Human Cell-Adhesion Molecule CD2 Binds CD58 (LFA-3) with a Very Low Affinity and an Extremely Fast Dissociation Rate but Does Not Bind CD48 or CD59†

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ABSTRACT: CD2 is a T lymphocyte cell-adhesion molecule (CAM) belonging to the immunoglobulin superfamily (IgSF) which mediates transient adhesion of T cells to antigen-presenting cells and target cells. Reported ligands for human CD2 include the structurally-related IgSF CAMs CD58 (LFA-3) and CD48 as well as, more controversially, the unrelated cell-surface glycoprotein CD59. Using surface plasmon resonance technology, which avoids several pitfalls of conventional binding assays, we recently reported that rat CD2 binds rat CD48 with a very low affinity (K_d 60–90 μM) and dissociates rapidly ($k_{off} \geq 6$ s^{-1}) [van der Merwe, P. A., Brown, M. H., Davis, S. J., & Barclay, A. N. (1993) *EMBO J. 12*, 4945–4954]. In contrast, a study using conventional equilibrium binding methods reported a much higher affinity (K_d 0.4 μM) for human CD2 binding CD58 which suggested that the weak binding of rat CD2 to CD48 may not represent a typical CAM interaction. In the present study we have used surface plasmon resonance to obtain definitive affinity and kinetic data on the interactions of a soluble, recombinant form of human CD2 with soluble forms of CD58, CD48, and CD59. Binding of CD2 to CD58 was readily detected but we were unable to detect any direct interaction between CD2 and either CD59 or CD48 under conditions in which very low affinity interactions ($K_d \sim 0.5$ mM) would have been detected. In contrast to previous reports we found that human CD2 bound CD58 with a very low affinity (K_d 9–22 μM) and dissociated with an extremely fast dissociation rate constant ($k_{off} \geq 4$ s^{-1}). The association rate constant (k_{on}) could not be measured directly but was calculated to be ≥400 000 M^{-1} s^{-1}. Taken together, these results provide conclusive evidence that CAM interactions can have very low affinities and extremely fast dissociation rate constants.

The transient adhesion of leucocytes to other cells is involved in processes such as lymphocyte recirculation, antigen presentation, and inflammation (Springer, 1990). Adhesion is mediated by cell-surface glycoproteins termed cell-adhesion molecules (CAMs), and in recent years large numbers of such CAMs have been identified on leucocytes (Springer, 1990; Barclay et al., 1993). One of the most intensively studied of these CAMs is the immunoglobulin superfamily (IgSF) molecule, CD2, which is found on virtually all T lymphocytes as well as on natural killer cells and which mediates the adhesion of these cells to antigen presenting cells and infected target cells (Springer et al., 1987; Bierer & Burakoff, 1989; Moingeon et al., 1989; Dustin & Springer, 1991). Rat CD2 is presently the only CAM for which the three-dimensional structure of the entire extracellular region is known (Jones et al., 1992), and this consists of two IgSF domains organized in such a way that the ligand binding face of domain 1 is maximally exposed at the top of the molecule (Jones et al., 1992; Davis et al., 1993). Human CD2 has long been known to bind to CD58 (Hunig, 1985; Selvaraj et al., 1987) but no murine CD58 homologue has been identified and recently murine CD2 has been shown to bind CD48 (Kato et al., 1992; van der Merwe et al., 1993b). Sequence comparisons indicate that both CD58 and CD48 are structurally-related to CD2 (Killeen et al., 1988) suggesting that CD48 may function as the murine equivalent of CD58.

In an attempt to gain insight into the functioning of CAMs we recently undertook the measurement of the affinity and kinetics of the rat CD2–rat CD48 interaction. Analysis of the interaction by sucrose-gradient sedimentation (van der Merwe et al., 1993b) indicated that the affinity was very low ($K_d \geq 2.5$ μM) but we were unable to obtain a precise affinity measurement and conventional equilibrium-binding studies were unsuccessful (P. A. van der Merwe, S. J. Davis, unpublished). However, when we used a novel technique based on surface plasmon resonance (van der Merwe et al., 1993a), we were able to demonstrate that rat CD2 binds rat CD48 with a very low affinity (K_d 60–90 μM) and that the low affinity was the result of an extremely fast off-rate constant ($k_{off} \geq 6$ s^{-1}). These results contrast with a previous study using a conventional equilibrium binding technique which reported that soluble, recombinant human CD2 bound CD58 on cells with a much higher affinity of $K_d = 0.4$ μM (Sayre et al., 1989). Taken together with recent semiquantitative binding studies which report that human CD2 binds far more very weakly to human CD48 than to CD58 (Arulanandam et al., 1993; Sandrin et al., 1993), this finding suggested that the

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binding of rat CD2 to rat CD48 may be unusually weak and may not be representative of a typical CAM interaction. It has also been reported that human CD2 may interact with the structurally-unrelated, complement regulatory protein CD59 (Deckert et al., 1992; Hahn et al., 1992) but it is not known how strong this interaction is, and a more recent study has failed to demonstrate any interaction (Arulanandam et al., 1993).

In order to undertake a definitive analysis of the interactions of human CD2 we have produced soluble, monomeric forms of human CD2, CD48, CD58, and CD59 and analyzed in detail the affinities and kinetics of their interactions using the BIAcore biosensor, which implements surface plasmon resonance technology (Jönsson et al., 1991; Karlsson et al., 1991; Chaiken et al., 1992; Granzow & Reed, 1992). Using the BIAcore we did not detect any direct interaction between soluble forms of human CD2 and either CD48 or CD59 under conditions in which interactions with a $K_d \leq 0.5$ mM should have been detectable. As found with rat CD2–rat CD48 interaction, the human CD2–human CD58 interaction had a very low affinity (K_d 9–22 μM) as a result of an extremely fast dissociation rate constant ($k_{off} \geq 4$ s^{-1}). These results suggest that the interactions of cell adhesion molecules may generally have much lower affinities than previously anticipated.

