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The MRC OX-45 antigen of rat leukocytes and endothelium is in a subset of the immunoglobulin superfamily with CD2, LFA-3 and carcinoembryonic antigens

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The MRC OX-45 cell surface antigen is a glycoprotein of 45 000 apparent mol. wt of rat leukocytes and endothelium. Antibodies against the antigen inhibit T lymphocyte responses by stimulation of suppression by accessory cells. We now report the immunochemical characterization of this antigen and its cDNA sequence. The predicted protein sequence contains 240 amino acids including a leader sequence of 22 residues and a carboxy-terminal sequence of 23 residues that is replaced in the processed molecule by a glycosyl-phosphatidylinositol anchor attached at serine 195. Two Ig-related domains are predicted to account for all of the processed sequence and the circular dichroism spectrum shows pure β-structure. The amino-terminal domain is V-like, but without a disulphide bond, while the second domain is C-like (C2-SET) with two disulphide bonds. The sequence matches particularly well with the extracellular parts of LFA-3 and CD2 antigens and the first two domains of carcinoembryonic antigen and non-specific, cross-reacting antigen.

Key words: MRC OX-45 antigen/leukocytes/endothelium/glycoprotein/Ig superfamily/glycosyl-phosphatidylinositol anchor

Introduction

The MRC OX-45 antigen is a glycoprotein of ~45 000 mol. wt that was identified with the MRC OX-45 and OX-46 mouse monoclonal antibodies which were raised against rat T lymphocyte blasts (Arvieux *et al.*, 1986a,b). Both the OX-45 and OX-46 antibodies inhibited stimulation of T lymphocyte DNA synthesis in a mixed lymphocyte response. However, this inhibition was unusual in that it occurred when spleen cells were used as stimulators, but not when activation was with allogeneic dendritic cells. It was shown that inhibition occurred via activation of non-specific suppression by accessory cells that are thought to be macrophages. The OX-45 antigen was found to be expressed weakly on all thymocytes and lymphocytes and in large amounts on activated T lymphocytes and macrophages. All endothelium appeared to be positive, as were erythrocytes (Arvieux *et al.*, 1986b).

Some of the properties of the rat OX-45 antigen were similar to those of human LFA-3 antigen which interacts with the T lymphocytes CD2 antigen to mediate adhesion between T lymphocytes and other cell types (Selvaraj *et al.*, 1987). On the basis of the immunochemical data it seemed possible that OX-45 was rat LFA-3 or a related antigen. To resolve these possibilities we have cloned and sequenced the OX-45 antigen and also determined the properties of the fully processed glycoprotein. The molecule is shown to have a carboxy-terminal glycosyl-phosphatidylinositol (GPI) hydrophobic anchor and shows sequence similarities to LFA-3 and other Ig-superfamily molecules.

Results and discussion

cDNA cloning and sequence of OX-45 antigen

The amino-terminal sequence of OX-45 antigen was reported previously (Arvieux *et al.*, 1986b) and further sequence was determined from tryptic peptides to yield the following total peptide data:

Amino-terminal: FQDQSVPNVNAITGSYV−L−KLK
Tryptic 1: LTWLHTTNQKILEYF
Tryptic 2: GDYYMR

The sequences GDYYMR and HTTNQK allowed the synthesis of oligonucleotide probes of 17 residues with a redundancy of 64 and 128 respectively, and these probes were used to screen a rat lymph node cDNA library constructed in the plasmid pATX (Barclay *et al.*, 1987). A clone was first isolated that encompassed all of the processed protein sequence but lacked some of the leader sequence. Then a full-length clone was obtained from a rat T blast library in the same plasmid (Figure 1a) and the composite sequence is shown in Figure 1b. The only unusual feature of the nucleotide sequence was the presence of five homologous segments of ~75 nt in length at the 3′ end of the clone. The sequence in segment 1 was:

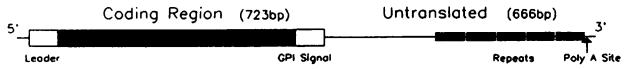
aagataaatgcattccttttaccaaaatatgtggctat
cttatactaatgttgtttatatcactctttttttata

with variations at the level of ~15% in the other repeats.

