) and (**B**) muOX40−Fc (solid line) or huIL-4R−Fc (dotted line). S49.1 cells stimulated for 18 h with PMA (**D**) were stained as described in (B) and OX49.4 cells (**C**) were stained as described in (A).

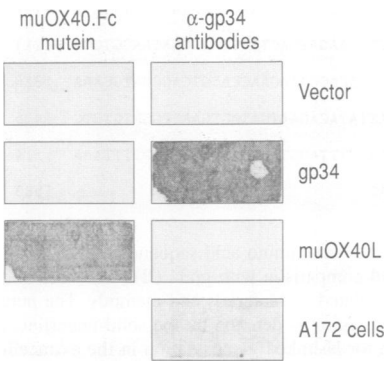

Fig. 2. Binding of muOX40−Fc mutein to cells transfected with muOX40L and human gp34. Slides containing adherent CV-1/EBNA cells were co-transfected with 500 ng of plasmid pSV3neo (Mulligan and Berg, 1981) and either 500 ng of pDC409 vector, 500 ng pDC409 containing a full-length gp34 cDNA or 2 ng pDC409 containing full-length muOX40L cDNA. Two days after transfection, binding was performed with either muOX40−Fc mutein followed by ^{125}I-labelled murine anti-human IgG F(ab)$_2$ fragment (first column), or a mixture of the anti-gp34 antibodies TAG34 and 6D1 followed by ^{125}I-labeled sheep anti-mouse IgG F(ab)$_2$ antibodies (second column). The slides were placed overnight on cassettes prior to analysis by a phosphoimager (Molecular Dynamics). Shown are the results with a grey scale setting of 0–5000 pixels. Included is a slide containing adherent A172 cells (last slide column 2) which served as a positive control for the anti-gp34 antibodies (Miura *et al.*, 1991). muOX40−Fc mutein was used at 20 μg/ml on the slides with vector and gp34 transfected cells, and at 1 μg/ml for slides with muOX40L transfected cells. To verify that the observed signal was due to binding to cells, all slides were dipped in autoradiographic emulsion, processed and viewed by a microscope as described previously (Gearing *et al.*, 1989).

mutein as the first step. Positive pools were subdivided and the procedure was repeated with sub-pool DNAs until single cDNAs conferring binding were identified (Figure 2).

The recombinant protein encoded by the longest cDNA, 69-9-2, was compared with the natural OX40L by affinity precipitation of labelled cell-surface proteins from CV-1/Epstein−Barr virus nuclear antigen (EBNA) cells transfected with the cDNA and from OX49.4 cells. (The OX49.4 line was derived from S49.1 cells by four rounds

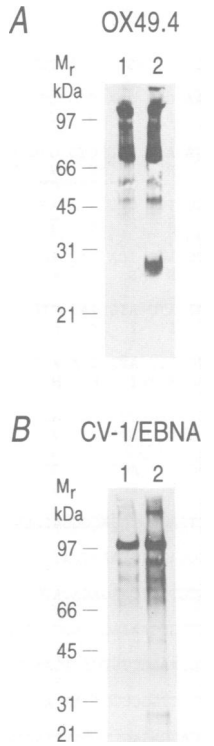

Fig. 3. Bioaffinity precipitation of muOX40L protein using soluble muOX40−Fc mutein. Ox49.4 cells (**A**) or CV-1/EBNA cells (**B**) expressing the muOX40L protein were surface-labelled as described in Materials and methods. Lysates were precipitated using CD40−Fc (lane 1) or OX40−Fc (lane 2) and protein A/G agarose, and analysed under reducing conditions on a 12.5% polyacrylamide−SDS gel.

of FAC sorting for increased OX40L expression using binding to muOX40−Fc mutein and having an ~5- to 10-fold increase in fluorescent staining; Figure 1C verses A.) When compared with a control Fc fusion protein, CD40−Fc, muOX40−Fc mutein specifically precipitated a M_r 26–28 kDa protein from both OX49.4 and the transfected CV-1/EBNA cells (Figure 3). This indicates that the recombinant protein encoded by the isolated cDNA is that detected on the lymphoma cells (Figure 3A and B).

Structure of muOX40L and a related human protein, gp34

The DNA sequence of cDNA clone 69-9-2 was found to consist of a 1586 bp insert with a single long open reading frame (ORF) encoding a 198 amino acid protein (Figure 4A). The encoded protein is predicted to be a type II membrane glycoprotein with a 28 amino acid cytoplasmic region, a 20 amino acid hydrophobic transmembrane domain and a 150 amino acid C-terminal extracellular domain with a single consensus site for N-linked glycosylation and four cysteine residues. The slightly higher apparent molecular weight of the protein precipitated by muOX40−Fc (26–28 kDa) than that predicted from the amino acid sequence (22 kDa) suggests that the N-linked glycosylation site is utilized by mammalian cells (Figure 3A and B).

When compared with sequences in the GENBANK database, significant identity for the nucleotide and predicted protein sequences was found to a cDNA encoding

A

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AGGCTTGACTTTGCCCTTATTGGCTCCTTTGTGGTGAAGAGCAGTCTTCCCCCAGGTTCCCCGCCACAGCTGTATCTCCTCTGCACCCCGACTGCAGAG   122

ATG GAA GGG GAA GGG GTT CAA CCC CTG GAT GAG AAT CTG GAA AAC GGA TCA AGG CCA AGA TTC AAG TGG AAG AAG   197
M  E  G  E  G  V  Q  P  L  D  E  N  L  E  N  G  S  R  P  R  F  K  W  K  K                          25

ACG CTA AGG CTG GTG GTC TCT GGG ATC AAG GGA GCA GGG ATG CTT CTG TGC TTC ATC TAT GTC TGC CTG CAA CTC   272
T  L  R  L  V  V  S  G  I  K  G  A  G  M  L  L  C  F  I  Y  V  C  L  Q  L                          50

TCT TCC TCT CCG GCA AAG GAC CCT CCA ATC CAA AGA CTC AGA GGA GCA GTT ACC AGA TGT GAG GAT GGG CAA CTA   347
S  S  S  P  A  K  D  P  P  I  Q  R  L  R  G  A  V  T  R  C  E  D  G  Q  L                          75

TTC ATC AGC TCA TAC AAG AAT GAG TAT CAA ACT ATG GAG GTG CAG AAC AAT TCG GTT GTC ATC AAG TGT GAT GGG   422
F  I  S  S  Y  K  N  E  Y  Q  T  M  E  V  Q  N  N  S  V  V  I  K  C  D  G                         100

CTT TAT ATC ATC TAC CTG AAG GGC TCC TTT TTC CAG GAG GTC AAG ATT GAC CTT CAT TTC CGG GAG GAT CAT AAT   497
L  Y  I  I  Y  L  K  G  S  F  F  Q  E  V  K  I  D  L  H  F  R  E  D  H  N                         125

CCC ATC TCT ATT CCA ATG CTG AAC GAT GGT CGA AGG ATT GTC TTC ACT GTG GTG GCC TCT TTG GCT TTC AAA GAT   572
P  I  S  I  P  M  L  N  D  G  R  R  I  V  F  T  V  V  A  S  L  A  F  K  D                         150

