Affinity and Kinetics of the Interaction between Soluble Trimeric OX40 Ligand, a Member of the Tumor Necrosis Factor Superfamily, and Its Receptor OX40 on Activated T Cells*

(Received for publication, August 13, 1996, and in revised form, November 15, 1996)

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OX40 ligand (OX40L) and OX40 are members of the tumor necrosis factor and tumor necrosis factor receptor superfamilies, respectively. OX40L is expressed on activated B and T cells and endothelial cell lines, whereas OX40 is expressed on activated T cells. A construct for mouse OX40L was expressed as a soluble protein with domains 3 and 4 of rat CD4 as a tag (sCD4-OX40L). It formed a homotrimer as assessed by chemical cross-linking and gel filtration chromatography. Radiolabeled sCD4-OX40L bound to activated mouse T cells with a high affinity (K\text{D} = 0.2–0.4 nM) and dissociated slowly (\kappa_{\text{off}} = 4 \times 10^{-5} \text{ s}^{-1}). The affinity and kinetics of the OX40L/OX40 interactions were studied using the BIAcore™ biosensor, which measures macromolecular interactions in real time. The extracellular part of the OX40 antigen was expressed as a soluble monomeric protein and immobilized on the BIAcore sensor chip. sCD4-OX40L bound to the OX40 with a high affinity (K\text{D} = 3.8 nM), although this was lower than that determined on the surface of activated T cells (K\text{D} = 0.2–0.4 nM), where there is likely to be less restriction in mobility of the receptor. In the reverse orientation, sOX40 bound to immobilized sCD4-OX40L with a stoichiometry of 3.1 receptors to one ligand, with low affinity (K\text{D} = 190 nM) and had a relatively fast dissociation rate constant (\kappa_{\text{off}} = 2 \times 10^{-2} \text{ s}^{-1}). Thus if the OX40 receptor is cleaved by proteolysis, it will release any bound ligand and is unlikely to block re-binding of ligand to cell surface OX40 because of the low monomeric affinity.

The OX40 antigen was defined in the rat as an antigen with a highly restricted distribution being present only on activated rat CD4+ T lymphocytes and absent from resting lymphocytes and other tissues (1). In the mouse OX40 is present on both CD4+ and CD8+ activated T cells (2, 3). It is a transmembrane glycoprotein whose extracellular portion contains three cysteine-rich repeats of approximately 40 amino acids (4). Similar repeats are found in the extracellular parts of several other membrane glycoproteins, including the low affinity nerve growth factor receptor, two receptors for tumor necrosis factor (TNFR) (1) and the leukocyte antigens CD40, CD27, CD30, 4–1BB, and Fas (CD95) that make up the TNFR superfamily (reviewed in Refs. 5 and 6). The structure of the TNFR I shows that the cysteine-rich repeats form a linear array of small domains, which comprise the binding site for TNF (7).

The ligands of the TNFR superfamily members, with the exception of nerve growth factor and other neurotrophins, also share sequence similarity (~15–36%) in what is now known as the TNF superfamily. These proteins are type II membrane proteins, and their similarity is confined to the COOH-terminal extracellular domains, which are often released as soluble proteins by proteolysis (reviewed in Refs. 6 and 8). Structural studies on TNF-α (9, 10), TNF-β (7), and CD40 ligand (11) show that they form homotrimers with a characteristic “jelly roll” β-sandwich. The stoichiometry of the interaction between TNFR I and TNF-β trimer is three to one (7).

The OX40 ligand (OX40L) is expressed on the surface of activated B (2) and T (12) lymphocytes and has been shown recently to be present on endothelial cell lines (13). The OX40L is involved in T cell help for B cells in the development of IgG responses (14, 15). The quaternary organization of OX40L is unknown, although sequence similarity with other members of the TNF superfamily suggests that it is likely to form a homotrimer. However, another member of this superfamily, 4–1BB ligand, forms a disulfide-linked homodimer (16), indicating that there exists heterogeneity in the quaternary structure among members of this superfamily.