The translated protein sequence is shown in Figure 1c and the peptides match this with the exception of two residues towards the end of the amino-terminal sequence. The discrepancies are in regions where the protein sequence assignments were becoming unclear.

The sequence in Figure 1c shows a typical leader, five potential *N*-linked glycosylation sites and a carboxy-terminal sequence of the type that suggests the presence of a GPI anchor (Ferguson and Williams, 1988). Sequence patterns suggest the presence of two Ig-related domains in the molecule taking in sequences 1−106 and 109−191 respectively. Before OX-45 was sequenced it was thought that this might be the rat LFA-3 antigen, but sequence comparison showed that this was not the case (see below). However,

A. cDNA Clone pX45.2



B. Nucleotide Sequence

```
gtctccagtgtcacaggcagcttctcaaagtattatgtacttcaaaaaacggagatggtt   60
tctgatcctggaatcgcttttgctgtctttggtaactggatttcaagatcaatcagtacc  120
aaatgtaaatgccataaccggcagcaacgtaaccctgacaatcctgaagcacccacttgc  180
atcgtatcaacgtctcacctggcttcatactaccaaccagaagattttagagtacttccc  240
taatggtaaaaaaactgtcttcgagtctgtatttaaagacagggtcgatcttgacaaaac  300
aaatggtgcacttcgtatctataatgtctcgaaagaggacagaggtgactactacatgag  360
aatgttgcacgaaactgaggaccagtggaagataaccatggaagtatacgatcttgtgtc  420
caagcctgccatcaaaatcgagaagactaaaaatttgactgactcctgtcacctgaggct  480
atcatgtaaggtagaggaccaaggtgttgactatacttggtatgaggactcggggccctt  540
tccccaaaggaatccaggatatgtactcgaaatcaccatcactccacacaacaagtctac  600
attttacacctgccaagtcagcaatcctgtaagcagcgagaacgacacactgtactttat  660
tccaccttgtacgctggccagatcttctggagtccattggattgcagcttggctagtggt  720
cacgttatccatcattcccagcatcctgctagccctgacaagatctctcctcagtcaagaa  780
ggaaacatcaaagccgtatcttgccttcatcccctgcactgctcctaaccattgacgctg  840
ctctggctccgtggagcaaaggaaagtgtgttattgttatctgtgctggtttgaatgcat  900
gctctatggagtaagcacaggacctagtacagtgctacatcactgatctttacaaagatt  960
ctaagctaatttttttaaaaactgggggtagcatctaatttttatataccctagttgtttcc 1020
taacattcattgaagataaatgcattccttttaccaaaatatgtggctatcttatactaa 1080
tgttgtttatatcactctttttttataaagataaatgcattccttttaccaaaatatgtga 1140
ctatatcatgctaatgttgtttatatcactccttttttgtgaagataaatgcattccttttt 1200
accaaaatatgtgactatgtcatgctaatgttgtttatatcactcttttttataaagata 1260
aatgcattccttttaccaaaaacatgtggctatattatactaatgttgtttatatcactc 1320
ttttttataaagataaatgcattccttctaccaaaatatgtgactatatcatgctaatgt 1380
tgtttatatcacctttttaaaaataaaatcttttcacatactaaaa                1426
```