EXPERIMENTAL PROCEDURES

Expression of shCD2, sCD58, and shCD59. Soluble human CD59 (shCD59) comprised amino acids 1–70 of the mature protein (Davies et al., 1989). The expression and purification of shCD59 is described elsewhere (Kieffer et al., 1994). The polymerase chain reaction (PCR) was used to produce DNA encoding soluble human CD2 (shCD2) and soluble human CD58 (sCD58). The 5′ oligonucleotides were complementary to the CD2 and CD58 leader sequences (Seed, 1987; Seed & Aruffo, 1987; Wallner et al., 1987; Sewell et al., 1988) but added, upstream from the leader sequences, an XbaI restriction site and the 25 bp immediately 5′ of the initiating codon of the rat CD4 cDNA sequence (Clark et al., 1987). The sequences of these 5′ oligonucleotides were 5′tagtagtctagatccccatccgctcaagcaggccaccatgagctttcca3′ for shCD2 and 5′tagtagtctagatccccatccgctcaagcaggccaccatggttgctg3′ for sCD58. The 3′ oligonucleotides introduced termination codons immediately after the codons for lysine-182 of CD2 and serine-180 of CD58 cDNA [numbered from the NH_2-terminus of the mature polypeptides (Wallner et al., 1987; Moingeon et al., 1989)], followed by a BamHI site to facilitate subcloning. The sequences of the 3′ oligonucleotides for generating shCD2 and sCD58 were 5′ctactaggatcctcatttctctggaca3′ and 5′ctactaggatcctcagcttgggataca3′, respectively. The CD2 cDNA template used in the PCR reactions was the kind gift of Dr. David Wootton of Dr. Michael J. Owen's laboratory (Imperial Cancer Research Fund, Lincolns Inn Field, London, U.K.) and the human CD58 cDNA template was kindly provided by Dr. Brian Seed (Department of Molecular Biology, Massachusetts General Hospital, Boston). The entire coding sequence of each PCR product was sequenced by dideoxy sequencing after subcloning into M13. For the expression of shCD2 and sCD58, the PCR fragments were subcloned into the polylinker of pEE6.hcmv-GS (Bebbington & Hentschel, 1987; Davis et al., 1990). The expression constructs (20 μg) were then transfected using calcium phosphate into CHO-K1 cells, and methionine sulfoximine-resistant clones were selected as described previously (Davis et al., 1990). To detect shCD2 expression a solid phase, competitive binding assay was used (Barclay & Ward, 1982). This assay utilized as a binding target human CD2 domain 1 expressed as a fusion protein with glutathione-S-transferase (Driscoll et al., 1991; J. G. Cyster, unpublished) adsorbed onto vinyl microtiter plates and it monitored the inhibition of the binding of the human CD2 mAb X/3 (Table 1) to the plate. The bound mAb was detected with [I^{125}]-labeled F(ab′)$_2$ from rabbit anti-mouse IgG. sCD58 expression was detected by western-blotting of tissue culture supernatants with the CD58 antibody TS2/9 (Table 1) under standard conditions. For large scale production the highest-expressing cell lines were grown to confluence in cell factories (Nunc) prior to the addition of 2 mM sodium butyrate. The cultures were then left for a further 3–4 weeks prior to harvesting. The shCD2 and sCD58 were purified from the spent tissue culture supernatant by affinity chromatography (Arvieux et al., 1986) using X/3 and TS2/9 antibody affinity columns, followed by gel filtration on Sephacryl S-200. In some experiments (Figures 7–10) shCD2 and sCD58 were fractionated by gel-filtration on a Superdex 75 HR10/30 FPLC column (Pharmacia) immediately before use and the fractions were used without concentration. The extinction coefficients of shCD2, sCD58, and shCD59 were determined by amino acid analysis to be 1.5, 1.0, and 1.4 cm^2/mg, respectively.

Table 1: Monoclonal Antibodies Which Bind the Recombinant Proteins Used in This Study

protein	monoclonal antibody
CD2[a]	9.6, 7E10, MT110, MT910, 95-5-49, T11/3PT2H9, 9.2, T11/3T48B5, NU-TER, CLB-T11/1, TS1/8.1.1, F92-3A11, OCH.217, X/3
CD58[b]	TS2/9, 1A3, 1A2, 1C4, PAK, 1A10, 2B3
CD48[c]	MEM 102, 6.28
CD59[d]	MEM 43, MEM 43/5, H19 p282, YTH53.1

[a] All CD2 antibodies were obtained from the Third International Workshop on Human Leukocyte Differentiation antigens except for X/3 which was from Dr. David Y. Mason (Knapp et al., 1989). [b] All obtained from Dr. Stefan C. Meuer (Dengler et al., 1992) except TS2/9 which was obtained from Dr. Timothy A. Springer (Sanchez-Madrid et al., 1982). [c] Antibodies obtained from Dr. Václav Horejsí [MEM 102, (Korinek et al., 1991)] and Dr. David Thorley-Lawson [6.28 (Yokoyama et al., 1991)]. [d] Antibodies obtained from Dr. Václav Horejsí [MEM 43, MEM 43/5 (Stefanova et al., 1989)], Dr. Alain Bernard [H19 p282 (Groux et al., 1989)], and Professor Herman Waldmann [YTH53.1 (Davies et al., 1989)].

Expression of shCD48. Soluble human CD48 (shCD48) was produced by engineering a stop codon at tryptophan-199 (of the mature protein) by oligonucleotide-directed mutagenesis of the full-length CD48 cDNA 2A1 (Yokoyama et al., 1991) subcloned in the vector CDM8 (Seed, 1987). The mutating oligonucleotide also created an XbaI site which required converting glutamate-198 to valine. The entire coding sequence was subsequently checked by dideoxy sequencing. For expression in the baculovirus system (Piwnica-Worms, 1990) this mutated cDNA fragment (2A1.sec) encoding a soluble form of human CD48 was subcloned into the XbaI site of the baculovirus transfer vector pVL1393, creating the construct pVL2A1.sec. This construct was cotransfected, using cationic liposomes, together with linearized AcMNPV DNA into Sf9 insect cells to produce recombinant baculoviruses, one of which (Ac2A1.sec) was isolated and used for protein expression. shCD48 was produced in large quantities by infection of Sf21 insect cells with Ac2A1.sec and was purified from tissue culture supernatants by 6.28 mAb affinity chromatography and gel-filtration on a Superdex 75 HR10/30 FPLC column (Pharmacia). The extinction coefficient

of shCD48 was determined by amino acid analysis to be 1.6 cm^2/mg.