AAA GTT TAC CTG ACT GTA AAT GCT CCT GAT ACT CTC TGC GAA CAC CTC CAG ATA AAT GAT GGG GAG CTG ATT GTT   647
K  V  Y  L  T  V  N  A  P  D  T  L  C  E  H  L  Q  I  N  D  G  E  L  I  V                         175

GTC CAG CTA ACG CCT GGA TAC TGT GCT CCT GAA GGA TCT TAC CAC AGC ACT GTG AAC CAA GTA CCA CTG TGA ATT   722
V  Q  L  T  P  G  Y  C  A  P  E  G  S  Y  H  S  T  V  N  Q  V  P  L  *                            198

CCACTCTGAGGGTGGACGGGACACAGGTTCTTTCTCGAGAGAGATGAGTGCATCCTGCTCATGAGATGTGACTGAATGCAGAGCCTACCCTACTTCCTC   821

ACTCAGGGATATTTAAATCATGTCTTACATAACAGTTGACCTCTCATTCCCAGGATTGCCTTGAGCCTGCTAAGAGCTGTTCTGGGAATGAAAAAAAAA   920

TAAATGTCTCTTCAAGACACATTGCTTCTGTCGGTCAGAAGCTCATCGTAATAAACATCTGCCACTGAAAATGGCGCTTGATTGCTATCTTCTAGAATT  1019

TTGATGTTGTCAAAAGAAAGCAAAACATGGAAAGGGTGGTGTCCACCAGCCAGTAGGAGCTGGAGTGCTCTCTCCAGGTTAAGGTGATAGAAGTTTACA  1118

TGTTGCCTAAAACTGTCTCTCATCTCATGGGGGGCTTGGAAAGAAGATTACCCCGTGGAAAGCAGGACTTGAAGATGACTGTTTAAGCAACAAGGTGCA  1217

CTCTTTTCCTGGCCCCTGAATACACATAAAAGACAACTTCCTTCAAAGAACTACCTAGGGACTATGATACCCACCAAAGAACCACGTCAGCGATGCAAA  1316

GAAAACCAGGAGAGCTTTGTTTATTTTGCAGAGTATACGAGAGATTTTTACCCTGAGGGCTATTTTTATTATACAGAATGATAGTGAACTGGATGTCTC  1415

AGGATAAAGGCCAAGAAGGATTTTTCACAGTCTGAGCAAGACTGTTTTTGTAGGTTTCTCTCTCCAAAACTTTTAGGTAAATTTTTGATAATTTTTAAA  1514

TTTTTATATTTTTGGACCATTTTCAATAGAAGATTGAAACATTTCCAGATGGTTTCATATCCCCACAAG                                1583
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B

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  1 MEGEGVQPLDENLENGSRPRFKWKKTLRLVVSGIKGAGMLLCFIYVCLQL  50
    |  ||||:||::|:.||||.:.| | ||.|.|.| |:||||.|:||::
  1 ..MERVQPLEENVGNAARPRFERNKLL.LVASVIQGLGLLLCFTYICLHF  47

 51 SSSPAKD..PPIQRLRGAVTRCEDGQLFISSYKNEYQTMEVQNNSVVIKC  98
    |. ....  |.||.::...| :..:. || . ..| :.|.||||||:|.||
 48 SALQVSHRYPRIQSIKVQFTEYKKEKGFILTSQKEDEIMKVQNNSVIINC  97

 99 DGLYIIYLKGSFFQEVKIDLHFREDHNPISIPMLNDGRRIVFTVVASLAF 148
    ||:|:| ||| | |||.|.||:..|.:|:    |...|.:   :||||.:
 98 DGFYLISLKGYFSQEVNISLHYQKDEEPLF..QLKKVRSVNSLMVASLTY 145

149 KDKVYLTVNAPDTLCEHLQINDGELIVVQLTPG.YCAPEGSYHSTVNQVP 197
    ||||||.|...:|  :.:::|:||||::: .|| :||.
146 KDKVYLNVTTDNTSLDDFHVNGGELILIHQNPGEFCVL..............  183
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Fig. 4. Nucleotide and amino acid sequence of muOX40L cDNA (**A**) and amino acid comparison with gp34 (**B**). (A) Nucleotide sequence was derived as stated in Materials and methods. The putative transmembrane region is denoted by the solid underline, and the single consensus site for N-linked glycosylation in the extracellular region is shaded. (B) Amino acid sequences of muOX40L and gp34 were compared using the GCG program GAP (University of Wisconsin, Madison, WI). Vertical lines between sequences indicate identity, while double or single dots between the two sequences indicate conserved or similar residues, respectively. The boxed cysteine residues conserved in each molecule are predicted to form a disulfide bond (see Figure 5 and Results). GenBank accession number for this sequence is U12763.

a human protein, gp34, a predicted type II membrane protein of 183 amino acids (Miura *et al.*, 1991). The identities over the entire ORF of the smaller gp34 consisted of 68% at the nucleotide level and 46% at the amino acid level (Figure 4B). The overall amino acid similarity was 67%.

Because rat OX40 shares a cysteine-rich extracellular domain structure with other TNF receptor family members, we investigated whether the tertiary structures of muOX40L and gp34 are similar to that of TNF. The crystal structure of TNF is known, and its tertiary structure consists of β-extended regions wrapped in the form of a jelly roll (Eck and Sprang, 1989). Using TNF as a template, as well as comparison with other ligands for the TNF receptor family, homology-based secondary structures (Rost and Sander, 1992) for muOX40L and gp34 were generated (Figure 5, left panel). The predictions suggest that like other family members, both muOX40L and gp34 consist of β-extended regions connected by short loops. However, the two proteins are unique in that they do not share most amino acid motifs conserved in the other ligand family members. muOX40L and gp34 do, however, contain a signature region common to all members of this family of proteins in the D strand forming the central buried β-sheet (Figure 5, right panel).

The 3-D models generated for muOX40L and gp34 also show considerable differences from that of TNF, in spite of folding it on the TNF template. muOX40L and gp34 still possess extended β-segments but have fewer amino acids in the loops facing away from the membrane that connect segments D−E and F−G. Therefore, the predicted tertiary structure is much more compact than the elongated structure of TNF (Figure 5, right panel, green trace). The topology of TNF is such that the residues at the C-terminus are in close proximity with the residues at the beginning of the folding domain. Among membrane-bound family members, muOX40L and gp34, in particular, are predicted to have few amino acids between those in the transmembrane domain and those beginning the folding domain, leaving little room for a long unstructured C-terminal region near the beginning of the domain. Instead, our modelling predicts that the C-termini of muOX40L and gp34 fold back onto the jelly roll to form a disulfide bond that crosslinks the conserved cysteine after the I β-