A recombinant soluble OX40L-Fc fusion protein binds OX40 on activated T cells (3, 12), but the strength of binding could not be quantified, because the effect on the avidity brought about by the dimeric Fc portion of the OX40L-Fc construct could not be estimated. We have expressed a soluble recombinant protein containing the COOH-terminal extracellular domain of OX40L fused to domains 3 and 4 of rat CD4. The CD4 portion of the fusion protein has been used previously as a tag to generate monomeric fusion proteins, including several different domain types (17). Using the soluble CD4-OX40L (sCD4-OX40L) fusion protein, we have studied the affinity and kinetics of the OX40L binding to OX40 using (i) conventional radiolabeled ligand binding studies to activated T cells and (ii) the BIAcore™ biosensor, which detects macromolecular interactions in real time.
time using the phenomenon of surface plasmon resonance (18–20). We have also investigated the affinity and kinetics of sOX40 binding to immobilized OX40L, as soluble forms of several TNFR superfamily members, including both TNFR1 and TNFR2 (21, 22), CD27 (23), and CD30 (24) are released from the cell surface by proteolysis, and any functional effects of the soluble receptors will be limited by their monomeric affinities.

MATERIALS AND METHODS

Preparation of Constructs for Expression of Soluble Forms of OX40L and sCD4-OX40L—Rat CD4 and the NH2 terminus of OX40L were linked as a fusion protein with domains 3 and 4 of rat CD4. Rat CD4 leader sequence together with domains 3 and 4 was amplified by PCR using the plasmid pEE14/CD4L34 as a template (17). The 3′ antisense oligonucleotide used (5′-gctgctgccgtggtcactggtgg, in the leader sequence and tga-cggatcctattcacagactgtttccaagctgt-3′) introduced an EcoRI site and a downstream BamHI site (underlined). The PCR fragment was digested with XbaI and BamHI and ligated into the pEE14 vector at the XbaI and BclI sites to produce pEE14/CD4L34-RI. The extracellular domain of OX40L (121 residues 51–198) was amplified by PCR using single strand cDNA prepared from 3-day concanavalin A-stimulated mouse splenocytes. The PCR product, which contained an additional EcoRI site introduced with the sense oligonucleotide (5′-ggttgaattcttcctctccggcaaagga 3′), was digested and cloned into the EcoRI and HindIII sites of pEE14/CD4L34-RI. Rat CD4 leader sequence (5′-CACGCGGATCCTATTCACTGACTGTGGTCTATAACAAGTGATTGAACCACTGACTGTTCTCAGGAATTCAACCCTTTGGATAAAC-3′) was amplified using the oligonucleotides and cloned into the RI and HindIII sites to produce pEE14/CD4L34-RI. The predicted amino acid sequence at the junction between domain 4 of rat CD4 and the NH2 terminus of OX40L is SKGLN/SSSPA.

Truncated mutants of OX40 were prepared by PCR amplification from the cDNA, where the antisense oligonucleotide primer introduced a stop codon. The sequences of the 3′ oligonucleotides were as follows: sOX40(1–50)h, gctgctgccgtggtcactggtgg, in the leader sequence and tga-cggatcctattcacagactgtttccaagctgt; sOX40d1

were determined by usingcompetition binding experiments using unlabeled sCD4-OX40L to be 1100 Ci/mmol. Binding assays were carried out as described (28) using 72-h concanavalin A-activated mouse spleen cells (2 × 106/ml) were incubated at 4°C for 45 min with sCD4-OX40L (20 μg/ml) or soluble CD4 (26) as a control, washed once in cold PBS, 0.2% (v/v) bovine serum albumin (BSA) and incubated at 4°C with biotinylated MRC OX68 mAb (Serotec, Kidlington, UK) for 30 min, washed again, and incubated for 30 min with CD4 and CDS-FITC mAbs (Sigma Ltd., Poole, UK). After washing the cells were analyzed by flow cytometry.