C. Deduced Protein Sequence

```
                -1                                          Leader
MYFKKRRWFLILESLLLSLVTG
                                                50
FQDQSVPNVNAITGSNVTLTILKHPLASYQRLTWLHTTNQKILEYFPNGK          Domain 1
                                               100
KTVFESVFKDRVDLDKTNGALRIYNVSKEDRGDYYMRMLHETEDQWKITM
                                               150
EVYDLVSKPAIKIEKTKNLTDSCHLRLSCKVEDQGVDYTWYEDSGPFPQR          Domain 2
                                          195
NPGYVLEITITPHNKSTFYTCQVSNPVSSENDTLYFIPPCTLARS
                    218                                      GPI Signal
SGVHWIAAWLVVTLSIIPSILLA*
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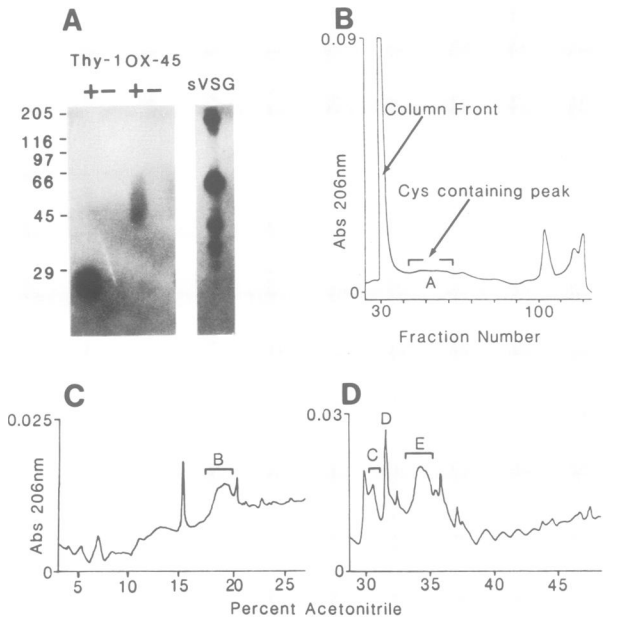
Fig. 1. (**a**) Diagrammatic representation of pX45.2, a cDNA clone encoding the precursor of the rat OX-45 antigen. (**b**) Composite nucleotide sequence derived from pX45.1 and pX45.2. The initiation, termination and poly(A) addition signals are boxed. (**c**) Predicted protein sequence of the precursor of the OX-45 antigen. Potential *N*-linked glycosylation sites are underlined.

identities at the level of ~50% are seen with the recently published sequence for human Blast-1 antigen (Staunton and Thorley-Lawson, 1987) and it is most likely that OX-45 and Blast-1 are species homologues.

The GPI anchor

To test for a GPI anchor and determine its position of attachment, pure OX-45 glycoprotein was reduced and alkylated and digested for 72 h with α-chymotrypsin. The digested material was then fractionated by gel filtration on Biogel P-30 in the presence of Brij 96 non-ionic detergent. In this system the peptides are retarded by the column while any peptide with a GPI group will bind to the large non-ionic detergent micelle and be recovered at the gel front (Campbell *et al.*, 1981). Analysis of the front should then reveal the amino acids of the peptide plus ethanolamine and glucosamine of the GPI anchor. Galactosamine may also be detected if the relevant anchor has an *N*-acetyl galactosamine side chain (Tse *et al.*, 1985; Homans *et al.*, 1988). The protein was fully digested since virtually no radioactivity from [^{14}C]iodoacetic acid used in the alkylation was seen at the void volume (not shown). The amino acid analysis of the front fraction gave the composition: Ala (0.91), Arg (1.00), Ser (1.09), ethanolamine (2.37), glucosamine (1.06), galactosamine (0.13), Asp (0.24), Glu (0.22), Phe (0.09),



E

Amino Acid Analysis (nmoles of residue)

Residue	Peak B	Peak C	Peak D	Peak E
C	0.17	0.08	0.08	0.02
D/N	0.72	0.30	0.25	0.19
E/Q	0.59	0.22	0.21	0.10
GlucNH2	0.57	0.19	0.06	0.38
S	0.47	0.21	0.12	0.13
G	0.23	0.28	0.14	0.08
H	0.05			0.10
R	0.05	0.01		0.02
T	0.32	0.14	0.06	0.26
A	0.04	0.03	0.02	0.02
P	0.21	0.09		0.13
Y	0.16	0.03	0.04	0.07
V	0.48	0.16	0.16	0.07
M				
I	0.14			0.15
L	0.21	0.01		
F	0.03	0.01	0.01	0.11
K	0.15	0.03	0.06	0.11

F

Sequence Obtained from Peak B

```
S - - V E D Q G V
T - Q V - N P V -
I T - T
```

Assignment for Peaks C, D and E Based on Amino Acid Analysis and cDNA Sequence

