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Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule related to nerve growth factor receptor

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The antigen recognized by the monoclonal antibody (mAb) MRC OX40 is present on activated rat CD4 positive T lymphocytes but not other cells. cDNA clones were isolated from an expression library using the MRC OX40 mAb and the protein sequence for the OX40 antigen deduced. It contains a typical signal sequence and a single putative transmembrane sequence of 25 predominantly hydrophobic amino acids giving an extracellular domain of 191 amino acids and a cytoplasmic domain of 36 amino acids. The sequence of the extracellular domain includes a cysteine-rich region with sequence similarities with the low affinity nerve growth factor receptor (NGFR) of neurons and the CD40 antigen present on human B cells. Within this region three cysteine-rich motifs can be recognized in OX40 compared with four similar motifs in both NGFR and CD40. OX40, CD40 and NGFR constitute a new superfamily of molecules with expression including lymphoid cells (OX40, CD40) and neuronal cells (NGFR). This is reminiscent of the immunoglobulin superfamily whose molecules are variously found at the surface of lymphoid or brain cells or both.

Key words: activated T lymphocyte/membrane glycoprotein/nerve growth factor receptor/OX40

Introduction

When T lymphocytes are activated they change from metabolically quiescent small lymphocytes to large, metabolically active lymphoblasts. This is accompanied by many changes in expression of their cell surface molecules. Some of these molecules reflect the cells' increased requirement for metabolites, e.g. the transferrin receptor is expressed on activated T cells and is also found on other dividing cells (Larrick and Cresswell, 1979). Other surface molecules are found specifically on activated T cells, e.g. the interleukin-2 (IL-2) receptor which, together with IL-2 produced by the activated T cells, plays a key role in the control of T cell activation (reviewed in Smith, 1988). Other controlling factors are likely and receptors for these are possible candidate functions for new T cell activation markers.

The MRC OX40 monoclonal antibody (mAb) (MRC OX40 or OX40 are used to name the antibody, antigen or cDNA clone) recognizes a membrane glycoprotein with an apparent M_r of 47 000–51 000 that is present on activated rat T cells but not known to be on any other cell type (Paterson *et al.*, 1987). The OX40 antigen is of particular interest as it is present only on the CD4 positive subset of activated T cells and we are not aware of an antigen with similar properties in another species. CD4 positive T cells play a key role in the control of T cell responses and it seems possible that the OX40 antigen may be an important cell surface molecule involved in these responses. The OX40 mAb has been tested for effects on *in vitro* assays of T cell function (Paterson *et al.*, 1987). The mAb is not mitogenic like some other antibodies recognizing T cell markers (reviewed in Weiss and Imboden, 1987) but it does cause enhancement of some T cell responses, e.g. the mixed lymphocyte response and in T cell activation induced by the lectin concanavalin A (Paterson *et al.*, 1987). We now report the isolation and sequence of a cDNA clone for the OX40 antigen and its similarity to the low affinity nerve growth factor receptor (NGFR).

Results

Isolation and characterization of cDNA clones for the OX40 antigen

The cDNA clone for the OX40 antigen was isolated using the COS cell expression system developed by Seed and co-workers (Seed, 1987; Aruffo and Seed, 1987; Seed and Aruffo, 1987). Poly(A)$^+$ RNA was isolated from activated T cells prepared by stimulation of rat lymph node cells by concanavalin A and used to synthesize cDNA. The cDNA was cloned into the expression vector CDM8 and a cDNA library was prepared. Purified plasmid from the library was transfected into COS cells using DEAE–dextran and cells expressing OX40 antigen were separated with the OX40 mAb and magnetic Dynal beads coated with sheep anti-mouse immunoglobulin (Ig) antibody. Plasmid was recovered from these COS cells and used to transfect fresh COS cells by spheroplast fusion followed by Dynal bead selection and plasmid isolation. After two more cycles of selection, 50% of the cDNA clones gave OX40 antigen expression following transfection of plasmid DNA into COS cells by the DEAE–dextran method. All the positive clones contained cDNA inserts of 1.2 kb.