Purification of uCD59. Fresh human urine was filtered through glass wool and then concentrated (Amicon, 10 kDa cutoff), filtered again (0.2 μm), and applied to a 10-mL affinity column comprising the CD59 mAb MEM 43 (Stefanova et al., 1989) coupled to sepharose beads. The column was washed with phosphate-buffered saline (PBS) and then PBS plus 0.5 M NaCl. Urinary CD59 (uCD59) was eluted in 50 mM diethylamine (pH 11.5) and then dialyzed into PBS. The purity and identity of the uCD59 was confirmed by SDS–PAGE (single band of 18.5 kDa, not shown) followed by western blotting as well as by NH_2-terminal sequencing.

Expression of sCD58-Hμ and sCD59-Hμ. Soluble chimeric protein comprising CD58 (sCD58-Hμ) or CD59 (sCD59-Hμ) fused to the immunoglobulin μ heavy chain were expressed by modifying the vector pCD2-hCμ (Rutschmann & Karjalainen, 1991). A Sal I site (underlined) was engineered 5 bp upstream of the splice donor site which follows the CD2 exon by mutating the sequence to GTC GAC CAG G*GT AAGTA (* shows the position of splicing), thus allowing the sequence encoding mouse CD2 to be cut out of pCD2-hCμ with the restriction enzymes Sst I (Sac I) and Sal I. The polymerase chain reaction was used to synthesize DNA fragments encoding the signal peptide and extracellular domains of CD58 (amino acids 1–187) or CD59 (amino acids 1–77) flanked by a 5′ Sac I site and a 3′ Sal I site. The sequences of both synthesized fragments were subsequently confirmed. The Sac I–Sal I fragment encoding CD2 was then replaced by the CD58 and CD59 Sac I–Sal I DNA fragments, thus creating the expression plasmids pCD58-Hμ and pCD59-Hμ. The resulting vectors encoded the full extracellular portions of CD58 and CD59 followed by the sequence VDN (introduced by the engineered Sal I site) and then the Igμ heavy chain domains 2–4 (CH2 to CH4). The vectors were transfected into the J558/L myeloma cell line as described (Rutschmann & Karjalainen, 1991) and the secreted sCD58-Hμ and sCD59-Hμ was concentrated by ammonium sulfate precipitation, followed by dialysis into PBS. sCD58-Hμ and sCD59-Hμ expression were confirmed using the mAbs TS2/9 and YTH 53.1 mAbs (Table 1), respectively, as well as an anti-human-IgM antibody (Sigma; M. Tone and A. K. C. Krishnam, unpublished).

BIAcore Analysis. These experiment were performed on a BIAcore biosensor (Pharmacia Biotech AB, Uppsala, Sweden) (Jönsson et al., 1991; Karlsson et al., 1991; Chaiken et al., 1992; Granzow & Reed, 1992). The BIAcore software was modified as recommended by the manufacturers to increase the maximum data aquisition rate from 5 to 10 s^{-1}. All experiments were performed at 37 °C at the indicated buffer flow-rates, and the buffer used was Hepes buffered saline (HBS) which contained (in mM): NaCl 150, $MgCl_2$ 1, $CaCl_2$ 1, Na azide 10, 0.005% Surfactant P-20 (Pharmacia), and Hepes 10 (pH 7.4). Na azide was omitted during the immobilization procedures. The proteins injected were all purified in HBS and diluted in HBS for injection, and thus all binding on the BIAcore was assayed in the presence of physiological $MgCl_2$ and $CaCl_2$ concentrations. The only exceptions to this were uCD59, sCD59-Hμ, and sCD58Hμ which were prepared in PBS without $MgCl_2$ and $CaCl_2$ (see above). To ensure that binding of these molecules was assayed in the presence of divalent cations uCD59 (Figure 6B) was diluted 1:3 in HBS so that the final $MgCl_2$ and $CaCl_2$ concentrations were 0.67 mM each and $MgCl_2$ and $CaCl_2$ (1 mM each) were added to sCD59-Hμ and sCD58Hμ (Figure 6C).

Proteins were covalently coupled to the carboxymethylated dextran matrix on a CM5 sensor chip (Pharmacia Biosensor AB) by amine coupling (Johnsson et al., 1991), which utililizes primary amine groups on the protein, or thiol coupling (O'Shannessy et al., 1992), which utililizes free thiol groups on the protein. shCD2 (Figures 3, 6A, 6B, 6C, and 10), shCD48 (Figure 5A), and the mAb BU1 (Figure 6D) were amine-coupled using the Amine Coupling Kit (Pharmacia Biosensor AB) as described (Johnsson et al., 1991) with the following modifications. The proteins were diluted to 20–70 μg/mL in 10 mM Na acetate (pH 5), and the activation period was varied from 3 to 7 min to optimize the immobilization level. After the immobilization of shCD2 the surface was conditioned with 10 mM HCl for 3 min but, in order to avoid inactivation, this was not done for the shCD48 (Figure 5A) and the anti-IgM mAb BU1 (Figure 6D) immobilizations. The thiol coupling of shCD2 (Figures 5B, 8, and 9) and sCD58 to the CM5 sensor chip (O'Shannessy et al., 1992) was performed as follows. Recombinant sCD58 or shCD2 (50 μg) were reduced in nondenaturing conditions by incubation in 200 μL of 2% dithiothreitol (w/v) in 0.5 M Tris (pH 6.8) for 30 min at 22 °C. This reaction was cooled on ice, and then the buffer was immediately changed to 50 mM Na formate (pH 4) using a 10-mL PD10 Sephadex G-25M desalting column (Pharmacia LKB Biotechnology AB, Uppsala). The eluted reduced protein (~20 μg/mL) was frozen in aliquots and stored at −20 °C until immobilization. The immobilization reagents were all injected at a flow-rate of 5 μL/min. After activation of the sensor surface by injection of 10 μL of 50 mM *N*-hydroxysuccinimide/200 mM *N*-ethyl-*N*′-[(dimethylamino)propyl]carbodiimide, 20 μL of freshly prepared 2-(2-pyridinyldithio)ethanamine hydrochloride (Pharmacia Biotech AB, 80 mM in 0.1 M borate, pH8.5) was injected to introduce reactive disulfides on the surface, followed by 35 μL of the reduced protein ligand. This was followed by 20 μL of 50 mM l-cysteine/1 M NaCl in 100 mM Na formate (pH 4.3) which blocked the remaining reactive groups and removed noncovalently bound material.