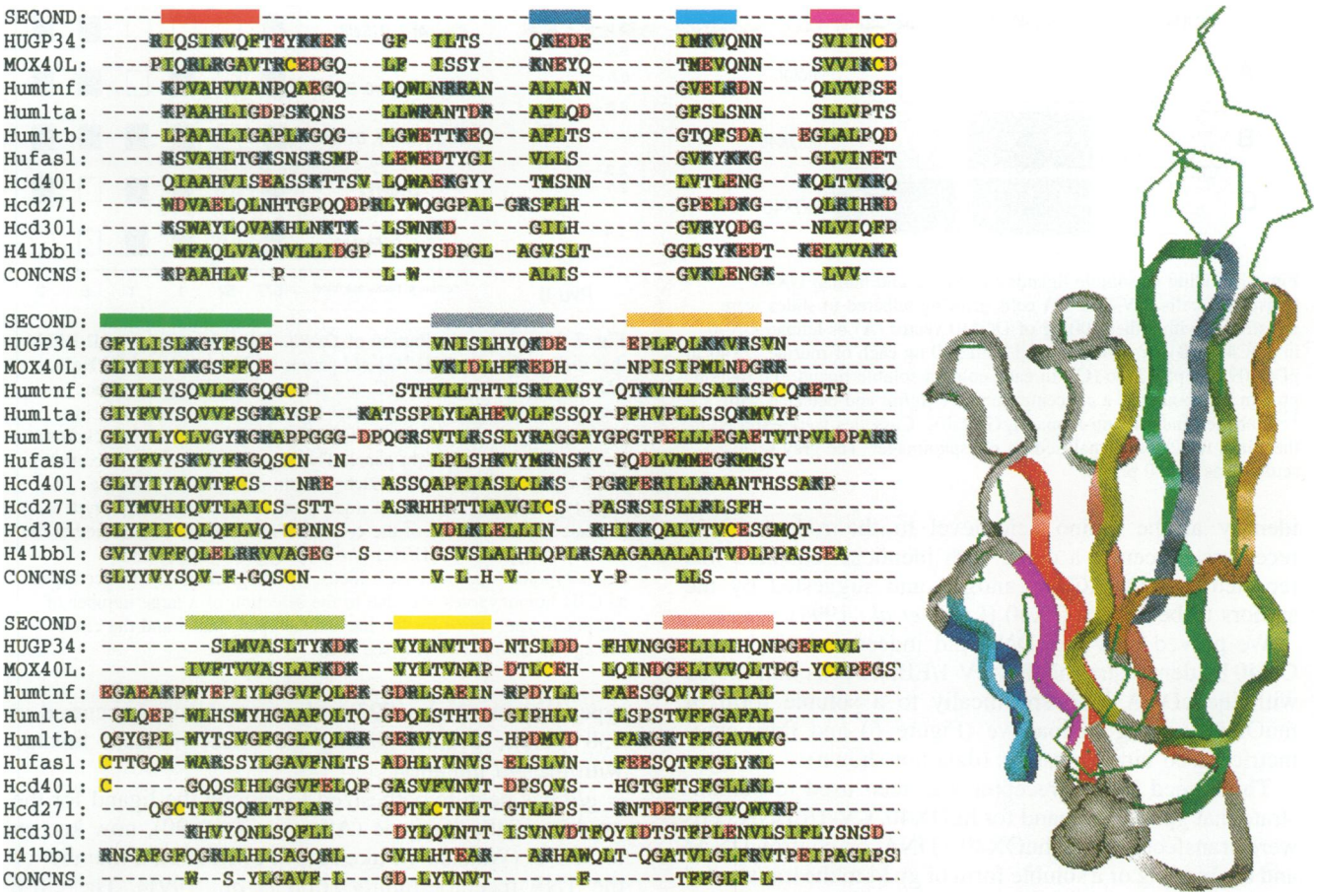

Fig. 5. Family sequence alignment and predicted secondary structure of gp34/muOX40L (left) and the predicted tertiary structure of gp34 (right). On the left, predicted extended β-strands for ligands [B, b′, c′, C-I in the terminology of Eck and Sprang (1989)] are shown by coloured bars on the top line (SECOND) over the alignment of the OX40Ls with human members of the TNF family. Conserved amino acid motifs are shown by the line CONCNS. Amino acids are colour-coded as follows: green, hydrophobic; blue, basic; red, acidic; and yellow, Cys. On the right, a model of a gp34 molecule (ribbons) superimposed on the crystal structure of TNF (green trace). β-Strands are coloured to correspond with the appropriate strand in SECOND in the left panel, while loops between strands are grey. The predicted disulfide bond at the bottom of the molecule is shown in CPK.

strand with the conserved cysteine at the end of the C strand (Figure 5, right panel).

TNF and the closely related LTα exist as trimers and it has been shown recently that the LTα trimer interacts with three receptor chains (Banner *et al.*, 1993). In spite of the weak primary sequence homologies with TNF/LTα, the conservation in the buried signature region and the hydrophobic nature of the amino acids for muOX40L or hugp34 (Figure 5B) at regions analogous to those in TNF/LTα at the trimer interface suggests that they may also oligomerize as trimers.

gp34 is an OX40L

To test the possibility that gp34 is a human OX40L, a cDNA encoding gp34 was cloned into an expression vector, transfected into cells and examined for binding of muOX40−Fc fusion protein to the cells. No binding was detectable using the slide-based (Figure 2, column 1) or flow cytometry-based (data not shown) binding assays. This was the case even though high concentrations of the muOX40−Fc fusion protein were used and the transfected cells were shown to be expressing high levels of gp34 with the anti-gp34 monoclonal antibodies TAG34 and 6D1 (Figure 2, column 2) (kindly provided by Dr Kazuo Sugamura, Tohoku University, Sendai, Japan) (Tanaka *et al.*, 1985; Tozawa *et al.*, 1988; Miura *et al.*, 1991).

Because there is precedence within the TNF family that a ligand from one species does not bind, or binds poorly, to its cognate receptor(s) from another species (Smith *et al.*, 1990; Alderson *et al.*, 1994), we cloned the previously undescribed human OX40 receptor to test better the possibility that gp34 binds OX40. Rodent OX40 receptors are expressed on activated T cells; therefore, using riboprobes consisting of the coding region of muOX40 we performed Northern analysis on RNAs derived from human peripheral blood lymphocytes (PBL) or peripheral blood T (PBT) cells that had been activated with PMA and ionomycin. Strong hybridization was detected to an ~1.5 kb mRNA species even under stringent conditions (Figure 10). To clone a cDNA representing this RNA, pools of cDNA clones from a library constructed with the PBL RNA were screened with radioactive muOX40 probes by blot hybridization. Single clones were then isolated from positive pools by colony hybridization with the muOX40 probe. When the cDNAs from these clones were analysed by DNA sequencing a single long ORF of 274 amino acids was found, resembling a typical type I transmembrane protein with highly significant 63%

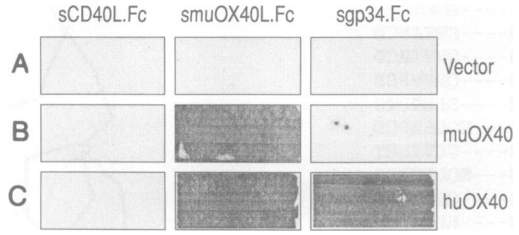

Fig. 6. Binding of soluble ligands by murine and human OX40 transfected cells. CV-1/EBNA cells growing adhered to slides were transfected with either 500 ng of DC410 vector (**A**) or human OX40 in pDC410 (**B**), or co-transfected with 500 ng each of murine OX40 in pDC409 and pSV3neo (**C**). In each column soluble ligand−Fc mutein protein was bound at a concentration of 1 μg/ml and detected with ^{125}I-labelled murine anti-human IgG F(ab)$_2$. Cassettes were exposed to the slides for 2 h and analysed by phosphoimager. The grey scale setting was 0–250 pixels.

identity at the amino acid level to the rodent OX40 receptors. Recently a clone with identical sequence was reported for the ACT35 antigen and suggested by the authors to be human OX40 (Latza *et al.*, 1994).