```
D  S C K V E D Q G V D
C  T C Q V S N P V S S E N D
E  I T I T P H N K S T F Y
```

Fig. 2. (**a**) Pure Thy-1 and OX-45 glycoproteins were digested with *S.aureus* PI-PLC and then analysed by Western blot with a rabbit anti-VSG-specific antiserum (Zamze *et al.*, 1988). The blot was developed with a radioactive antiserum against rabbit immunoglobulins. + and − refer to whether or not the samples were digested with PI-PLC. (**b**) Gel filtration of OX-45 peptides on a Biogel P-30 column. Peak A was subjected to reverse-phase HPLC and the trace is shown in (**c**). Peak B was oxidized with performic acid and further fractionated by HPLC (**d**). The amino acid analysis of 30% of peak B and 60% of peaks C, D and E is shown in (**e**). The assignment for peaks C, D and E is given in (**f**) and is based on the sequence obtained from 15% of peak B, the amino acid analysis and the known protein sequence.

Gly (0.38), His (0.03), Ile (0.13), Lys (0.13), Leu (0.19), Met (0.02), Pro (0.16), Thr (0.18), Val (0.11), Tyr (0.09). Sequencing of this material gave Ala-Arg-Ser. These results show that a GPI anchor is attached to the protein on the carboxyl group of Ser 195. This excludes from the fully processed molecules the amino acid residues 196−218 that are predicted from the cDNA. This sequence is hydrophobic but lacks basic residues at the carboxy-terminal side of the hydrophobic region. Sequences of this type are believed to function as signal sequences that are cleaved off in the process of GPI anchor attachment (Ferguson and Williams, 1988).

To show the presence of phosphatidylinositol (PI), pure OX-45 was digested with *Staphlyococcus aureus* phospholipase C that is specific for phosphatidylinositol (PI-PLC). Cleavage of the PI was detected with an antibody against the cleaved GPI anchor of *Trypanosoma brucei*

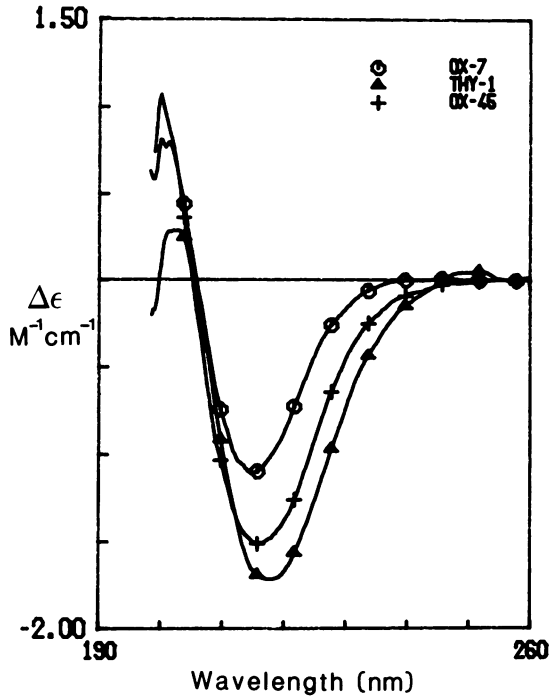


Fig. 3. CD spectra of OX-45 and Thy-1 glycoproteins compared with OX-7 monoclonal IgG. The spectra were measured on a Jasco J41 CD spectrometer using a cell of 0.1 mm pathlength. OX-45, Thy-1 and OX-7 were at 0.86, 0.98 and 1.43 mg protein/ml in 10 mM phosphate buffer, pH 8.2.

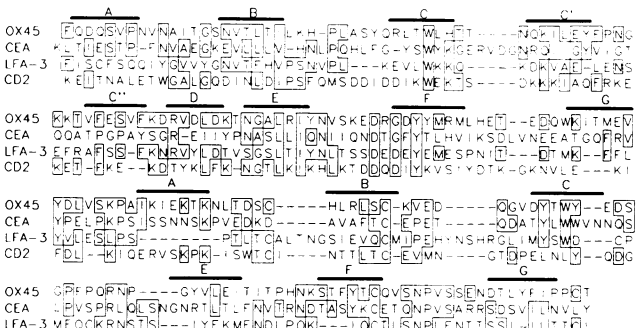


Fig. 4. Sequence alignment of rat OX-45 with human CEA (domains I and II), LFA-3 and CD2. Identities are boxed and the dashes indicate gaps to maximize alignments. The bars and letters above the sequences indicate the position of postulated β-strands based on comparisons with immunoglobulin structures.

variant surface glycoprotein (VSG) by Western blotting. In the anti-VSG serum a sub-fraction of the antibody is specific for the glucosamine and myoinositol with the cyclic phosphate that results from PI-PLC cleavage. This determinant appears to be revealed in all GPI anchors on cleavage with PI-PLC (Zamze *et al.*, 1988). With OX-45 glycoprotein the antibody reacted with material that had been digested with the enzyme but not in the control track where the enzyme was omitted (Figure 2a).

Disulphide bonds