Data Analysis. The equilibrium binding data (Figures 7 and 8) were analyzed by (1) nonlinear curve-fitting of the Langmuir binding isotherm to the primary data and (2) linear curve fitting of the Scatchard plots. The dissociation phases were analyzed by first normalizing them so that the response before dissociation was 100% and the base-line response was 0%. Dissociation times for each dissociation phase were then determined by fitting a monoexponential decay function to the data. Plots of the effect of varying the flow-rate (Figure 9C) were analyzed by linear curve fitting. All curve fitting was performed using the curve-fitting functions of the program Origin v 2 (MicroCal SoftwareInc, Northampton, MA) which was run on a Compaq PC. Linear curve fitting was by linear least-squares regression. Nonlinear curve fitting employed iterative least-squares curve fitting using the Marquardt–Levenberg algorithm.

RESULTS

Protein Constructs. Soluble, monomeric forms of human CD2 (shCD2), CD48 (shCD48), CD58 (sCD58), and CD59 (shCD59) contained the entire extracellular portions of each protein (Figure 1). Multimeric forms of CD58 and CD59 were produced by constructing chimeras comprising the extracellular portions of CD58 or CD59 NH_2-terminal to part of the constant region (including domains CH2, CH3, and

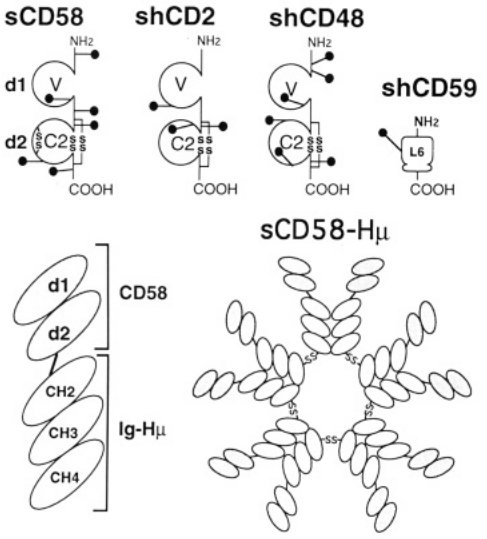

FIGURE 1: Forms of soluble human CD58, CD2, CD48, and CD59 used in the present study shown schematically. Ig domains are depicted as incomplete circles. V and C2 refer to V-set and C2-set IgSF domains. L6 refers to a Ly-6 domain (Williams, 1991a). Predicted N-glycosylation sites (●) and disulfide bonds (SS) are shown. The sCD58-Hμ chimera, which comprises the two IgSF domains (d1 and d2) of CD58 (shaded ovals) and the second, third, and fourth constant domains (CH2, CH3, and CH4) of the μ immunoglobulin heavy chain (Ig-Hμ), is shown as a monomer (bottom left) and as the secreted disulfide-linked decamer (bottom right). The sCD59-Hμ chimera is similar and is not shown.

CH4) of the immunoglobulin μ heavy chain (Figure 1). When expressed in myeloma cells these chimeras (sCD59-Hμ and sCD58-Hμ) form decamers (Figure 1) (Rutschmann & Karjalainen, 1991).

SDS–PAGE analysis of shCD2, shCD48, sCD58, and sCD59 (Figure 2A) showed that all four proteins run as broad bands and are larger than their calculated protein molecular weights (21.4, 22.7, 20.6, and 8.1 Kd, respectively). This is presumably because of variable glycosylation at N-glycosylation consensus sites present in all 4 proteins (Figure 1). sCD58 is noticably larger on SDS–PAGE than shCD2 presumably because it is more highly glycosylated. Nonreducing SDS–PAGE analysis confirmed that these proteins were not secreted as disulfide-linked multimers (not shown). Gel-filtration showed that shCD2 eluted well after soluble rat CD4 (srCD4), which has 4 IgSF domains, and just after soluble rat CD2 (srCD2), which has 2 IgSF domains (Figure 2B). sCD58 eluted just after srCD4, well ahead of shCD2 and srCD2 (Figure 2B). The apparently larger size of sCD58 is consistent with the SDS–PAGE result (Figure 2A). It is noteworthy that sCD58 has six potential N-glycosylation sites whereas shCD2 has three (Figure 1). Together these results indicate that the shCD2 and sCD58 are predominantly monomeric in solution.

In order to confirm that the recombinant proteins were correctly folded into the same tertiary structure as their native counterparts each protein was tested for binding to several monoclonal antibodies known to be specific for the native protein (Table 1). We confirmed that recombinant shCD2, sCD58, shCD48, and shCD59 bound to all these mAbs (data not shown), and sCD58-Hμ and sCD59-Hμ were shown to bind TS2/9 and YTH 53.1, respectively (M. Tone and A. K. C. Krishnam, unpublished data). It is noteworthy that 12 out of the 14 CD2 mAbs tested (Peterson & Seed, 1987) and five out of seven of the CD58 antibodies (Dengler et al., 1992) bind to the ligand binding NH_2-terminal IgSF domains of

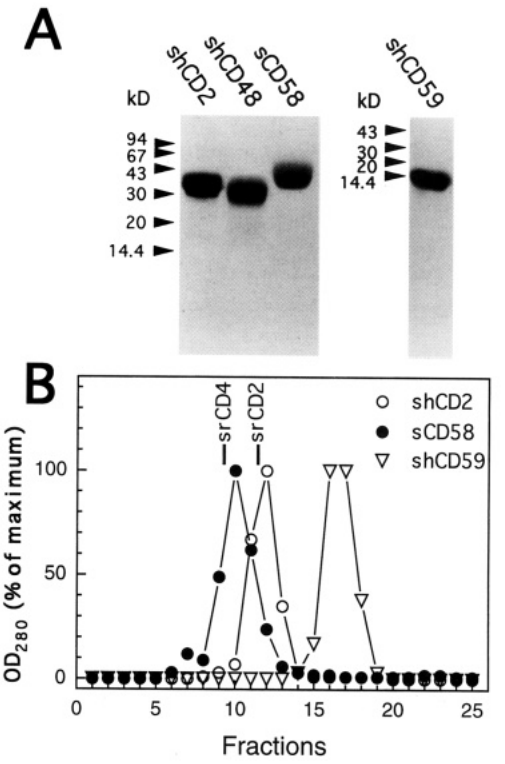

FIGURE 2: (A) Reducing SDS–PAGE of shCD2, shCD48, and sCD58 (15% gel, left) and shCD59 (20% gel, right). Lanes were loaded with ~8 μg of each protein. The positions of the molecular weight standards are shown (kD) (B) Gel-filtration of purified shCD2, sCD58, and shCD59 on a Superdex 75 HR10/30 FPLC column (Pharmacia) in HBS with a fraction size of 0.5 mL. The fraction collection began after 4 mL of buffer had passed through the column. The elution positions of recombinant forms of the extracellular portions of rat CD4 (srCD4) and rat CD2 (srCD2) are shown (Davis et al., 1990; Gray et al., 1993).

these molecules. Recently, shCD2 has been demonstated to be correctly folded by the determination of its tertiary structure by X-ray diffraction (Bodian et al., 1994). Similarly, the tertiary structure of shCD59 has been solved by nuclear magnetic resonance (Kieffer et al., 1994).