We proved that the cDNA did indeed encode human OX40 by demonstrating that CV-1/EBNA cells transfected with the cDNA bind specifically to a soluble form of muOX40L using radioactive (Figure 6) and flow cytometric-based binding assays (data not shown).

The cloned human receptor was then used to demonstrate that gp34 is a ligand for huOX40. CV-1/EBNA cells were transfected with huOX40 cDNA or control DNAs and the binding of a soluble form of gp34 to the transfected cells was examined. Soluble gp34 bound specifically to the huOX40 transfected cells but not to the muOX40 transfected cells (Figure 6), demonstrating that gp34 is a human OX40L and that its binding, unlike that of muOX40L, is species-restricted.

muOX40L maps near the gld gene and gp34 maps to a homologous region

To determine the chromosomal location of the *Ox40l* gene we analysed a panel of DNA samples from an interspecific cross that has been characterized for over 700 genetic markers throughout the genome. Initially, DNA from the two parental mice [C3H/HeJ-*gld* and (C3H/HeJ-*gld*×*Mus spretus*)F$_1$] was digested with various restriction endonucleases and hybridized with the cDNA probes to determine restriction fragment length variants to allow haplotype analyses. Informative RFLVs were *Pvu*II-restricted parental DNAs; C3H/HeJ-*gld*, 1.7 kb; *M.spretus*, 3.8 and 1.4 kb (Figure 7).

Comparison of the haplotype distribution of the *Ox40l* with those determined for loci throughout the mouse genome indicated that in all the 638 meiotic events examined this locus co-segregated with the markers *D1Sel10* and *D1Sel11* (Figure 7), loci previously mapped to distal mouse chromosome 1 (Hunter *et al.*, 1993). The best gene order (Bishop, 1985) ± the standard deviation (Green, 1981) indicated the following relationships: (centromere) *At-3/D1Sel8*, 0.16 ± 0.16 cM; *Ox40l/D1Sel10/D1Sel11*, 0.16 ± 0.16 cM; *D1Mit106/D1Mit107*, 0.78 ± 0.35 cM; *D1Sel12*, 0.31 ± 0.22 cM; *Sell/D1Mit108*. In addition, in backcross mice that had the *gld/gld* phenotype (Watson *et al.*, 1992) only one crossover occurred in 638 meiotic events, placing the *Ox40l* in close linkage with the *gld* mutation.

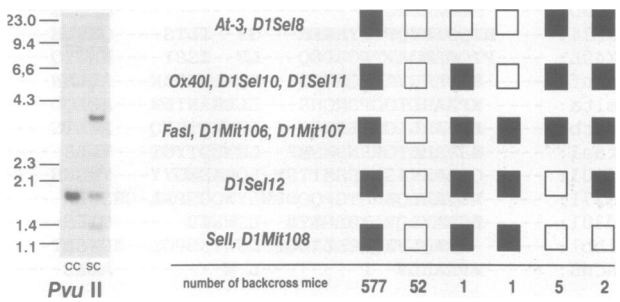

Fig. 7. Haplotype distribution of *Ox40l*. The informative *Pvu*II RFLV that distinguish the C3H/HeJ-*gld* parent (CC) from the (C3H/HeJ-*gld*×*M.spretus*)F$_1$ parent (SC) are shown in the left panel. The segregation of *Ox40l* among distal mouse chromosome 1 loci in [(C3H/HeJ-*gld*×*M.spretus*)F$_1$×C3H/HeJ-*gld*] interspecific backcross mice is shown in the right panel. The loci are listed from proximal to distal on the left-hand side. Each column represents a possible haplotype, and the number of mice observed with each haplotype is indicated at the bottom of the column. The boxes indicate whether the mice were typed as C3H/HeJ-*gld* homozygotes (filled) or F$_1$ heterozygotes (open) for each locus. The large number of mice typed as C3H homozygotes was due to the selection of a large number of *gld* phenotype$^+$ mice for the backcross typing panel and the close linkage of *Ox40l* with *gld* (see text).

gld has been hypothesized to encode the ligand for the product of the *lpr* gene (Allen *et al.*, 1990), now known to be Fas (Watanabe-Fukunaga *et al.*, 1992), a member of the TNF receptor family (Itoh *et al.*, 1991). The close linkage of muOX40L to *gld* raised the possibility that this ligand is encoded by *gld*. However, the single crossover between the ligand and the mutation, and our findings of no defects in the expression of muOX40L mRNA or in the coding region (data not shown), rule out muOX40L as the product of the *gld* gene. Indeed, *gld* was shown recently to encode a distinct TNF ligand family member, FasL, which is defective in *gld* mice (Lynch *et al.*, 1994; Takahashi *et al.*, 1994). The single crossover for *gld* and *Ox40l* in the 638 meiotic events examined indicates the close linkage of these two genes.

To map gp34 in the human genome, a biotin-labeled probe covering the coding region and the FISH technique were employed on metaphase chromosomes from two normal males. Most metaphases showed signal on one or both chromatids of chromosome 1 in the region 1q22−q31.1; 71% of this signal was at 1q25 (data not shown). Previous work has shown this region in the human genome to be homologous with the portion of mouse chromosome 1 bearing the *OX40L* locus (Watson *et al.*, 1992). This suggests that gp34 is the homologue of muOX40L.

Lymphocytic expression of muOX40L and muOX40

Using flow cytometry, cell-surface expression of muOX40L was measured by the binding of muOX40−Fc mutein, and of muOX40 by the binding of a soluble form of muOX40L (muOX40L−Fc). It has been reported previously that expression of the rodent OX40 receptors is restricted to activated CD4$^+$ T cells, so we began our investigations by examining ligand expression in murine splenocytes and thymocytes with or without brief treatment

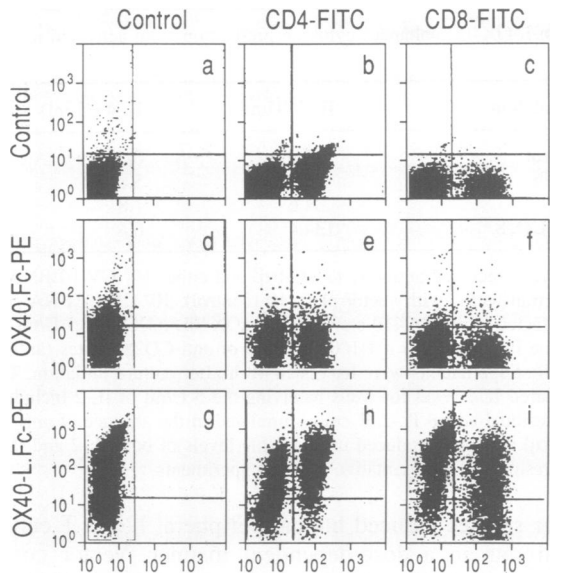

Fig. 8. Expression of OX40 ligand and OX40 receptor on activated murine T cells. Purified C57BL/6 splenic T cells were cultured for 18 h in medium with 1 μg/ml immobilized TCRαβ mAb. No detectable ligand or receptor expression was observed on cells cultured in medium alone (data not shown). To detect ligand, the activated T cells were double-stained with OX40−Fc mutein and either IgG1-FITC (**d**), CD4-FITC (**e**) or CD8-FITC (**f**). To detect receptor, the cells were double-stained with OX40L−Fc and either IgG1-FITC (**g**), CD4-FITC (**h**) or CD8-FITC (**i**). As controls, cells were double-stained with HuIL-4R−Fc and either control IgG1-FITC (**a**), CD4-FITC (**b**) or CD8-FITC (**c**). Binding of Fc fusion proteins was detected with an anti-huFc-biotin Ab followed by streptavidin-phycoerythrin (PE). Results are representative of the three experiments performed.