Northern blot analysis showed that the OX40 cDNA reacted with a single mRNA species of ~1.4 kb present in both concanavalin A activated thymocytes and lymph node cells, but not resting lymph node cells, thymocytes or liver (Figure 1). This is in agreement with the known distribution of the OX40 antigen (see Introduction) and approximates the size of the cDNA clone. The cDNA of 1.2 kb contained a translational start site, ATG, surrounded by residues consistent with the consensus sequence found around initiator ATGs (Kozak, 1987). This was followed by an open reading frame coding for 271 amino acids. The 3′ non-coding

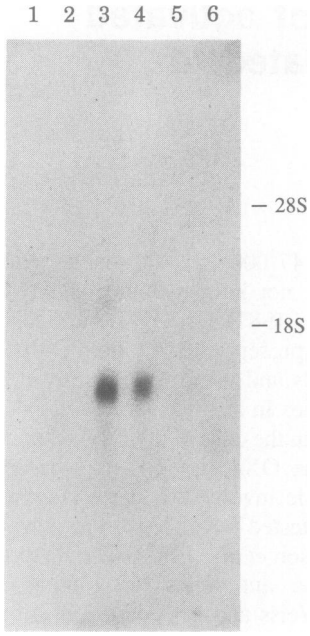

Fig. 1. Distribution of OX40 mRNA by Northern blot analysis. The migration positions of 28S and 18S RNA are indicated. The numbers above the tracks represent RNA prepared from the following: (**1**) thymocytes, (**2**) lymph node, (**3**) activated thymocytes (3 day concanavalin A), (**4**) activated lymph node cells (3 day concanavalin A), (**5**) spleen, (**6**) liver. A clear band of ~1.4 kb is present only in lanes containing T cells activated from thymus and lymph node.

sequence contained a typical polyadenylation site 33 residues from a poly(A) tail of more than 80 residues (Figure 2A). The initiator methionine was followed by a sequence containing a high proportion of hydrophobic residues typical of a signal sequence. The most likely signal cleavage point, predicted on the basis of other signal sequence cleavage sites (von Heijne, 1986), is after residue 19 which could give a mature protein of 252 amino acids. There is a single putative transmembrane domain of 25 predominantly hydrophobic amino acids, dividing the protein into an extracellular domain of 191 amino acids and a cytoplasmic region of 36 amino acids (Figure 2B).

The extracellular part contains a cysteine-rich region in which 18 out of 140 residues are cysteines and, adjacent to the membrane, a 46 amino acid hinge-like region which includes a 25 residue sequence of which 17 residues are threonine, serine or proline. This latter type of sequence is often glycosylated with *O*-linked sugars (McMullen and Fujikawa, 1985; Killeen *et al.*, 1987; Jackson and Barclay, 1989). This seems likely for OX40 as the apparent M_r on SDS−PAGE is 47 000−51 000 (Paterson *et al.*, 1987) but the M_r predicted for the mature protein from the cDNA sequence is 27 777 and there are only two potential sites for *N*-linked glycosylation. One of these is NCTP and may not be glycosylated as the sequences NTTP and NCTP in an Ig and the leukocyte common antigen, respectively, have been shown not to be glycosylated (Frangione *et al.*, 1980; Barclay *et al.*, 1987) presumably due to the juxtaposition of the proline residue to the NXS/T sequence.

Sequence similarities between OX40, NGFR and CD40

A search of the protein databases with the amino acid sequence of OX40 using the program FASTP gave a good

(A)