Binding of sCD58 to Immobilized shCD2. The BIAcore biosensor (Jönsson et al., 1991; Karlsson et al., 1991; Chaiken et al., 1992; Granzow & Reed, 1992) uses an optical method to detect changes in the refractive index within a thin dextran matrix (1.6 mm × 0.1 mm × ~ 0.0001 mm) which lies on the floor of a rectangular flow-cell (2.4 mm × 0.5 mm × 0.05 mm). The binding of a soluble protein to a ligand immobilized within the matrix increases the protein concentration within the matrix. This, in turn, alters the refractive index within the matrix, and it is this refractive index change which is detected and quantified in arbitrary response units (RU). The response is linearly related to the total (free *plus* bound) protein concentration within the matrix (Stenberg et al., 1991)—increasing the total protein concentration by 1 mg/mL leads to an increase in the response of approximately 100 RU. Consequently, the injection of high concentrations of protein will result in a nonspecific increase in RU even if no protein binds to the matrix. In order to control for this as well as other factors which can lead to refractive index changes within the matrix, samples were injected through a control flow-cell in which either no protein or an irrelevant protein was immobilized.

In order to analyze the interaction of sCD58 with shCD2 on the BIAcore shCD2 was coupled directly to the dextran matrix using a coupling chemistry which uses primary amine

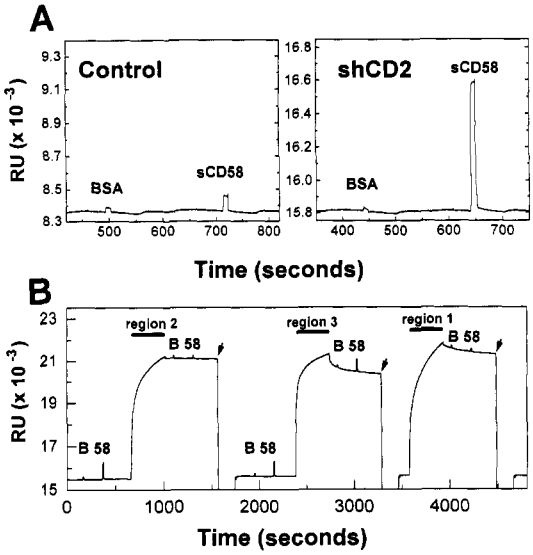

FIGURE 3: Binding of sCD58 to immobilized shCD2 and blocking by CD2 mAbs. (A) BSA and sCD58 (both at 0.5 mg/mL) were injected for 9 s through a control flow-cell (left panel) or a flow-cell with immobilized shCD2 (right panel). Flow-rate 20 μL/min. (B) BSA (B) and sCD58 (58) (both at 0.5 mg/mL) were injected for 12 s through a flow-cell with shCD2 immobilized with no mAbs bound ($t \sim 250$ and $t \sim 2000$ s) or after injection of region 2 (TS1/8.1.1), 3 (OCH.217), or 1 (T11/1PT2H9) CD2 mAbs (1:100 of ascites) where indicated for 360 s. The response seen with mAb binding is large because mAbs are big and the signal is proportional to the mass bound. Bound mAbs were eluted with 10 mM HCl (arrows) for 180 s. Flow-rate 5 μL/min.

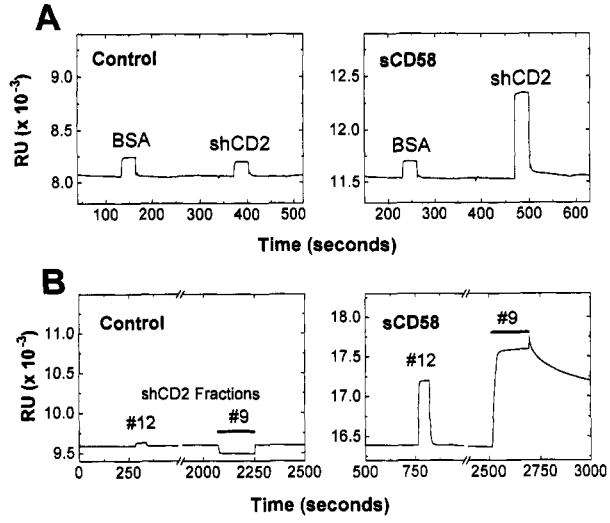

FIGURE 4: Binding of monomeric and multimeric, aggregated shCD2 to immobilized sCD58. (A) BSA and shCD2 (both at 0.5 mg/mL) were injected for 30 s over a control flow-cell (left panel) or a flow-cell with immobilized sCD58 (right panel). Flow-rate 20 μL/min. (B) shCD2 used in A was fractionated by FPLC (Figure 2B), and then fractions 12 (50 μg/mL) and 9 (60 μg/mL) were injected for 60 and 180 s, respectively, over a control flow-cell (left panel) or a flow-cell with immobilized sCD58 (right panel). Flow-rate 5 μL/min.

groups on the protein (Johnsson et al., 1991). Previous results with a soluble rat CD2 construct coupled in this way showed that ~80% of the CD2 retains binding activity for monoclonal antibody (mAb) and rat CD48 (van der Merwe et al., 1993a). The activity of amine-coupled shCD2 was analyzed using three noncompeting CD2 monoclonal antibodies (Figure 3B). All three mAbs bound to similar levels (for example, see Figure 3B). The mAb binding activity of the immobilized shCD2 was routinely >30% and as high as 77–88% when immobilization conditions were optimized (not shown). Furthermore, 10 other CD2 mAbs all bound to a high proportion (>70%) of immobilized shCD2 (P. A. van der Merwe and S. J. Davis, manuscript in preparation). These mAb binding results indicate that the recombinant shCD2 is correctly folded and that the covalent immobilization procedure does not disrupt the shCD2 structure.