To determine the arrangement of the disulphide bonds in OX-45 the pure molecule was digested with pepsin in the presence of Brij 96 and fractionated on a Biogel P-30 column in 80 mM acetic acid. Amino acid analysis of the eluted material suggested that cleavage had not occurred between Cys 123 and Cys 129. Thus, the majority of the Cys was located at the column front where it was still covalently attached to the GPI anchor which was in turn attached to the Brij 96 micelles. In an effort to liberate Cys-containing peptides from the material at the column front further digests were done with trypsin and chymotrypsin followed by gel filtration. In both cases the majority of the Cys again ran at the column front but from the chymotrypsin digest small amounts of Cys were also detected in a broad retarded peak (Figure 2b). This material was subjected to reverse-phase HPLC (Figure 2c) and the resulting, broad, Cys-containing peak gave the sequence shown in Figure 2f. This indicated the presence of a dipeptide containing Cys 129 and Cys 171. Oxidation of this material and further fractionation by reverse-phase HPLC (Figure 2d) allowed for the isolation of two peptides containing Cys 129 and Cys 171 respectively (Figure 2e and f). Thus, a disulphide bond is established between these two residues. It also seems likely that Cys residues 123 and 190 are disulphide-bonded since this would account for all of the Cys peptides eluting with the Brij 96 micelles after the pepsin digestion.

Circular dichroism spectra

Since the OX-45 molecule has a GPI anchor, the secondary structure of the extracellular portion can be assessed by determination of the circular dichroism (CD) spectrum. This was determined for pure OX-45 in comparison with Thy-1 and the OX-7 monoclonal mouse IgG_1 antibody that is specific for the Thy-1.1 determinant. The results are shown in Figure 3 and were identical for readings taken in phosphate buffer alone in which Thy-1 and OX-45 will exist as oligomers, or in phosphate buffer plus 0.33% deoxycholate in which the molecules will be in the monomeric from (Kuchel *et al.*, 1978). The spectra for all three molecules are compatible with pure β-structure with no evidence of α-helix. The intensity of negative signal per amino acid is about the same for Thy-1 and OX-45, both of which are believed to consist solely of Ig-related domains with virtually no interconnecting or hinge sequences. For OX-7 IgG_1 the negative signal was less intense and this was previously seen for a human myeloma IgD versus Thy-1 (Campbell *et al.*, 1979). Possibly this difference is due to the presence of more random sequence in the immunoglobulins than the other structures.

Glycopeptides

Glycosylation at Asn residues 16, 75, 164 and 181 was established by isolation of peptides that contained glucosamine by analysis. The sequence assignment was made by sequencing (Asn 16 and 75) or by composition where this gave unambiguous identification of an expected peptide (Asn 164 and 181). A glycopeptide including Asn 118 was not isolated but nor has the unglycosylated peptide been isolated. Thus glycosylation or otherwise at this position remains to be established.

Ig superfamily relationships

It is suggested that the extracellular part of OX-45 consists solely of two Ig-related domains encompassing residues 1–106 (domain I) and 109–191 (domain II). Domain I has the hallmarks of IgV and V-related domains (called V-SET