Cell Binding Assays—sCD4-OX40L was radiolabeled with [125]iodo-
dide (Amersham International plc, Little Chalfont, UK) using the chloro-
amine-T method as described (27). The specific activity of [125]I-sCD4-
OX40L was determined by competition binding experiments using unlabeled sCD4-OX40L to be 1100 Ci/mmol. Binding assays were car-
rried out as described (28) using 72-h concanavalin A-activated mouse spleen cells (2 × 106/ml). Nonspecific binding was measured in the presence of at least 100-fold molar excess of unlabeled sCD4-OX40L. For dissociation kinetics, cells were incubated in 150 μl of binding medium containing [125]I-sCD4-OX40L (4.6 nM) for 2 h. The cells were then pelleted and resuspended in 1.2 ml of binding medium with or without unlabeled sCD4-OX40L (550 nM). Samples (180 μl) were removed at various times, and cell-bound radioactivity was determined as described (28). The values given for the dissociation and association rates are the mean of two independent experiments.

BIAcore™ Analysis—All experiments were performed at 25°C at the indicated flow rates in HBS buffer containing 150 mM NaCl, 1 mM, MgCl2, 0.005% surfactant P20 (Pharmacia Biosensor Ltd., Uppsala, Sweden) and 10 mM Hepes, pH 7.4. Proteins were covalently bound to the carboxylated dextran matrix by amine coupling using the amine coupling kit (Pharmacia) with the following modifications. Proteins were diluted to 20–45 μg/ml in 10 mM sodium acetate pH 5, and the activation period was varied from 1 to 7 min to obtain the desired level of immobilization after which the surface was conditioned with 0.1 M glycine/HCl buffer, pH 2.5, for 3 min. Recombinant proteins were purified by gel filtration on Superdex 200 (in HBS) prior to injection and were used immediately or after storage at 4°C for no longer than 1 week. Preliminary experiments showed no measurable difference in the behavior of the stored material.

The association and dissociation rate constants (k on and k off, respectively) for the interaction between sOX40 and immobilized scD4-OX40L were determined using the BIAevaluation 2.1 program (Phar-
macia-Biosensor). The dissociation kinetics can be described by a mono-
exponential decay, defined as

\[ R = R_0 e^{-k_{\text{off}}t} + R_1 \] (Eq. 1)

where t 0 is the start time for the dissociation and R0 is the response at the start of the dissociation. The k off was calculated using the model A + B = AB and fitting the data to the following equation,

\[ R = R_{\text{eq}} = 1 - e^{-k_{\text{off}}(t - t_0)} \] (Eq. 2)

where C is the molar concentration of the analyte (sOX40) and R eq is the steady state response level.

The following equation was used to analyze the dissociation kinetics for the interaction between sCD4-OX40L (0.38–1.53 μM) and immobilized sOX40,

\[ R = R_0 e^{-k_{\text{off}}(t - t_0)} + R_1 e^{-k_{\text{on}}(t - t_0)} \] (Eq. 3)

and

\[ R_1 + R_2 = R \text{ (observed)} \] (Eq. 4)

where t 0 is the start time for the dissociation, R2 is the amplitude of the dissociation with a rate constant k on, and R1 is the amplitude of the dissociation with a rate constant k off.

A model (A + B1 + B2 = AB1 + AB2) was used to describe the
The association kinetics of the binding of sCD4-OX40L to sOX40, where one analyte can interact with two independent binding sites. The $k_{on}$ values were calculated by fitting the association data to the equation,

$$R_{eq} = \frac{1}{1 + C} \left( e^{2k_{on}} - 1 \right) \left( e^{-k_{off}} - 1 \right)$$

(Eq. 5)

where $k_{on(1)}$ and $k_{off(1)}$ are rate constants describing one type of interaction with a steady state response level $R_{eq(1)}$, and $k_{on(2)}$ and $k_{off(2)}$ are rate constants describing a second type of interaction with a steady state response level $R_{eq(2)}$. $C$ is the molar concentration of the analyte.