Injections of bovine serum albumin (BSA) and sCD58 (both at 0.5 mg/mL) through a control flow-cell gave small nonspecific responses because of the change in the buffer refractive index caused by the high protein concentrations (Figure 3A). When the same samples were injected through a flow-cell with shCD2 immobilized the control BSA response was unchanged whereas the sCD58 response was dramatically increased, indicating binding (Figure 3A). The specificity of this binding was tested using three CD2 mAbs which bind to nonoverlapping regions on CD2 (Peterson & Seed, 1987; Wallace et al., 1987). The region 1 and 2 mAbs (T11/3PT2H9 and TS1/8.1.1, respectively) bind to distinct regions (Peterson & Seed, 1987) on the GFCC′C″ face of the NH_2-terminal IgSF domain of CD2 (Withka et al., 1993) and block CD58 binding (Wallace et al., 1987). The region 3 antibody (OCH.217) binds to the carboxy-terminal IgSF domain and does not block CD58 binding. In agreement with this, when immobilized shCD2 was saturated with the region 1 and 2 antibodies the response to sCD58 was reduced almost to the level seen in the control flow-cell (compare Figure 3B with A) whereas the region 3 antibody had little effect (Figure 3B).

Binding of shCD2 to Immobilized sCD58. The interaction was analyzed in the reverse orientation with sCD58 immobilized. The immobilization of sCD58 through amine groups led to its inactivation, as determined by mAb and shCD2 binding (not shown), and so an alternative coupling chemistry was used which utilizes protein thiol groups (O'Shannessy et al., 1992). The carboxy-terminal IgSF domain of CD2 contains two disulfide bonds (Killeen et al., 1988; Jones et al., 1992) one of which [cys 110-cys 174 in human CD2] lies in an exposed position (Jones et al., 1992), suggesting that it may be susceptible to mild reduction under nondenaturing conditions. Since a sequence comparison of CD58 with CD2 suggested that it to has a disulfide bond in this exposed position (Killeen et al., 1988) thiol-coupling of sCD58 (following mild reduction) was attempted and was successful (see Experimental Procedures).

Injection of BSA and shCD2 through a control flow-cell and then over immobilized sCD58 demonstrated that shCD2 bound to the thiol-coupled sCD58 (Figure 4A). Furthermore, this binding was abolished by saturating the sCD58 with the mAb TS2/9 (not shown), which blocks the CD2–CD58 interaction (Selvaraj et al., 1987). Following injection of shCD2 over sCD58 the response dropped rapidly but did not fall completely to the base line, as evidenced by a small, but distinct, shoulder (Figure 4A, right panel). This indicates that a small proportion of the bound shCD2 dissociates very slowly and suggests that the shCD2 preparation may contain multimeric aggregates. This was confirmed by gel-filtration of the shCD2 preparation (Figure 2B) and analysis of the fractions on the BIAcore (Figure 4B). shCD2 from the monomeric peak contained no slowly dissociating binding (Figure 4B, fraction 12) whereas a small shoulder of shCD2 running ahead of the monomeric peak (≤2% of the total shCD2) contained slowly-dissociating shCD2 (Figure 4B, fraction 9). These results demonstrate that, as found with other soluble IgSF proteins (van der Merwe et al., 1993a),

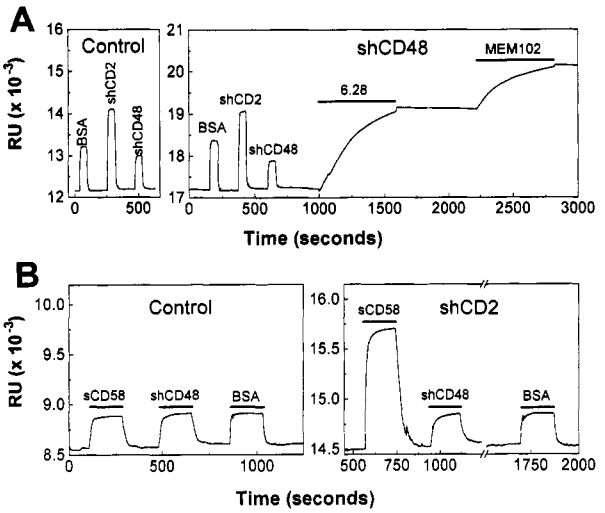

FIGURE 5: No binding of shCD48 to itself or to shCD2. (A) BSA (7 mg/mL), shCD2 (7 mg/mL), and shCD48 (4 mg/mL) were injected for 60 s over a control flow-cell, with immobilized soluble mouse I-E^k (Wettstein et al., 1991) (left panel) or a flow-cell with immobilized shCD48 (right panel). Purified mAbs 6.28 and MEM 102 (both at 10 μg/mL) were injected for 600 s over shCD48. Flow-rate 3 μL/min. (B) sCD58 (1.5 mg/mL), shCD48 (1.4 mg/mL), and BSA (1.5 mg/mL) were injected for 180 s over a control flow-cell (left panel) or a flow-cell with immobilized shCD2 (right panel). Flow-rate 1 μL/min.

shCD2 tends to form small amounts of multimeric aggregates in solution.

Testing for Binding of shCD2 to shCD48 and shCD59. Injection of a high concentration of shCD2 (7 mg/mL, ~300 μM) over shCD48 immobilized onto the dextran matrix gave the same nonspecific response as when injected over a control flow-cell, indicating that there was no binding (Figure 5A). The immobilized shCD48 bound two CD48 antibodies, MEM 102 (Korinek et al., 1991) and 6.28 (Yokoyama et al., 1991), suggesting that at least some of the immobilized shCD48 retained activity (Figure 5A). In order to exclude an artifact due to the immobilization process shCD48 was injected over immobilized shCD2 (Figure 5B), which retains ligand- and mAb-binding activity when immobilized (see above). Whereas the sCD58 responses indicated that it clearly bound shCD2, shCD48 at 1.4 mg/mL (~70 μM) showed no evidence of binding (Figure 5B). It has been suggested that CD48 may bind to itself (Yokoyama et al., 1991). However, there was no evidence for self-association when shCD48 was injected over immobilized shCD48 (Figure 5A).