Align Scores for OX45 Domain I Versus V and V-Related Domains

	V_λ	V_K	V_H	Tcr α	Tcr β	Tcr γ	CD4 I	CD8 I	CD8 II	PIR I	OX2 I	Thy1	P_0	CD2 I
Rat OX45 I	3.2	3.8	4.4	3.9	3.4	3.3	4.3	3.9	3.2	1.9	3.4	3.1	3.0	5.3

Align Scores for Various Two Domain Structures

	Rat OX45	Blast 1	CEA	NCA	LFA-3	Rat CD2	Human CD2	NCAM1	NCAM2	NCAM3	NCAM4	ClassII β	Ig_λ	Ig_K
Rat OX45		26	8	8	8	6	7	4	6	5	4	3	5	6
Blast 1	26		9	9	6	5	6	4	3	8	4	3	5	4
CEA	8	9		51	2	3	4	7	5	7	4	3	3	4
NCA	8	9	51		3	3	4	7	5	6	3	3	4	4
LFA-3	8	6	2	3		6	6	1	3	3	4	1	3	3
Rat CD2	6	5	3	3	6		30	4	2	3	7	1	4	2
Human CD2	7	6	4	4	6	30		4	2	4	7	1	3	2
NCAM1	4	4	7	7	1	4	4		8	9	9	3	6	5
NCAM2	6	3	5	5	3	2	2	8		12	10	4	7	6
NCAM3	5	8	7	6	3	3	4	9	12		12	3	7	8
NCAM4	4	4	4	3	4	7	7	9	10	12		3	6	6
ClassII β	3	3	3	3	1	1	1	3	4	3	3		9	7
Ig_λ	5	5	3	4	3	4	3	6	7	7	6	9		26
Ig_K	6	4	4	4	3	2	2	5	6	8	6	7	26	

Fig. 5. The single domains used in the first part were defined by taking inclusive sequences beginning 20 residues before and ending 20 residues after the Cys residues (or equivalent) postulated to be in the position of the conserved Ig-like disulphide bond. The two domain sequences used in the second part were contiguous and included sequences starting 20 residues before the amino-terminal Cys position of domain I and ending 20 residues after the carboxy-terminal Cys of domain II in the two domain pair. The domain pairs are obvious except for CEA, NCA, NCAM and class II β. NCAM 1 includes NCAM domains I and II; NCAM 2, II and III; NCAM 3, III and IV and NCAM 4, IV and V. For class II β there is only one Ig-like domain but the whole extracellular sequence was used in the comparisons. Scores are in SD units rounded upwards to the nearest whole number and are derived from the ALIGN program (Dayhoff *et al.*, 1983) with a bias of 6 and gap penalty of 6 with 200 random comparisons. Scores of >3 are considered to be of significance (Williams, 1987). Sequences are either referred to in the text or are from the references below or NBRF (Protein Indentification Resource, 1986, Release 8.0) database codes: human V_λ [L1MS4E]; human $V_\varkappa$ [K1HURY]; human V_H [G1HUNM]; mouse TCRα [RWMSAV]; human TCRβ [RWHUVY]; mouse TCRγ [RWMSV1]; human CD4 [RWHUT4]; rat CD8 chains 1 and 2 (Johnson and Williams, 1986); rabbit poly(Ig) receptor (PIR) [QRRBG]; rat OX-2 [TDRTOX]; rat Thy-1 [TDRT]; rat P_0 [MPRTO]; chicken NCAM (Cunningham *et al.*, 1987); MHC class II β [HLHU3D]; human Ig ϰ [K1HUEU]; human Ig λ [L4HUKN].

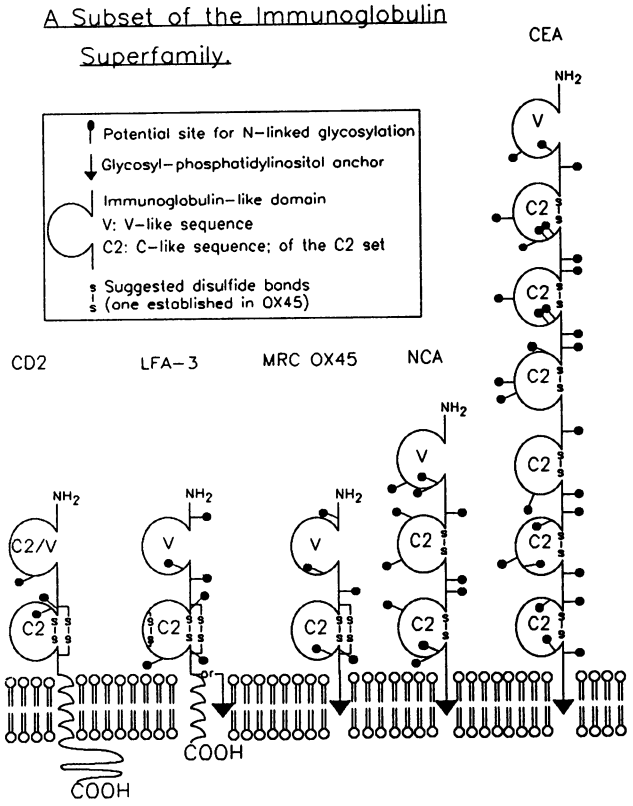


Fig. 6. A subset of the immunoglobulin superfamily. The diagram shows models for rat OX-45 and human CD2, LFA-3, NCA and CEA. The presence of a GPI anchor has been established for OX-45, LFA-3 (Seed, 1987; Wallner *et al.*, 1987) and CEA (Hefta *et al.* 1988) and is suggested for NCA on the basis of the carboxy-terminal protein sequence.