RESULTS

Expression of Soluble Forms of OX40 and OX40 Ligand—In the absence of antibodies recognizing mouse OX40L, a chimeric protein was designed in which the extracellular domain of OX40L was fused to the COOH terminus of domains 3 and 4 of rat CD4 (sCD4-OX40L) (Fig. 1A). This allowed purification of the recombinant protein by immunoaffinity chromatography using the CD4 mAb MRC OX68. This chimeric protein system had been used previously to express domains from type I proteins where the CD4 domains are engineered COOH-terminal to the domains to be expressed (17). In this case the CD4 domains are NH2-terminal in order to mimic the orientation of OX40L at the cell surface, and the sCD4-OX40L was expressed at high levels in CHO cells (approximately 80 mg/liter). When analyzed by SDS-PAGE the sCD4-OX40L ran as a broad band of molecular mass 48 kDa presumably as a result of variable glycosylation at the two predicted N-glycosylation sites (one in OX40L and one in CD4 d3; Fig. 1B). This value for the apparent molecular mass is higher than that calculated from that of the polypeptide (42,237 Da) but consistent with the presence of two typical N-linked carbohydrates.

Four constructs encoding the extracellular regions of rat OX40 were transfected into CHO cells to provide proteins to analyze the binding of OX40 to OX40L. These consisted of domain 1 (amino-terminal), domain 1-2, all the cysteine-rich soluble recombinant forms of OX40 and OX40L. $T$ and TR indicate TNF and TNFR superfamily domains and 3 and 4 the IgSF domains of CD4 used in the chimeric proteins. Predicted N-glycosylation sites (filled lollipop symbols), and O-linked sites (—) are indicated. B, SDS-PAGE (12% gel) of sCD4-OX40L and sOX40. Lanes were loaded with 5 μg of each protein and gave similar molecular masses under reducing and nonreducing conditions showing that both proteins are not secreted as disulfide-linked oligomers. C, gel filtration of purified sOX40 and sCD4-OX40L on Superdex 200. Proteins were eluted with HBS at a flow rate of 0.5 ml/min. The elution positions of molecular mass standards are shown (alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; and carbonic anhydrase, 29 kDa).
domains (sOX40) and the complete extracellular region (sOX40h) (Fig. 1A). The two latter constructs containing all the cysteine-rich domains were expressed, but the single and double domain constructs were not, suggesting that the synthesis of a single domain is dependent on interactions with adjacent domains as might be expected by analogy with the TNFR structure (7). sOX40 was expressed at about 3 mg/liter and was purified from tissue culture supernatant by immunoaffinity chromatography using MRC OX40 mAb. Amino-terminal protein sequencing gave two sequences (TVKL and KLNC) corresponding to cleavage after residue 20 and 22. The proportion of each form varied with 80% after residue 20 in sOX40h and 55% in sOX40. It seems unlikely that the heterogeneity is due to proteolytic cleavage, as this site does not conform to any known protease specificity. It probably results from imprecise signal cleavage as both sites conform to consensus sequences (29).

sOX40 gave bands of 28 and 29 kDa on SDS-PAGE under reducing and nonreducing conditions, respectively (Fig. 1B). This is higher than the calculated molecular mass of the polypeptide (15,931 Da), even after taking into account the additional molecular mass of carbohydrate structures at the two predicted N-linked glycosylation sites of sOX40. The relative molecular mass of sOX40 was determined by laser desorption mass spectrometry to be 20,117 Da, which is consistent with the polypeptide mass plus two typical N-linked carbohydrates.

Gel filtration analysis showed that sOX40 migrated as a single peak corresponding to an apparent molecular mass of 29 kDa when calibrated with globular proteins (Fig. 1C). This is consistent with sOX40 being monomeric in solution, and the slightly higher apparent molecular masses obtained by SDS-
PAGE, and gel filtration compared with mass spectrometry, are likely to be due to its elongated structure. Sucrose gradient centrifugation analysis was also consistent with sOX40 being monomeric.²

On gel filtration the sCD4-OX40L eluted slightly before the 150-kDa protein marker (Fig. 1C). This suggests that sCD4-OX40L associates in solution to form non-covalent multimers and probably trimers given the molecular mass of 48 kDa determined by SDS-PAGE (Fig. 1B). This was confirmed by treating purified sCD4-OX40L with disuccinimidyl suberate, a homobifunctional cross-linker which reacts with primary amine groups of proteins. The proteins were separated by SDS-PAGE and detected by immunoblotting using a Cd4 mAb, MRC OX68. Fig. 2 shows that treatment with the cross-linker produced two major bands with molecular masses of 88 kDa and 130 kDa, corresponding to a dimer and a trimer, respectively. As no forms larger than the trimer were detected, this confirms the suggestion that sCD4-OX40L is a homotrimer, which would be consistent with the gel filtration data. A similar approach showed that TNF-α and FasL were homotrimers (30–32).