The putative interaction of CD2 with CD59 was tested by the injection of a soluble form of human CD59 (shCD59) at a high concentration (~1.5 mM) over immobilized shCD2 (Figure 6A). The response to shCD59 was the same as in a control flow-cell, indicating that there was no detectable binding (Figure 6A), whereas in the same experiment sCD58 bound to the immobilized shCD2 (Figure 6A). In order to exclude the possibility that the failure of shCD59 to bind CD2 is the result of either altered glycosylation or truncation of recombinant CD59 at asparagine-70, the experiment was repeated using endogenous CD59 purified directly from human urine (uCD59) but, once again, there was no detectable binding (Figure 6B). In this experiment the buffer $MgCl_2$ and $CaCl_2$ concentrations (0.67 mM each) were slightly below the physiological levels, but identical results were obtained in the presence of 1 mM $MgCl_2$ and $CaCl_2$ (not shown). To increase the likelihood of detecting a very low affinity interaction a multimeric CD59 chimera (sCD59-Hμ) was used which

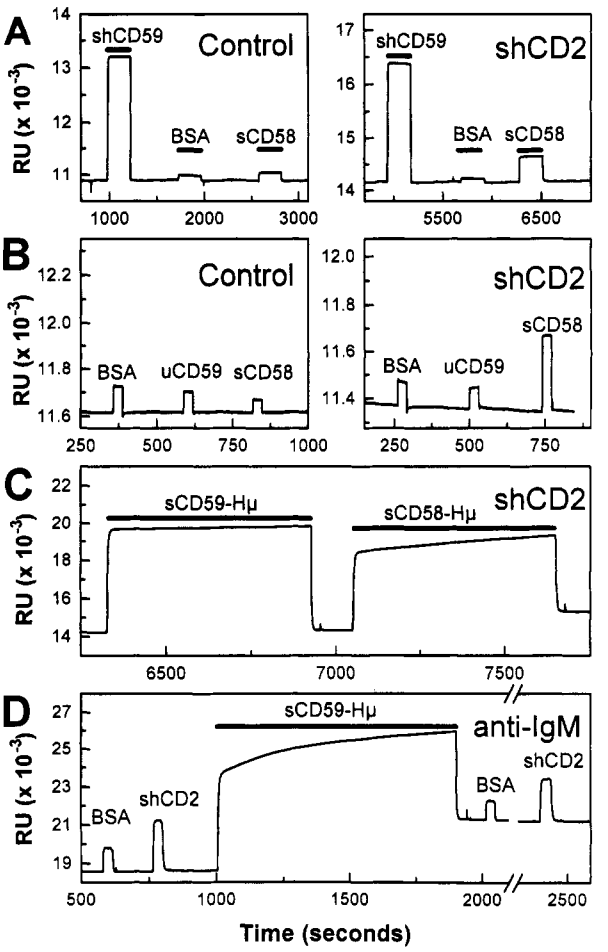

FIGURE 6: No binding of human CD59 to shCD2. (A) shCD59 (12.6 mg/mL), BSA (0.5 mg/mL), and sCD58 (0.5 mg/mL) were injected for 240 s over a control flow-cell, with immobilized soluble mouse I-E^k (Wettstein et al., 1991) (left panel) or a flow-cell with immobilized shCD2 (right panel). Flow-rate 5 μL/min. (B) BSA (1 mg/mL), uCD59 (1 mg/mL), and sCD58 (0.5 mg/mL) were injected for 30 s over a control flow-cell (left panel), with immobilized rat soluble CD2 (Gray et al., 1993), or a flow-cell with immobilized shCD2 (right panel). Flow-rate 20 μL/min. (C) Concentrated TCS preparations containing sCD59-Hμ or sCD58-Hμ were injected for 600 s over immobilized shCD2. $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) were added to the preparations before injection. Flow-rate 3 μL/min. (D) BSA (5 mg/mL) and shCD2 (10 mg/mL) were injected for 36 s over immobilized anti-IgM mAb before and after injection of sCD59-Hμ TCS for 360 s. Flow-rate 5 μL/min.

contains 10 CD59 domains on one molecule. When sCD59-Hμ in concentrated tissue culture supernatant (TCS) was injected over immobilized shCD2 (Figure 6C) there was a large nonspecific response due to the TCS but no evidence of specific binding. In contrast a sCD58 chimera (sCD58-Hμ, Figure 1) clearly bound with a high avidity (Figure 6C), dissociating with a k_{off} of 10^{-5} s^{-1} (not shown). One unlikely explanation for these results is that the covalent coupling of shCD2 abolishes CD59 binding without affecting CD58 or mAb binding. In order to exclude this possibility the sCD59-Hμ chimera was immobilized, via an anti-IgM mAb BU1 (Knapp et al., 1989), and shCD2 was injected over the sCD59-Hμ (Figure 6D). sCD59 was immobilized indirectly because direct covalent coupling invariably led to its inactivation as determined by mAb binding (not shown). The response to injection of shCD2 at 10 mg/mL (~450 μM) did not increase following the binding of sCD59-Hμ to the flow-cell (Figure 6D), indicating that there was no detectable binding. In conclusion, we were unable to demonstrate any binding of