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gaacacagacaaggATGtatgtgtgggttcagcagcccacagcatttctgctcctgggac     60
tctcacttggagttacagtgaagctcaactgtgttaaagatacctaccccagtggtcaca    120
agtgctgtcgtgagtgccagccaggccatggtatggtgagccgctgtgatcacaccaggg    180
acactgtatgtcatccatgtgagcctggcttctacaatgaggctgtcaattacgacacct    240
gcaagcagtgtacacagtgcaaccaccgaagtggaagtgaactcaagcagaactgcacac    300
ctactgaggatactgtctgccagtgtagaccaggcacccaaccccggcaagacagcagcc    360
acaagcttggagttgactgtgttccctgccccccctggccactttttctccaggcagcaacc    420
aagcctgcaagccctggaccaattgtaccttatctggaaagcagatccgccacccagcca    480
gtaacagcttggacacagtctgtgaagacagaagcctcctggccacactgctctgggaga    540
cccagcgcactacattcaggccaaccactgtcccgtccaccacagtctggcccaggactt    600
ctcagttgccctctacacccaccttggtggctcctgagggcccctgcatttgctgttatcc    660
taggcctaggcctgggcttgctggctcccttgactgtcctgctagccttgtacctgctcc    720
gaaaggcttggagatcgcccaacactcccaaaccttgttggggaaacagcttcaggaccc    780
ctatccaggaggagcagaccgacacacactttactctagccaagatcTGAgcaataccac    840
aggagtggatttatggggcacagacagcccatatcctgatgcctgcctgccagggccct    900
ccacaccgttctaggcgctgggctggctgtgcactctcccatgtatgctgtgcatactac    960
ctgcctggtggcactcctAATAAAcatgctcgcagctgtgagtctgtcactggccctaaa   1020
aaa
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(B)

```
MYVWVQQPTAFLLLGLSLGVTVKLNCVKDTYPSGHKCCRECQPGHGMVSR     50
                                  1
CDHTRDTVCHPCEPGFYNEAVNYDTCKQCTQCNHRSGSELKQNCTPTEDT    100
                        2
VCQCRPGTQPRQDSSHKLGVDCVPCPPGHFSPGSNQACKPWTNCTLSGKQ    150
          3                   4
IRHPASNSLDTVCEDRSLLATLLWETQRTTFRPTTVPSTTVWPRTSQLPS    200

TPTLVAPEGPAFAVILGLGLGLLAPLTVLLALYLLRKAWRSPNTPKPCWG    250

NSFRTPIQEEQTDTHFTLAKI
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Fig. 2. (**A**) The nucleotide sequence of OX40 cDNA. The initiator ATG, the termination signal and the polyadenylation signal are shown in capitals and bold face. These sequence data will appear in the EMBL/GenBankDDBJ Nucleotide Sequence Databases under the Accession number X17037. (**B**) Protein sequence of OX40 antigen deduced from the cDNA sequence. The probable leader sequence is 19 residues giving a predicted NH_2 terminus at valine residue 20. The putative transmembrane sequence is boxed and the extracellular cysteine-rich region divided into four segments (see Figures 3 and 5).

score with the low affinity NGFR (Johnson *et al.*, 1986; Radeke *et al.*, 1987) and an intermediate score with the B2 chain of laminin (Sasaki and Yamada, 1987). The human B cell antigen CD40 has recently been shown to have sequence similarities to NGFR (Braesch-Andersen *et al.*, 1989; Stamenkovic *et al.*, 1989) and OX40 shows sequence similarities in the NH_2-terminal cysteine-rich region of both NGFR and CD40 as shown in Figure 3. Statistical analysis using the ALIGN program (Dayhoff *et al.*, 1983) showed that these similarities were highly significant, indicating that the cysteine-rich regions were related in evolution (Figure 3). The remainder of the molecules showed no significant sequence similarities although both OX40 and NGFR have a serine/threonine/proline-rich hinge-like region in the 46 and 61 amino acids, respectively, between the cysteine-rich domains and the transmembrane region, whereas CD40 enters the transmembrane region only seven amino acids after its last cysteine. The cytoplasmic