Binding of sCD4-OX40L to OX40 on the Surface of Activated Mouse T Cells—The sCD4-OX40L gave good labeling by flow cytometry of both CD4+ and CD8+ cells in cultures of mouse spleen cells activated with concanavalin A (Fig. 3). These results are consistent with the pattern of expression of the receptor, OX40, on mouse T cells (3, 12). The recombinant sCD4-OX40L was labeled with 125I and gave saturating binding to OX40 (Fig. 4). A Scatchard plot of the transformed binding data is shown (Fig. 5). This suggests that sCD4-OX40L is a homotrimer, which would be consistent with the gel filtration data. A similar approach showed that TNF-α and FasL were homotrimeric (30–32).

Binding of sCD4-OX40L to Immobilized SOX40—The interaction between OX40 and its ligand was further examined with a BIAcore™ using recombinant soluble mouse CD4-OX40L and rat sOX40 proteins (Fig. 1A). sOX40 was immobilized directly to the carboxylated dextran matrix (1373 response units (RU)) through the primary amine groups on sOX40. Purified sCD4-OX40L (22 μg/ml) (fractions 11–13; Fig. 1C) was injected and gave good binding (285 RU) to immobilized sOX40 compared to the small increase in the signal (34 RU) when BSA (22 μg/ml) was passed over immobilized sOX40 (Fig. 6). When the injection was completed, the trimeric sCD4-OX40L dissociated very slowly (Fig. 6). The specificity of the interaction was demonstrated using the MRC OX40 mAb, which was shown previously to block the binding of OX40L to OX40 on cells (3). Injection of MRC OX40 mAb reduced the binding to sCD4-OX40L to the level observed with an equivalent amount of BSA (Fig. 6), while injection of a control mAb (MRC OX21) had no effect. Elution of the bound mAb with glycine/HCl (0.1 M, pH 2.5) restored the ability of sCD4-OX40L to bind to immobilized sOX40 (Fig. 6).

The kinetics of binding of sCD4-OX40L to OX40 on activated T cells were examined. A value for $k_{\text{on}}$ of $1.9 \times 10^5$ M$^{-1}$ s$^{-1}$ was determined from the initial reaction rates of 125I-sCD4-OX40L binding to activated cells (Fig. 5A; Table I) as described (27, 33). The dissociation rate was measured by incubating activated cells with near-saturating concentrations of 125I-sCD4-OX40L until binding reached equilibrium (2 h). Cells were pelleted and then resuspended in a relatively large volume of medium. As a result the rate of ligand re-association during the dissociation phase was negligible (not shown). The dissociation rate was also examined in the presence of excess unlabeled sCD4-OX40L. The dissociation of 125I-sCD4-OX40L at 4 and 23 °C in the absence of competitor and at 4 °C in the presence of competitor appeared to follow first order kinetics (Fig. 5B; Table I). The $k_{\text{on}}$ values were as follows: 4 °C = 3.8 $\times 10^{-5}$ s$^{-1}$, 23 °C = 4.4 $\times 10^{-5}$ s$^{-1}$, and 4 °C with competitor = 12 $\times 10^{-5}$ s$^{-1}$. As the sCD4-OX40L is a trimer the first order kinetics are compatible with the rate-limiting step being the association of the ligand to one receptor, whose half-life is then sufficiently long ($t_{1/2} = 35$ s; see below) to enable the ligand to associate with further receptors. The dissociation kinetics of 125I-sCD4-OX40L at 23 °C in the presence of competitor were complex (Fig. 5B; Table I), displaying an initial fast $k_{\text{off}}$ ($k_{\text{off}(1)} = 900 \times 10^{-5}$ s$^{-1}$) followed by a slow $k_{\text{off}}$ ($k_{\text{off}(2)} = 11 \times 10^{-5}$ s$^{-1}$). The faster $k_{\text{off}}$ values obtained in the presence of the competitor were presumably due to competition of unlabeled sCD4-OX40L with 125I-sCD4-OX40L for re-binding when one or two binding sites dissociate. A value of 0.2 mM for the $K_D$ was calculated from measurements of the $k_{\text{on}}$ and $k_{\text{off}}$ (at 4 °C), which is in agreement with the $K_D = 0.4$ mM obtained from the equilibrium binding data (Fig. 4; Table I). Thus the OX40 ligand binds with high affinity to its receptor, and this is consistent with a multimeric interaction.