sequences, Williams, 1987) except that it lacks Cys residues that could account for the conserved disulphide bond that is commonly seen in Ig superfamily domains. In Figure 4, suggested β-strands corresponding to those of a V domain are shown for OX-45 aligned with LFA-3, carcinoembryonic antigen (CEA) and CD2, which are discussed below. Cys residues equivalent to those of a conserved disulphide bond would be expected at residues 21 and 86 in the OX-45 sequence, which instead shows Ile and Met at these positions. The hydrophobic side chains of these amino acids would be suitable as in-pointing groups to help stabilize the hydrophobic interaction between the two postulated β-sheets. Results of statistical tests to evaluate the similarity of OX-45 domain I to V-SET sequences are shown in Figure 5 and, with few exceptions, significant scores were obtained.

OX-45 domain II has similarities to Ig-related domains that are of a truncated C domain type with some similarities to the sequence patterns of V domains in the second half of the domain. Sequences of this type are referred to as C2-SET sequences (Williams, 1987); Figure 4 shows postulated β-strand positions. The Cys residues in β-strands E and F would correspond to those that form the conserved disulphide bond of the Ig-fold, and in OX-45 these were shown to be disulphide-linked. The probable disulphide bond between Cys 123 and 190 is also compatible with the Ig-fold and is similar to the second disulphide bond seen in Thy-1 (Campbell *et al.*, 1981). In the Blast-1 antigen, which is the human equivalent of OX-45, two further Cys residues are seen at positions equivalent to Phe 168 and Tyr 185 of OX-45. A disulphide bond between these positions would also be compatible with an Ig-like fold.

Taking OX-45 domains I and II together it can be seen from Figure 6 that the whole molecule shows similarities to the structure proposed for the extracellular parts of CD2 (Sewell *et al.*, 1986; Williams, 1987) and LFA-3 (Seed, 1987) and the first two domains of non-specific, cross-reacting antigen (NCA; Tawaragi *et al.*, 1988, Neumaier *et al.*, 1988) and CEA (Oikawa *et al.*, 1987). Statistical analysis by the ALIGN program (Dayhoff *et al.*, 1983) (Figure 5) supports the argument that the molecules shown in Figure 6 can be regarded as a subgroup within the

Ig-superfamily. This group is characterized by an amino-terminal domain that lacks a disulphide bond but otherwise would be characterized as having a V-SET sequence pattern. The V-SET assignment is clear for OX-45, CEA and LFA-3, but for CD2 the choice between a V-SET or C2-SET assignment is less clear-cut. Previously a C2 categorization was suggested (Williams, 1987) but the alignments in Figure 4 suggest that a V-fold may be present. Other features of the molecules in Figure 6 are the occurrence of the GPI anchor in all of the molecules except CD2 and of two proposed disulphide bonds that are similar in domain II of OX-45, LFA-3 and CD2. It seems likely that NCA and CEA evolved from a structure like OX-45 firstly by gene duplication of an equivalent of domain II of OX-45 to give NCA, followed by two rounds of pairwise duplications of the two C2-SET sequences of an NCA-like structure to give CEA.