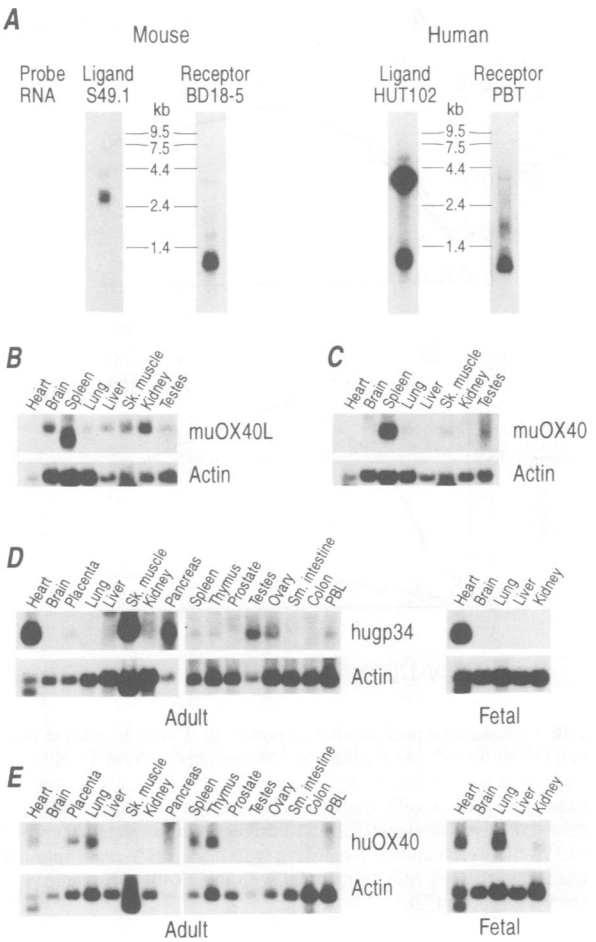

Fig. 9. Analysis of ligand and receptor mRNAs by Northern blot. (**A**) Autoradiographic results for gel lanes containing 2 μg of poly(A)$^+$ RNA (sources indicated on line two) after hybridization with radioactive riboprobes (from cDNAs indicated on the first line). RNA sizes were determined by comparison with lanes with 2 μg marker RNAs (Gibco-BRL, Gaithersburg, MD). (**B**−**E**) Blots containing RNA from the indicated tissues (⩾2 μg/lane) were hybridized with riboprobes generated from the cDNA indicated to the right of the autoradiographic results. Bands of appropriate size, as determined from (A), are shown. All exposures are overnight, except that filters probed with muOX40L were exposed for 4 days. To determine relative RNA levels for lanes on blots, filters were probed with a human β-actin probe and the film was exposed for 2 h.

with anti-TCRαβ antibodies. Although undetectable on resting cells, both the ligand and receptor were found on splenocytes and lymph node cells following activation (data not shown). Detectable expression of the ligand was found to be restricted to T cells in the spleen, as muOX40−Fc mutein binding was observed only on purified splenic T cells activated with CD3 mAb or PMA and ionomycin, but not on purified splenic B cells even after activation with lipopolysaccharide (LPS; data not shown). Therefore, as with the receptor, expression of OX40L is induced on activated T cells.

To determine whether muOX40L expression was restricted to either the CD4$^+$ or the CD8$^+$ T cell subset, two-colour flow cytometry was performed on activated splenic T cells by staining with muOX40−Fc and either anti-CD4 or anti-CD8 mAbs. Similarly, to determine the levels of muOX40 on the T cell subsets, staining with soluble muOX40L−Fc in conjunction with either anti-CD4 or anti-CD8 mAbs was analysed. The results demonstrated that detectable levels of muOX40L were found on some CD4$^+$ and CD8$^+$ T cells, although the levels on CD8$^+$ cells tended to be lower (Figure 8e and f). As expected, the receptor was found on most activated CD4$^+$ cells, but more surprisingly we found a large subpopulation of CD8$^+$ cells (60%) that had detectable levels of OX40 (Figure 8h and i). The levels of staining on the CD8$^+$ cells were ~5- to 10-fold lower than those found on CD4$^+$ cells.

Expression of the ligands and receptors in other tissues

To investigate expression of muOX40L and muOX40 in other tissues, blot hybridization was performed on a panel of poly(A)$^+$ RNAs from various murine tissues. In the case of muOX40L, hybridization was found to either an ~2.5 kb mRNA, a 3.0 kb mRNA or a combination of the two in all the tissues examined (Figure 9B). (These mRNAs correspond in size to the two muOX40L mRNAs detected in S49.1 cells; Figure 9A.) muOX40L mRNA was detected in all tissues examined but, except for spleen, the levels were quite low. On the other hand, murine receptor mRNA was found only in spleen, testes, lung and skeletal muscle (Figure 9C).

Similar blot hybridization analysis was performed for gp34 and huOX40 using a panel of mRNAs from various

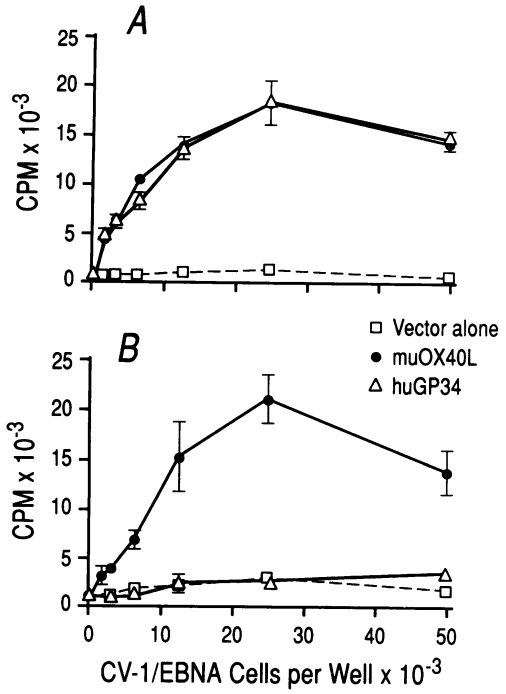

Fig. 10. Comparative proliferative responses of T cells to murine and human OX40 ligand. (**A**) Purified human peripheral blood T cells (1×10^5/well) were cultured for 3 days with 0.1% PHA and a titration of fixed CV-1/EBNA cells transfected with either muOX40L (●), hugp34 (Δ) or vector alone (□). (**B**) Purified murine splenic T cells (2×10^5/well) were cultured for 3 days with 0.5% PHA and a titration of fixed CV-1/EBNA cells transfected with muOX40L (●), hugp34 (Δ) or vector alone (□).

adult and fetal organs. In the case of gp34, two mRNAs of different size were found that correspond to those shown previously to encode the same gp34 protein and differ only in their 3′ untranslated region (Miura *et al.*, 1991; Figure 9A). We found high levels of expression of the 1.3 kb mRNA in heart (both adult and fetal), skeletal muscle and pancreas with lower levels in placenta. Only low levels of the 3.5 kb mRNA were detected in spleen, thymus, PBL, ovary and testes (Figure 9D). As was the case with gp34, mRNA for the human receptor was found in thymus, spleen and both fetal and adult heart (Figure 9E). There was also receptor mRNA detected in lung and at lower levels in placental and fetal kidney.