regions of the OX40, NGFR and CD40 proteins are dissimilar in length spanning 36, 152 and 62 amino acids, respectively. CD40 and NGFR each have four repeating sequence homology units that usually contain six cysteine residues although two repeats contain only four cysteines. OX40 contains only three clear repeats as domain 3 is much shorter than the rest (Figure 3). The domains differ slightly in length and the first domain in all three sequences shows a shorter sequence and a slightly different pattern, e.g. the second and third cysteines are adjacent whereas in all other domains they are separated by two amino acids.

Thus, between these three proteins there are a total of 11 domains containing the cysteine-rich motifs. All 11 domains

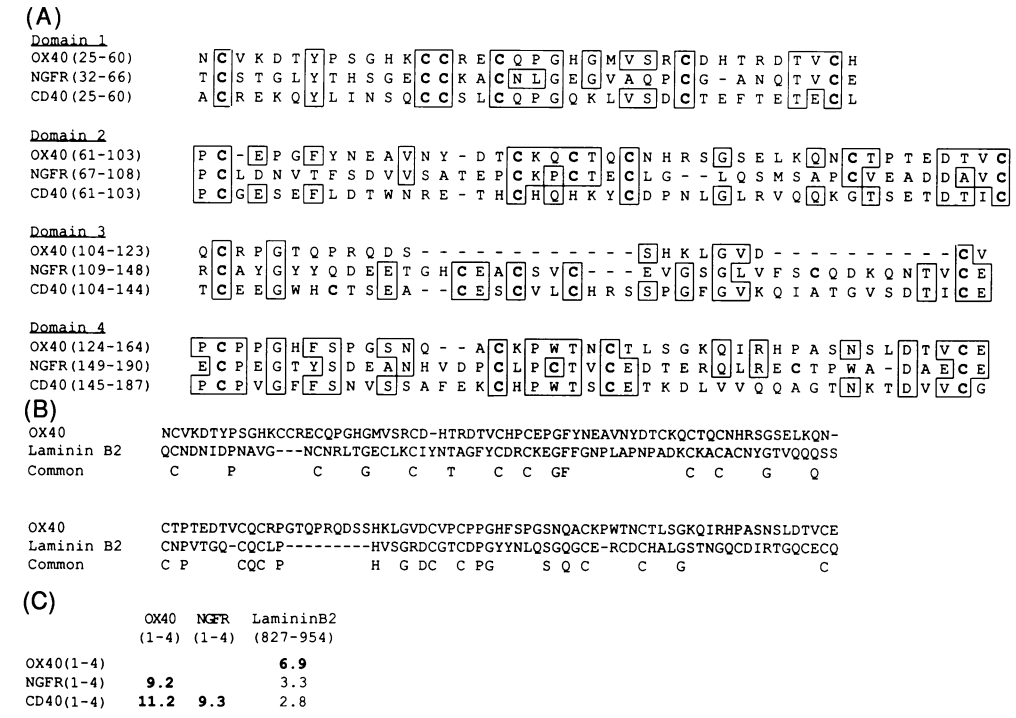
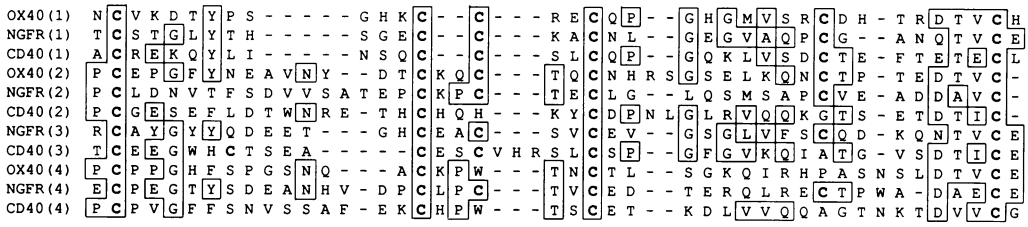


Fig. 3. Alignment of the cysteine-rich extracellular segment in OX40, NGFR and CD40 and laminin. (**A**) The alignment of four cysteine-rich segments in CD40 and NGFR aligned with three clear segments in OX40 and the short unrelated segment designated domain 3; residues common between two of the sequences are boxed and cysteine residues are shown in bold. (**B**) Alignment of OX40 with mouse laminin B2 precursor; residues common between the two sequences are indicated. (**C**) ALIGN scores in SDs for the alignments in (A) and (B). Assuming a normal distribution and no effect of sequence selection, scores of 3.1, 4.3, 5.2 and 6.0 SDs indicate probabilities of occurrences by chance of 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}, respectively. Data for human CD40 are from Stamenkovic *et al.* (1989), rat NGFR from Radeke *et al.* (1987) and laminin B2 (residues 827–954) from Sasaki and Yamada (1987). The number of residues is for the precursor forms of the four proteins.



		OX40 Domains				NGFR Domains				CD40 Domains			
		1	2	3	4	1	2	3	4	1	2	3	4
OX40	1												
	2	**4.2**											
	3	0.6	2.4										
	4	**4.1**	**6.7**	3.4									
NGFR	1	**7.0**	**5.2**	0.5	**6.3**								
	2	**5.9**	**6.1**	1.3	**4.9**	**4.6**							
	3	**6.1**	**5.7**	0.1	**5.0**	**6.4**	**4.6**						
	4	3.6	**6.0**	1.3	**4.9**	**4.9**	**5.5**	**5.7**					
CD40	1	**7.1**	3.0	2.0	2.8	**5.3**	3.3	3.9	**5.7**				
	2	3.2	**4.6**	0.5	**4.6**	3.2	**4.0**	3.9	2.8	3.7			
	3	3.8	**4.3**	0.6	**4.7**	**4.5**	1.9	3.4	1.8	2.7	**5.2**		
	4	3.0	**5.8**	2.2	**8.8**	**4.2**	**4.7**	**4.8**	**4.9**	1.5	**7.1**	3.5	

Fig. 4. Alignment of NGFR repeats and statistical analysis using the ALIGN program. The three cysteine-rich domains of OX40 are aligned with the four present in NGFR and CD40 (as defined and numbered in Figure 3). Cysteine residues are shown in bold. Residues identical in four or more segments are boxed. The scores for the alignments shown are given in SDs and those >4.0 are shown in bold. Domain 3 in OX40 (see Figure 3) is not included in the alignment of sequences but is included in the ALIGN analysis and as expected gave low scores confirming that there is no evidence for a relationship of this segment with NGFR repeats. The other domains gave generally high scores.

are aligned in Figure 4 and it can be seen that in addition to the regular pattern of cysteine residues there are also further residues common to many of the domains. The similarities are reflected in the statistical analysis using the ALIGN program (Figure 4). Most of the domains gave highly significant scores with each other. Domain 3 of OX40 has also been compared in this analysis. As mentioned above this is a short sequence and does not have the key residues