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### Table I

**Summary of kinetics of the interaction between OX40 and OX40L.**

| Reactant 1   | Reactant 2    | Temperature | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_D$ |
|-------------|--------------|-------------|----------------|-----------------|------|
| sCD4-OX40L  | OX40 on cells| 23          | ND             | 4.4             | 900  |
| +Competitor  | OX40 on cells| 23          | ND             | 12              | 900  |
| sCD4-OX40L  | sOX40 (BIAcore) | 25        | 0.7            | 27              | 3.8  |
| (Higher concentration) | sOX40 (BIAcore) | 25    | 1.5, 0.12      | 880, 52         | 180  |
| sCD4-OX40L  | sCD4-OX40L (BIAcore) | 25 | 1.1            | 2000            | 190  |

$^a$ Determined from equilibrium binding.

$^b$ ND, not determined.

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² A. Al-Shamkhani, S. Mallett, M. H. Brown, W. James, and A. N. Barclay, unpublished data.
The fast dissociating component was ~10% at immobilization level equivalent to 1373 RU of sOX40, and ~30% of the total dissociation process at lower immobilization levels (281 RU) (see "Materials and Methods"). These results suggest that at low concentrations of sCD4-OX40L the interaction is dominated by multimeric binding which is characterized by a slow $k_{off}$, whereas at high concentrations competition between sCD4-OX40L molecules for binding to immobilized receptors will result in some sCD4-OX40L molecules binding monomerically and thus dissociating relatively quickly. The determined association rate constants were: $k_{on(1)} = 1.5 \pm 0.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ and $k_{on(2)} = 0.12 \pm 0.05 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ (see "Materials and Methods", Table I).

**Binding of sOX40 to Immobilized sCD4-OX40L**—A CD4 mAb
MRC OX68 was covalently coupled to the carboxylated dextran matrix of the BIAcore™ and used to bind sCD4-OX40L (1708 RU bound; Fig. 8). When sOX40 was injected over the sCD4-OX40L, there was clear binding (717 RU). This compares with background value of 40 RU when the sOX40 was passed over the flow cell prior to the addition of sCD4-OX40L. This background value corresponds to an increase in the bulk refractive index and can be seen when BSA at a similar concentration is passed over the cell before and after the addition of the ligand (17 and 16 RU, respectively, Fig. 8A). The specific binding of sOX40 to immobilized sCD4-OX40L was calculated as the difference between the response generated before and after immobilization of sCD4-OX40L. The affinity of sOX40 binding to immobilized sCD4-OX40L was estimated by determining the equilibriumbinding levels for a range of sOX40 concentrations (0.03–1.29 μM). A Scatchard plot of the transformed data was linear and gave a $K_D$ of 190 nM and a maximal binding of 767 RU (Fig. 8B). An independent determination of the $K_D$ was calculated from the $k_{off}/k_{on}$ to be 180 nM, which is close to the value of 190 nM obtained from the equilibrium binding data (Fig. 8B). The majority of experiments were carried out using rat sOX40 and mouse sCD4-OX40L. However, mouse and rat OX40 share greater than 90% sequence identity and preliminary data on the homologous mouse reaction using sOX40-CD4 d3 and sOX40L-Fc chimeras gave a similar affinity (140 nM) and dissociation rate constant ($\sim 1200 \times 10^{-5}$ s$^{-1}$) (3). The relatively low affinity for the monomeric interaction ($K_D = 190$ nM) contrasts with the high affinity of the reverse multimeric interaction ($K_D = 0.2$ nM determined from binding to cells).