In conclusion, for both species OX40 and a ligand appear to be co-expressed in spleen and thymus suggesting a functional role for the OX40 system in these organs. In addition, both murine OX40L and OX40 mRNAs were found in testes, lung and skeletal muscle; and both gp34 and OX40 mRNAs were detected in heart. Further work will be required to determine if cell-surface protein and receptor signalling are found for non-lymphocytic cells.

Biological activity of the cloned ligands

To assess whether the recombinant ligands possessed biological function we first tested the ability of muOX40L and gp34 to stimulate the proliferation of T cells isolated from human peripheral blood or murine spleen during their activation with suboptimal levels of phytohemagglutinin (PHA). CV-1/EBNA cells transfected with ligand from either species induced human peripheral blood T cells to proliferate in a dose-dependent manner. Vector control transfected cells had no effect (Figure 10A). CV-1/EBNA cells expressing muOX40L induced potent proliferation of purified murine splenic T cells in the presence of suboptimal PHA. Cells transfected with gp34 or vector did not induce murine T cell proliferation (Figure 10B).

Table I. OX40L enhances cytokine production from activated murine T cells

Stimulation	IL-2 (U/ml)	IL-4 (U/ml)
Control	2.9	0.3
OX40L	32.6	5.8
IL-2	34.6	1.8
Anti-CD28	134.4	6.4

All wells received antibody to TCRαβ and either 10^4 CV-1/EBNA cells transfected with vector pDC410 (control), 10^4 CV-1/EBNA cells transfected with pDC410 containing muOX40L (OX40L), 5 U/ml murine IL-2 (IL-2) or a 1:1000 dilution of anti-CD28 ascites (anti-CD28). Supernatants were harvested 48 h after culture initiation. The measured IL-2 level for wells receiving the 5 U/ml of IL-2 includes any residual added IL-2. T cells stimulated in the absence of anti-TCRαβ antibody produced undetectable levels of both IL-2 and IL-4. The results are representative of four experiments.

We also measured cytokine production from murine lymph node cells stimulated by muOX40L in the presence of suboptimal concentrations of anti-TCRαβ monoclonal antibody. As positive controls, cytokine levels produced by the addition of anti-CD28 mAb or a low level of IL-2 were monitored as well. In this assay muOX40L, expressed in transfected CV-1/EBNA cells, induced secretion of IL-2 and IL-4 to high levels, with the IL-4 levels close to that produced by anti-CD28 administration (Table I). Thus, in certain settings the ligand may function to alter immune response by stimulating differential cytokine secretion.

Discussion

This work describes murine OX40L and a related human protein, gp34, which join the growing list of TNF family members that exist as type II membrane proteins with similar extended β-sheet−loop tertiary structures. While the overall structural similarity to other TNF family members is clear, as is the prediction that muOX40L and hugp34 molecules are more compact due to shorter inter-strand loops, the exact structures of the C-terminus and the I β-strand are less certain. An alternative model, derived by pairwise comparison of hugp34 to TNF, LTα and FasL, suggests that Phe180 of hugp34 and Tyr182 of muOX40L can be placed within the I β-strand, in alignment with the I strand Phe residue conserved in other family members. However, we do not favour this model for muOX40L and hugp34 because this placement disrupts the predicted disulfide bond and places strains on the packing of hydrophobic residues in the strands.

The close proximity in the genome of the genes for muOX40L and muFasL constitutes the third reported gene cluster for TNF-related ligands, the first consisting of TNF and the lymphotoxins (Nedospasov *et al.*, 1986; Browning *et al.*, 1993) and the second being CD27L and 41BBL (Alderson *et al.*, 1994). Among the known members of

the ligand family, rodent FasLs are most closely related to murine lymphotoxin α (Suda *et al.*, 1993; Lynch *et al.*, 1994; Takahashi *et al.*, 1994), raising the possibility that the OX40L/FasL and the TNF/lymphotoxin gene clusters are paralogous. If so, it is possible that functional relationships similar to those found in the TNF/lymphotoxin cluster might exist for the OX40L/FasL cluster. The TNF/lymphotoxin gene cluster contains ligands either with similar binding characteristics (TNF and LTα) or which can physically interact (LTα and LTβ). However, binding studies with recombinant muFasL and muOX40L and their respective soluble receptors have demonstrated that each ligand binds only to its cognate receptor (data not shown). In addition, preliminary evidence suggests that muOX40L and muFasL expressed in the same cell, unlike the lymphotoxins, retain normal binding properties and do not form hetero-oligomers (P.R.Baum, R.A.Sorensen, K.N.Clifford, W.C.Fanslow, R.G.Goodwin, M.R.Alderson, C.A.Smith and D.Lynch, unpublished data). Therefore, at present there is no evidence for a functional relationship between muOX40L and FasL as is found for the gene cluster on chromosome 17.

While this work was in progress it was reported that soluble murine OX40 bound to LPS-stimulated B cells, and it was proposed that B cells bear OX40L which might serve in generating a co-stimulatory signal for receptor-bearing T cells (Calderhead *et al.*, 1993). Using muOX40–Fc mutein we have not detected OX40L on B cells even after LPS stimulation. When we employed a non-mutein muOX40–Fc fusion protein similar to that used in the previous report, we detected binding to Fc receptors that could only be blocked with high concentrations of IgG (data not shown). Therefore, one explanation for our results is the lower affinity of the OX40–Fc mutein for Fc receptors. While our results indicate that there are negligible levels of muOX40L on B cells, expression of muOX40 receptor on B cells remains to be determined.

Our results demonstrate inducible expression of both muOX40L and muOX40 on activated $CD4^+$ and $CD8^+$ T cells. The slow kinetics of OX40 induction on activated T cells (Mallet *et al.*, 1990; Latza *et al.*, 1994; our data not shown) as compared with many other T cell accessory molecules suggest that the OX40 system plays a role late in the activation process, possibly altering some effector function such as homing or the eventual outcome of T cell activation (Janeway and Golstein, 1991; van Seventer *et al.*, 1991). While the latter could involve a number of possibilities, our results showing that OX40L can enhance significantly IL-2 and IL-4 production suggest that OX40L might function by altering cytokine levels important for the generation of T memory cells during the immune response.