of the NGFR repeats. This is reflected in the poor ALIGN scores obtained, confirming that OX40 has only three NGFR-like domains rather than the four present in NGFR and CD40.

The alignment of OX40 with laminin B2 shows a much lower level of similarity than it does with NGFR and CD40 (Figure 3). However, the alignment is good around the short non-NGFR-like domain 3 giving a score of 6.9 standard deviations (SDs) with the ALIGN program (Dayhoff *et al.*, 1983). NGFR and CD40 give lower scores for the same region (Figure 3) although somewhat higher scores of ~4–5 SDs were obtained against different regions of these cysteine-rich repeats present in laminin B2, the related proteins laminin B1 and laminin A (Sasaki *et al.*, 1987, 1988) and the basement membrane heparan sulphate proteoglycan (Noonan *et al.*, 1988). However, these cysteine-rich regions in the laminin chains and the proteoglycans contain a characteristic motif of eight cysteines, and the key residues in this repeat (Noonan *et al.*, 1988) do not correspond to those of the NGFR-like repeats (Figure 4). This is reflected in ALIGN scores for comparisons of NGFR-like repeats and laminin-like repeats where out of 60 alignments only two gave scores >2.0 SDs (3.7 and 2.6). Thus, the NGFR repeats and the laminin repeats could be distantly related but the evidence is equivocal.

Various other cysteine-rich motifs are found in receptors such as the LDL receptor (Yamamoto *et al.*, 1984; Bell *et al.*, 1986) and the EGF receptor (Ullrich *et al.*, 1984). However, the key residues found in these repeats are not conserved in the CD40, OX40 or NGFR repeats (data not shown). For example, comparison of the 11 NGFR-like repeats with the seven LDL repeats in the LDL receptor using the ALIGN program gave poor scores with only three scores >2 (2.2, 2.6 and 3.6) out of a total of 77 scores compared with typical scores of ≥5 or greater between the NGFR repeats shown in Figure 4. Thus, there is no evidence for a significant relationship between the NGFR repeats and the cysteine-rich motifs in the EGF and LDL receptors.

Discussion

The cDNA sequence for OX40 shows that it is a member of a family of cell surface proteins containing three or four extracellular domains with a characteristic pattern of cysteine residues originally noted in low affinity NGFR. So far this family consists of: (i) OX40, on activated T cells with a CD4 positive phenotype; (ii) CD40, restricted to resting and activated B cells, interdigitating cells and primary carcinomas (Clark and Ledbetter, 1986; Ledbetter *et al.*, 1987), and (iii) NGFR itself, present on various neural crest derived cells, e.g. neurones, melanomas and Schwann cells (reviewed in Hempstead *et al.*, 1988). Nerve growth factor is found in large amounts in the submaxillary gland and in its active form it is a homodimer of polypeptides of M_r 13 200. It acts on NGFR to stimulate neurite outgrowth from sympathetic and embryonic sensory ganglia *in vivo* and *in vitro* (reviewed in Davies, 1988). Less is known about CD40 although antibodies recognizing this antigen can augment B cell proliferation and it has been suggested that CD40 is a receptor for an unidentified growth factor (Braesch-Andersen *et al.*, 1989; Stamenkovic *et al.*, 1989).