**DISCUSSION**

We have studied the interaction between OX40 and its ligand using soluble recombinant proteins and a combination of biosensor technology and conventional radioligand binding studies. The finding that sCD4-OX40L is a trimer suggests that membrane-bound OX40L and native soluble OX40L, if it exists, will also form trimers. This is consistent with the determined stoichiometry of 3:1 for the interaction of sOX40 with immobilized trimeric sCD4-OX40L. The fact that sCD4-OX40L binds to immobilized sOX40 and to OX40 expressed on activated T cells.
cells with a much higher apparent affinity than when monomeric sOX40 binds immobilized sCD4-OX40L is also consistent with the existence of a trimeric form of the OX40L. The increase in the overall affinity of the trimeric sCD4-OX40L is primarily due to a decrease (−500-fold) in the $K_{D}$ of the apparent affinity of the interaction between sCD4-OX40L and OX40 expressed on the surface of activated T cells ($K_{D}$ of $2−0.2−0.4$ nM) obtained by either equilibrium binding or kinetic measurements at 4 °C is very similar to that of the TNF-$\alpha$ interaction with TNFR VII (34, 35), but is approximately 10-fold higher than that obtained from the BIAcore measurements. In the BIAcore experiments the direct immobilization of sOX40 on the dextran matrix may limit the possible orientations of sOX40 and hence the observed kinetics may represent a dimeric interaction. In contrast, the slower dissociation of sCD4-OX40L from cells is consistent with a trimeric interaction.

The soluble monomeric form of the rat OX40 molecule, containing the cysteine-rich domains, binds to immobilized trimeric mouse OX40L (sCD4-OX40L) with low affinity ($K_{D}$ of 190 nM) and dissociates relatively quickly ($\tau_{diss}$ of 35 s). To our knowledge, direct affinity and kinetic measurements of the interaction between soluble forms of other members of the TNFR superfamily and their ligands have not been reported, and thus we do not know whether these results are representative of other members of this superfamily. A soluble form of the TNFR II was estimated to be 1000-fold less effective than the dimeric TNFR II-Fc in inhibiting a functional assay for TNF (36). The sTNFR was only about 50-fold less effective in an inhibitory binding assay although kinetic analysis was not carried out.

The low dissociation rate of the trimer from cell surfaces makes reversal of the interaction very slow and it seems likely that a general mechanism for reversal is the cleavage of the receptor, together with any bound ligand, from the cell surface as observed for several members of the TNFR superfamily (21–24). If OX40L also released from the cell surface, our results suggest that sOX40 will not act as an antagonist of OX40L because of its low affinity. It is not known whether OX40L acts as a soluble protein and/or as a cell surface protein but this shedding mechanism would also provide a mechanism of terminating the interaction between membrane-bound OX40L and OX40 on activated T cells. The role of natural soluble forms of the TNFR superfamily members in vivo remains unknown but Mohler et al. (36) showed that injection of soluble TNFR II into mice did not protect them from the lethal effect of LPS, whereas a dimeric TNFR II-Fc chimeric protein gave good levels of protection. These results suggest that soluble TNFR II does not function as an antagonist in vivo.

In conclusion, our results indicate that the OX40/OX40L complex will have a similar overall structure to that of TNFR I/TNF-$\beta$ complex (7), namely, that three OX40 molecules interact with a single OX40L trimer. However, receptor dimerization rather than trimerization was shown to be sufficient for inducing the biological effects of TNF (37, 38). This will probably also apply to OX40 as cross-linking with the MRC OX40 mAb is known to enhance the proliferation of activated T cells in vitro (1). Furthermore, the kinetics of binding and dissociation of scd4-OX40L are comparable with those of Fab1/2 fragments of many known mAbs (35), but contrasts with the low affinity of the monomeric receptor binding to ligand. Thus the soluble receptor is unlikely to have functional effects in contrast to the high affinity ligand.