Co-expression of mRNA for both ligand and receptor was detected in the mouse for skeletal muscle and testes. In humans, transcripts are found for both OX40L and OX40 in fetal and adult heart and placenta. An important step in understanding what role OX40L and OX40 play in these tissues, and in others where only receptor or ligand mRNA was detected, will be to determine what regions and what cell type(s) express the molecules at the cell surface.

The finding that a human OX40L, gp34, is a protein regulated by the tax regulatory gene of the human pathogen, the retrovirus HTLV-1, and expressed at high levels on leukemic T cell lines bearing the retrovirus, suggests the possibility that the OX40 system plays a role in viral pathogenesis or tumorigenesis. To determine what role gp34 and OX40 play in the disease process it will be important to determine if OX40 is also expressed in virally infected cells, or is improperly regulated in virally infected individuals. Our generation of active soluble receptor and ligand reagents should help to address the function of OX40L in these systems.

Materials and methods

DNA sequencing was carried out using Taq DyeDeoxy Terminator Cycle Sequencing on an automated sequencer Model 373A (Applied Biosystems, Foster City, CA). Both strands for cloned cDNAs were sequenced. The nucleotide and predicted amino acid sequences were analysed with the GCG package (University of Wisconsin, Madison, WI) and compared with the GENBANK database with the programs fasta and tfasta, respectively.

Secondary structure predictions were generated by comparison with other ligand family members using a homology-based neural network algorithm (Rost and Sander, 1992).

A 3-D model of hugp34 was generated using the crystal structure of TNF as template by aligning the signature region and employing a homology modelling package, FOLDER (Srinivasan *et al.*, 1993). During modelling, the two cysteines of hugp34 predicted to form a disulfide crosslink were allowed to be in proximity.

Cloning of muOX40L and hugp34

To clone muOX40L, S49.1 cells (ATCC TIB 128) were incubated for 6 h with 10 ng/ml phorbol ester myristic acid and poly(A)$^+$ RNA was prepared by standard procedures (Sambrook *et al.*, 1989). The RNA was used to construct a cDNA expression library in pDC410 (Alderson *et al.*, 1994), essentially as described (Smith *et al.*, 1993). Using 1 μg/ml purified muOX40–Fc mutein in the first binding step, screening for muOX40L expression was carried out as described previously by a slide binding method (Gearing *et al.*, 1989; Goodwin *et al.*, 1993) until pure clones were derived.

A cDNA encoding gp34 was isolated from HUT102 (ATTC TIB 162) poly(A)$^+$ RNA (obtained from A.Larsen, Immunex Corporation) by performing PCR on first strand cDNA prepared with a commercial kit (Superscript cDNA kit, Gibco/BRL, Gaithersburg, MD) and cloning into expression vector pDC410.

Cloning of human OX40

Poly(A)$^+$ RNAs from human PBLs were subjected to Northern analysis on nylon membranes (Hybond N$^+$, Amersham, Arlington Heights, IL) with radioactive riboprobes for the full coding region of muOX40 prepared using a commercial *in vitro* transcription kit (Promega Gemini II, Madison, WI) and [^{32}P]UTP (Amersham). An ~1.2 kb RNA was detected after hybridization at 63°C in Stark's solution (Sambrook *et al.*, 1989), washing to high stringency (0.5× SSC at 63°C) and autoradiographic exposure (XAR5 film, Kodak, Rochester, NY). To isolate a cDNA for this RNA species, Southern analysis was performed using radioactive muOX40 probes generated by PCR with [^{32}P]dCTP on pools from a PBT cDNA expression library (kindly provided by B.Mosley, Immunex Corporation) after enzyme digestion to release cDNA inserts. A pure clone was isolated by colony hybridization from a positive cDNA pool.

Generation of Fc fusion proteins

To prepare muOX40–Fc mutein, RNA was prepared from murine T cell clone 7B9 (Mosley *et al.*, 1989) after 26 h of concanavalin A stimulation, reverse transcribed with oligo dT priming and muOX40 cDNA was amplified with muOX40-specific oligos with restriction enzyme sites for cloning into Bluescript SK$^+$ (Stratagene Cloning Systems, La Jolla, CA). The extracellular domain of muOX40 was amplified by PCR and ligated via a three-way reaction with Bluescript SK$^+$ and a fragment carrying the human IgG1 Fc region (Armitage

et al., 1992a). The region encoding the fusion protein was then cloned into the mammalian expression vector pDC406 (McMahan *et al.*, 1991).

To lower binding to Fc receptors *in vitro*, oligonucleotide site-directed mutagenesis was performed substantially as described (Deng and Nickoloff, 1992; Ray and Nickoloff, 1992) to modify the fusion protein encoded by this construct. Three amino acids in the Fc region were mutated (Leu234 to Ala, Leu235 to Glu, and Gly237 to Ala; amino acid numbering is based on Canfield and Morrison, 1991).

HuOX40–Fc mutein fusion protein was prepared by PCR amplification of the mature extracellular domain (residues 76–630) and a three-way ligation to a vector with the IL-7 signal sequence (Alderson *et al.*, 1994) and a fragment with the mutein version of human IgG1 Fc region. The region encoding the fusion protein was then excised and cloned into pDC410.

Soluble versions of the ligands were prepared by PCR amplification of the putative extracellular domains (amino acid residues 49–198 for muOX40L and 54–183 for gp34). The PCR products were then ligated to the mutein version of the Fc region of human IgG1 to form soluble ligand–Fc mutein fusions as described (Fanslow *et al.*, 1994c). Soluble muOX40L–Fc mutein was ligated into vector pDC406 (McMahan *et al.*, 1991) and soluble gp34–Fc mutein into vector pDC409 (Smith *et al.*, 1993).

Fusion proteins were prepared from transfected CV-1 EBNA cells as described previously (Armitage *et al.*, 1992a), except for elution from protein A agarose (BioRad, Richmond, CA) with 5 mM citrate pH 2.8, 75 mM NaCl. Protein concentration was determined by BCA analysis (Sigma Chemicals, St Louis, MO). Purity was assessed by SDS–PAGE (Laemmli, 1970). The fusion protein concentration in cell supernatants was determined by a human Fc-specific ELISA.

Slide binding assays

Assays were essentially as described (Goodwin *et al.*, 1993) except that: (i) lower amounts of DNA were used in transfections for pure clones as noted in the figure legends; (ii) cell supernatants containing 1 μg/ml fusion protein were sometimes used in place of purified fusion protein; and (iii) as binding reagents some slides received anti-gp34 antibodies TAG34, 6D1 or a combination at a total antibody concentration of 1 μg/ml followed by ^{125}I-labelled sheep anti-mouse IgG (New England Nuclear, Cambridge, MA).

Determination of expression by Northern analysis and flow cytometry

Filters containing poly(A)$^+$ RNAs for various tissues were purchased (Multiple Tissue Northerns, Clonetech, Palo Alto, CA; Figure 9B–E). RNAs in Figure 9A were isolated, separated by electrophoresis on 0.5× TBE agarose gels, blotted to nylon (Hybond N$^+$, Amersham, Arlington, IL) and hybridized with coding region riboprobes by standard procedures (Sambrook *et al.*, 1989). To assess RNA levels in the lanes, a human β-actin cDNA probe was random-primed and hybridized by standard procedures (Sambrook *et al.*, 1989).