The key structural features of OX40, CD40 and NGFR are illustrated in Figure 5 with the only sequence similarity

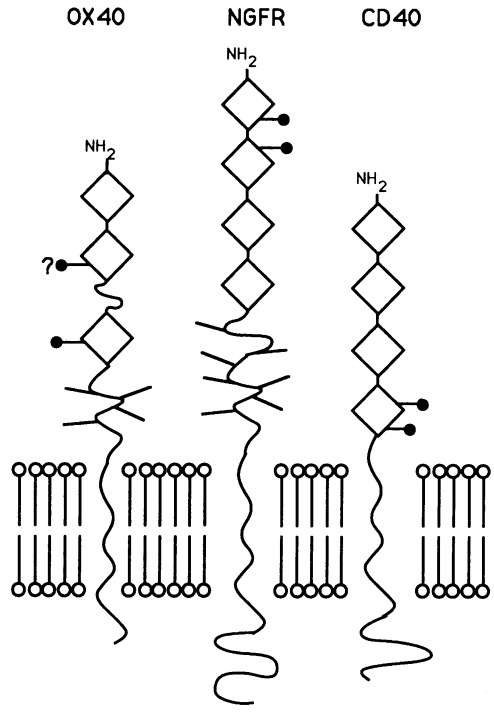


Fig. 5. Models for the NGFR family. The cysteine-rich NGFR repeats are represented by diamonds, possible *N*-linked glycosylation sites by (Y) and regions likely to be *O*-glycosylated by a number of (|). The '?' indicates one possible *N*-linked site in OX40 that is probably not glycosylated (see text). Data for rat NGFR are from Radeke *et al.* (1987) and for human CD40 from Stamenkovic *et al.* (1989).

confined to the cysteine-rich NGFR-like domains. In overall topology the three structures are similar to several other receptors which have cysteine-rich repeats, a hinge-like region and a single transmembrane sequence, e.g. EGF receptor, PDGF receptor, CSF-1 receptor (reviewed in Carpenter, 1987). The cytoplasmic regions of OX40, CD40 and NGFR show no sequence similarity to each other or to the tyrosine kinase sequences present in EGFR, PDGFR and CSF-1R, suggesting that if all three NGFR-like proteins act as growth factor receptors, then the cell signalling across the cell membrane may be via different mechanisms.

The sequence similarities between NGFR, CD40 and OX40 point to a common precursor in evolution. Presumably the multiple cysteine containing domains arose by gene duplication and divergence with the prototype for this for cell surface molecules being the Ig superfamily (Williams and Barclay, 1988). The first domains in all three molecules have additional similarities that distinguish them from the remaining motifs. This points to evolution of these three molecules from an intermediate containing more than one domain and possibly four domains. The gene structure is only known for NGFR, and the cysteine-rich domains are not contained in single exons which also argues against duplication of single domains as is the case in the Ig superfamily (Williams and Barclay, 1988). In NGFR the cysteine-rich motifs are coded for by two exons; one extends from within the leader to four residues into the second domain while the second exon extends to exactly the point defined as the end of the fourth repeat in Figures 3 and 4 (Sehgal *et al.*, 1988).

The only functional data for OX40 are that the OX40 mAb augments T cell responses already initiated. Can the known

functional data on NGFR and CD40 give any insight into the function of OX40? Examination of other superfamilies, such as the Ig superfamily, indicates their members can have quite diverse functions (Williams and Barclay, 1988). The finding that OX40 and CD40 are similar in the extracellular region to NGFR suggests that they could have a similar recognition role as NGFR, i.e. they may also bind soluble ligands involved in growth and differentiation. Interestingly, another brain growth factor called BDNF has recently been shown to have sequence similarities to the nerve growth factor although the nature of its receptor is unknown (Leibrock *et al.