Alternatives to the structural proposals in Figure 6 have been considered for CD2 by Clayton *et al.* (1987). They argued against the presence of Ig-related domains in the extracellular part of CD2 and suggested the presence of alternating α and β secondary structure. However, this suggestion was preferred on the basis of secondary structure predictions which were not decisively in contradiction to the β-strand predictions for Ig-folds as in Figure 4. The OX-45 antigen does not contain α-helix on the basis of the CD spectrum (Figure 3) and this, together with the similarities between OX-45 and CD2, argues against the possibility of a structure containing both α- and β-strands. These issues will ultimately be resolved by tertiary structure determination.

Materials and methods

Isolation of OX-45 glycoprotein and cDNA clones

OX-45 glycoprotein was purified from spleen or thymus as in Arvieux *et al.* (1986b) and tryptic peptides were produced and sequenced as for CD8 antigen (Johnson *et al.*, 1985). A rat lymph node cDNA library (Barclay *et al.*, 1987) was screened with degenerate oligonucleotide probes constructed using an Applied Biosystems 381A DNA Synthesizer. The hybridization conditions and sequencing strategy were as for leukosialin (Killeen *et al.*, 1987).

GPI anchor

Pure OX-45 glycoprotein was reduced and alkylated with [^{14}C]iodoacetic acid (Johnson *et al.*, 1985). After dialysis into 10 mM NH_4HCO_3, it was freeze-dried and resuspended at 1–4 mg/ml in the same buffer prior to digestion with 4% (w/w) α-chymotrypsin added in two aliquots over 72 h at 37°C. The resultant digest was made to 0.5% with Brij 96 detergent and chromatographed in 80 mM CH_3COOH on a 150 × 1 cm Biogel P-30 column. The effluent was monitored at 206 nm and 5% of each fraction was used to determine ^{14}C radioactivity. Further fractionation of peaks was by reverse-phase HPLC using an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) and a 4.6 mm i.d. Whatman Partisil-10 ODS-3 column. Amino acid analysis was performed using either an LKB Amino Acid Analyser or the Waters Pico-Tag system. The material running at the front of the gel filtration column and the glycopeptide HPLC peaks from this and the digests described below were identified on the basis of composition. When necessary, protein sequence was obtained using an Applied Biosystems 470A gas phase sequencer. *S.aureus* phospholipase C digestion and Western blotting with the anti-VSG-specific antiserum were as described by Zamze *et al.* (1988).

Disulphide bonds

OX-45 glycoprotein at 1 mg/ml in 0.5 M CH_3COOH was digested with 2% (w/w) pepsin added in two aliquots over 72 h at 37°C. The digest was then subjected to gel filtration in the presence of Brij 96 as above. The front-running material was freeze-dried, resuspended in 0.1 M NH_4CH_3COO containing 2% (w/w) trypsin and incubated at 37°C for 3.5 h before being reapplied to the gel filtration column. Again, the material running with the column front was freeze-dried and resuspended in the same buffer prior to an 8-h digestion with α-chymotrypsin, and a further round of gel filtration. Reverse-phase HPLC of an absorbance pool from the latter chromatogram allowed for the identification of one of the two OX-45 disulphides. For confirmation, the disulphide bond was oxidized with performic acid and the two constituent peptides were separated by reverse-phase HPLC using an acetonitrile gradient in 0.1% TFA and a 3 mm i.d. Shandon Hypersil-5 column.

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