Cell binding and flow cytometric analysis were performed at 4°C essentially as described (Armitage *et al.*, 1992b) in a binding medium consisting of 10% (v/v) normal goat serum (Sigma), 1% (v/v) fetal bovine serum, 50 μg/ml mouse IgG (Caltag, San Francisco, CA) and 0.1% NaN_3 in PBS. Bound muOX40–Fc mutein or other Fc constructs were detected with goat α human IgG (Fc-specific) biotin (Jackson Immunoresearch Laboratories Inc., Bar Harbor, ME) and subsequently incubated with streptavidin–phycoerythrin (1:15) (Becton-Dickinson, San Jose, CA).

To analyse cell-surface expression of T cell antigens, cells (5×10^5) were incubated with an optimal concentration of conjugated antibody (Fanslow *et al.*, 1994a). Binding of Fc fusion proteins and conjugated antibodies was analysed by single- and dual-colour flow cytometry (10 000 viable cells) on a single-laser FACScan instrument (Becton-Dickinson). Cells incubated with isotype-conjugated control antibodies (Becton-Dickinson) or with control Fc proteins were analysed to determine light scattering characteristics and the levels of non-specific binding.

Chromosome localization of ligands

Murine OX40L was mapped using C3H/HeJ-*gld* and *M.spretus* (Spain) mice and [(C3H/HeJ-*gld*×*M.spretus*)F_1×C3H/HeJ-*gld*] interspecific backcross mice which were bred and maintained as described previously (Seldin *et al.*, 1988).

DNAs from mouse organs were digested with restriction endonucleases and 10 μg samples subjected to Southern analysis on Nytran membranes (Schleicher & Schull, Inc., Keene, NH) by standard procedures (Sambrook *et al.*, 1989). For OX40L, a probe for the entire coding region was prepared by hexanucleotide random priming. Other clones used as probes in the current study and RFLPVs that detect the antithrombin 3 (*At-3*), anonymous DNA markers (*D1Sel8*, *D1Sel10* and *D1Sel11*) and L-selectin (*Sell*), were as described previously (Seldin *et al.*, 1988; Watson *et al.*, 1992; Hunter *et al.*, 1993). The *D1Sel12* marker was derived by end-clone recovery from a mouse yeast artificial chromosome (YAC M4-1.gld) that was subcloned by *Dpn*II digestion in agarose of YAC DNA followed by ligation into pBluescript SKII(-). For *D1Sel12*, *Pvu*II RFLV distinguished C3H/HeJ-*gld* (3.5 kb) and *M.spretus* (4.1 kb) derived chromosome segments. Additional molecular typing was performed using microsatellite markers (*D1Mit106*, *D1Mit107*, *D1Mit108*) obtained from Research Genetics (Huntsville, AL) and typed according to the methods of Dietrich *et al.* (1992). The sizes of amplified fragments *D1Mit106* (C3H/HeJ-*gld*, 118 bp; *M.spretus*, 114 bp), *D1Mit07* (C3H/HeJ-*gld*, 108 bp; *M.spretus*, 94 bp) and *D1Mit108* (C3H/HeJ-*gld*, 148 bp; *M.spretus* 155 bp) were used to type backcross mice.

Gene linkage was determined by segregation analysis (Green, 1981). Gene order was determined by analysing all haplotypes and minimizing crossover frequency, resulting in a most likely gene order (Bishop, 1985).

To map human gp34, the coding region of gp34 in Bluescript SK$^+$ (Stratagene, La Jolla, CA) was labeled by nick-translation with biotin-14-dATP and hybridized *in situ* at a final concentration of 15 ng/μl to metaphases from two normal males. The fluorescence *in situ* hybridization (FISH) method was modified from that described previously (Callen *et al.*, 1990) in that chromosomes were stained before analysis with propidium iodide (as counterstain) and DAPI (for chromosome identification).

Cell-surface labelling and bioaffinity precipitation of naturally occurring and recombinant OX40L

OX49.4 cells or transfected CV-1/EBNA cells were surface-labeled with biotin X-NHS as described previously (Fanslow *et al.*, 1994b). Immunoprecipitation, gel electrophoresis and analysis by chemiluminescence were then performed essentially as described (C.R.Maliszewski *et al.*, manuscript submitted) by incubating with muOX40–Fc mutein or CD40–Fc (Fanslow *et al.*, 1992) at 10 μg/ml for at least 2 h at 4°C with intermittent agitation. Light-emitting proteins on the blot were detected by exposure to Hyperfilm-ECL (Amersham) for variable exposure times of <5 min.

Mice

Female C57BL/6 mice (8–12 weeks old) were obtained from Charles River (Wilmington, MA). All mice were maintained in a specific pathogen-free environment until lymphoid organ harvest.

Media, cell lines, lymphokines and antibodies

The S49.1 cell line was obtained from the ATCC (TIB 128) and maintained in supplemented DMEM containing 5% FBS (Cerrottini *et al.*, 1974). All cell culture experiments with murine T cells were performed in this medium. Hybridoma supernatants containing mAbs to CD11b (Mac-1) (Springer *et al.*, 1979) and class II MHC (25-9-17) (Ozato and Sachs, 1981) were used for T cell purifications. The extent of T cell purification was determined with FITC-conjugated mAbs obtained from Pharmingen (San Diego, CA; 500A2 anti-CD3), Caltag (South San Francisco, CA; anti-CD4, anti-CD8) and Tago [Burlingame, CA; F(ab′)$_2$ anti-IgM]. The anti-TCRαβ mAb (H57-597) used in cell culture was obtained from Pharmingen. Hamster anti-CD28 (Gross *et al.*, 1992) was a gift from Dr James Allison. Recombinant murine IL-2 and IL-4 were expressed in yeast and purified as described (Urdal *et al.*, 1984; Mosley *et al.*, 1989).

T cell isolation and purification

Lymphoid organs were harvested aseptically and cell suspension was created as described (Fanslow *et al.*, 1991). Splenic and lymph node T cells were isolated exactly as described (Fanslow *et al.*, 1994a). The purity of the resulting splenic T cell preparations was routinely >95% CD3$^+$ and <1% sIgM$^+$.

PBT cells were purified from the blood of healthy donors (Armitage *et al.*, 1993). The resulting T cell population was routinely >98% CD3$^+$ as determined by flow cytometric analysis.

Proliferation and cytokine secretion assays

Proliferation of purified murine or human T cells was measured by culture with suboptimal concentrations of PHA, pulse-labelling and

harvesting as reported previously (Armitage *et al.*, 1993; Fanslow *et al.*, 1994a).

After purification from C57Bl/6 mice, murine lymph node T cells were incubated at 1×10^5/well in microtitre wells treated with medium with or without 0.2 μg/ml anti-TCRαβ. Further additions to the wells, including transfected cells expressing muOX40L, are described in Table I. Levels of cytokines in supernatants were determined by their ability to promote the growth of the IL-2- and IL-4-dependent cell lines CTLL-2 and CT.4S. The supernatants were titrated starting at a 1:2 dilution, with units determined from standard curves using recombinant cytokine. Proliferation of the CTLL-2 and CT.4S cells was measured at 24 h (Hu-Li *et al.*, 1989; Morrissey *et al.*, 1989).

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