*, 1989). The effect of the OX40 mAb *in vitro* could then be explained in one of two ways: either the antibody mimics a ligand that gives an activation signal or the antibody blocks the binding of a ligand that normally gives a negative signal, i.e. inhibiting growth. It is possible that the OX40 antigen binds to a ligand-like NGF although it is noticeable that NGF is very basic and the NGFR is rich in acidic residues (27% aspartate plus glutamate residues in domain 4) compared with only 5% in the comparable region of OX40. In conclusion, the OX40 antigen could well be a receptor for a cytokine and be involved in the control of immune responses at the level of the CD4 positive subset of activated T cells.

Materials and methods

cDNA library construction

Standard DNA methods were as in Maniatis *et al.* (1982).

Lymphocytes were prepared by teasing cervical and mesenteric lymph nodes from 15-week-old PVG × (PVG×DA) rats, filtered through cotton wool and cultured in RPMI−10% FCS containing concanavalin A at 5 μg/ml for 3 days, by which time 54% cells were positive for OX40 by cytofluorography. RNA was extracted from 1.5×10^9 cells by the guanidinium isothiocyanate method. Poly(A)$^+$ RNA was prepared by elution twice from an oligo(dT)−cellulose column (Collaborative Research, Uniscience Ltd, Cambridge, UK). cDNA was synthesized from 4 μg poly(A)$^+$ RNA and cloned into the *Bst*XI sites of the expression vector CDM8, according to Seed (1987) and Aruffo and Seed (1987) except for the following modifications. The 8mer oligonucleotide CTCTAAAG (1.6 μg) and the 12mer CTTTAGAGCACA (2.4 μg) were kinased separately for 1 h at 37°C, with 24 U of T4 polynucleotide kinase (Amersham International Ltd, Amersham, UK) in 70 mM Tris, pH 8.0, 10 mM $MgCl_2$, 7 mM DTT, 1 mM ATP. After phenol extraction and ethanol precipitation, the oligonucleotides were ligated to half the cDNA, at 16°C overnight in 70 mM Tris, pH 7.5, 7 mM $MgCl_2$, 8 mM DTT, 1 mM ATP, 1200 U T4 DNA ligase (New England Biolabs, Beverly, USA). The cDNA was size-fractionated on a 5−20% KOAc gradient in a Beckman SW50.1 rotor centrifuged at 45 000 r.p.m. for 3.75 h at 20°C. Fractions were collected from the base of the gradient and analysed by agarose gel electrophoresis. Fractions containing cDNA of between 1 and 6 kb were combined. Ratios of *Bst*XI cut CDM8 vector to cDNA were based on small scale ligations.

CDM8 vector from a CsCl preparation (20 μg) was digested with *Bst*XI (New England Biolabs, Beverly, USA) at 55°C for 2.5 h. After phenol extraction and ethanol precipitation, the 4.3 kb vector band was separated from the 350 bp stuffer band by gel filtration on a Sephacryl S1000 column (25 × 0.8 cm) (Pharmacia Ltd, Milton Keynes, UK). Fractions containing vector were detected by measuring optical density at 260 nm. Purity of fractions was checked on a minigel and by trial ligations.

The cDNA was transformed into competent MC1061/P3 (Seed and Aruffo, 1987), plated out and incubated overnight at 37°C on agar plates (22 × 22 cm) containing ampicillin at 12.5 μg/ml and tetracycline at 7.5 μg/ml. The complexity of the library was estimated to be 5×10^5 and the average cDNA insert size was 1.7 kb. The colonies were resuspended in L-broth by scraping, and plasmid purified by the standard CsCl method.

Selection of positive clones

The selection of positive clones was basically as in Seed and Aruffo (1987) except that Dynal beads (Dynal Ltd, Oslo, Norway) were used to select positive cells instead of panning. Purified plasmid from the cDNA library was transfected into ten 75 cm^2 flasks of 50% confluent monkey COS cells (Gluzman, 1981) by DEAE−dextran (Sussman and Milman, 1984). Cells were trypsinized at 24 h and replated in RPMI−10% FCS. After a further 48 h the cells were detached by incubation with EDTA−phosphate buffered saline (PBS) (0.2 g/l), centrifuged and resuspended in 200 μl OX40 spent tissue culture medium in a total of 2 ml at 4°C for 1 h. After three washes with 1 ml PBS, 0.2% bovine serum albumin, 3×10^7 cells were incubated at 4°C for 30 min, with 4×10^7 magnetic Dynal beads coated with sheep anti-mouse Ig antibodies (Dynal Ltd, Oslo, Norway). Cells expressing OX40 antigen were rosetted by the Dynal beads and separated against a magnet. Plasmid DNA was recovered from these cells by the Hirt procedure (Hirt, 1967) and used to transform competent *Escherichia coli* MC1061/P3. Three further rounds of selection were by transfection of COS cells by spheroplast fusion and positive selection by Dynal bead rosetting. Plasmid DNA was prepared from single colonies by the alkali lysis method and transfected into COS cells by DEAE−dextran. After 48 h, seven out of 12 clones gave OX40 expression as detected by cytofluorography with a FACScan (Becton Dickinson, Oxford, UK).

Northern blot analysis

RNA was prepared by the acid guanidine thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987) from rat liver, spleen cells, lymph node, thymocytes and activated T cells prepared by stimulation of lymph node cells and thymocytes by concanavalin A for 3 days (see above). Samples (10 μg) were electrophoresed on a 1.2% agarose gel in formaldehyde. The RNA was transferred to GeneScreen Plus membrane and hybridized according to the manufacturer's instructions (New England Nuclear, Drierich, FRG). The cDNA insert was labelled with [α-^{32}P]dATP with the multiprime labelling system (Amersham International, Amersham, UK). The filters were washed at a stringency of 0.1 × SSC at 60°C and exposed for 7 days at −70°C.

Nucleotide sequencing and sequence comparisons

Restriction enzyme fragments were subcloned into M13mp18 and M13mp19. Nucleotide sequences were obtained by the dideoxy-chain termination method using a Sequenase sequencing kit (US Biochemical Corporation, Cleveland, OH, USA). The complete nucleotide sequence was sequenced in both strands with an average of 3.8 determinations per base. Nucleic acid sequences were analysed with the computer programs of Staden (1986). The NBRF database (Protein Identification Resource, National Biomedical Research Foundation, Georgetown, USA) was searched using the FASTP program (Lipman and Pearson, 1985). Sequence alignments and statistical analysis were with the ALIGN program using the mutation matrix, a bias of 6 and a gap penalty of 6 (Dayhoff *et al.*, 1983). The results from the ALIGN program are given as SDs of the score of the two actual sequences relative to the average best score from 300 random permutations of the amino acid sequences of the two proteins. These scores can be interpreted in terms of the probability that the two sequences being compared could have arisen by chance by random mutation from two unrelated proteins. The relationship between the SD score and the probability values is shown in the legend to Figure 3.

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