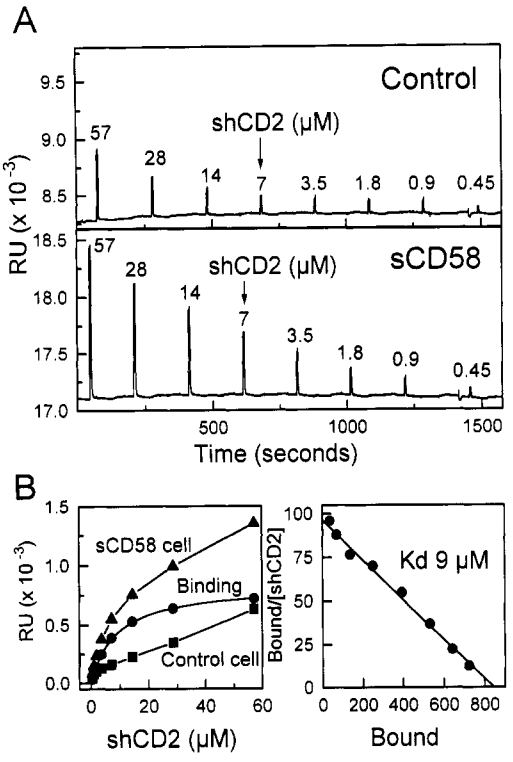

FIGURE 7: Saturation binding analysis of shCD2 binding to immobilized sCD58. (A) shCD2 was injected for 6 s at the indicated concentrations over a control flow-cell (top panel) or a flow-cell with immobilized sCD58 (bottom panel). Equilibrium binding levels were attained within 3 s (not shown). Flow-rate 20 μL/min. (B) Plot of data in A. (Left panel) specific binding (circles, binding) is the difference between the total response (triangles, sCD58 cell) and the nonspecific response (squares, control cell). A Langmuir binding isotherm was fitted to the specific binding data (circles) and gave a K_d of 9 μM. (Right panel) Scatchard plot of the specific binding data. The line was fitted by linear-regression and gave a K_d of 9 μM.

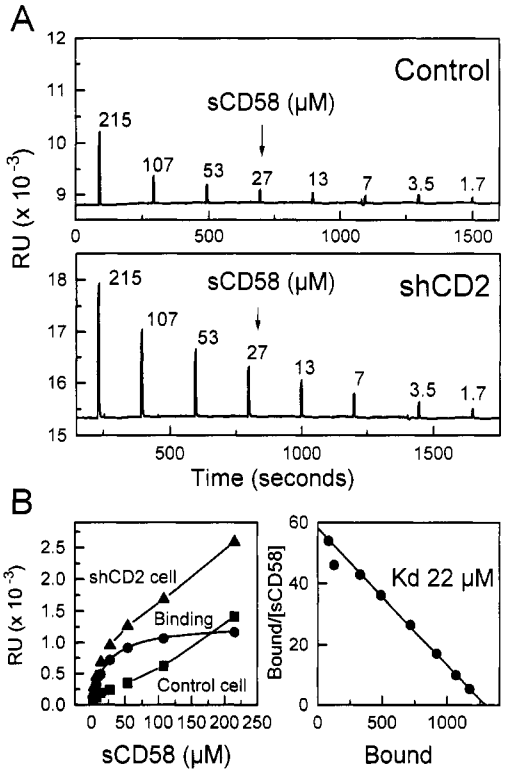

FIGURE 8: Saturation binding analysis of sCD58 binding to immobilized shCD2. (A) sCD58 was injected for 6 s at the indicated concentrations over a control flow-cell (top panel) or a flow-cell with immobilized shCD2 (bottom panel). Equilibrium binding levels were attained within 2 s (not shown). Flow-rate 20 μL/min. (B) Plot of data in A. (Left panel) specific binding (circles, binding) is the difference between the total response (triangles, shCD2 cell) and the nonspecific response (squares, control cell). A Langmuir binding isotherm was fitted to the specific binding data (circles) and gave a K_d of 22 μM. (Right panel) Scatchard plot of the specific binding data. The line was fitted by linear-regression and gave a K_d of 22 μM.

human CD2 to either CD48 or CD59 under conditions in which very low affinity interactions ($K_d \sim 0.5$ mM) would have been detected.

The Affinity of the CD2–CD58 Interaction. The affinity of CD2 binding CD58 was determined by equilibrium binding analysis on the BIAcore as previously done for the rat CD2–CD48 interaction (van der Merwe et al., 1993a). A range of concentrations of shCD2 were injected over immobilized sCD58 for 6 s each (Figure 7A, sCD58), and equilibrium binding levels were attained within 3 s (not shown). The nonspecific response, caused by the high protein concentrations used, was measured by the injection of the same sample through a control flow-cell (Figure 7A, control). The binding response was calculated by subtracting this nonspecific response from the response in the sCD58 cell. A plot of these data indicated that the binding was saturable (Figure 7B, left panel, circles). Nonlinear curve fitting of the Langmuir binding isotherm to these data gave a dissociation constant (K_d) of 9 μM. A Scatchard plot of the transformed data was linear and also gave a K_d of 9 μM (Figure 7B, right panel). Five independent determinations gave a K_d of 9 ± 1.8 μM (mean ± SD).

In order to confirm this very low affinity the experiment was performed with shCD2 immobilized and sCD58 in solution (Figure 8). The affinity obtained was similar, albeit slightly lower (K_d 22 μM). Four independent determinations gave a K_d of 22 ± 2 μM (mean ± SD). In this experiment shCD2, like sCD58, was also immobilized using thiol coupling in order to make the measurements directly comparable. However, a similar affinity was obtained using amine-coupled shCD2 [K_d of 23 ± 3 μM (mean ± range, n = 2)].

Determining the Off-Rate Constant for the CD2–CD58 Interaction. When shCD2 or sCD58 are injected over their immobilized counterparts equilibrium binding levels are attained very rapidly (within 3 s), and so it was not possible to determine directly the on-rate constant (k_{on}). For this reason we concentrated on measuring the off-rate constant (k_{off}) and calculated the k_{on} (= k_{off}/K_d). Preliminary observations indicated that the k_{off} was faster than 1 s^{-1}, as found with the rat CD2–CD48 interaction (van der Merwe et al., 1993a). One technical difficulty when measuring such fast off-rate constants on the BIAcore is that, at the flow-rates routinely used (~5 μL/min or 0.083 μl/s), the washing time of buffer from the flow-cell [which has a volume of 0.06 μL (Jönsson et al., 1991)] is on the order of 1 s (van der Merwe et al., 1993a). The washing time was quantitated using the control protein BSA since it does not bind to shCD58 (Figure 9C). Increasing the flow-rate resulted in faster washing times (Figure 9C). A plot of the washing time (or apparent dissociation time) of BSA against 1/flow-rate (Figure 9D) was linear with a slope of 0.057 ± 0.004 μL (mean ± SD, n = 4) which equals almost exactly the volume of the flow-cell. When this plot is extrapolated to the y-intercept it does not pass though the origin, suggesting that even at an infinite flow-rate (1/flow-rate = 0) there is a significant washing time (Figure 9D). Several independent determinations showed that this residual washing time was 0.022 ± 0.005 s (mean ± SD, n = 4). These results indicate that the washing time (T_{wash}) obeys the relationship

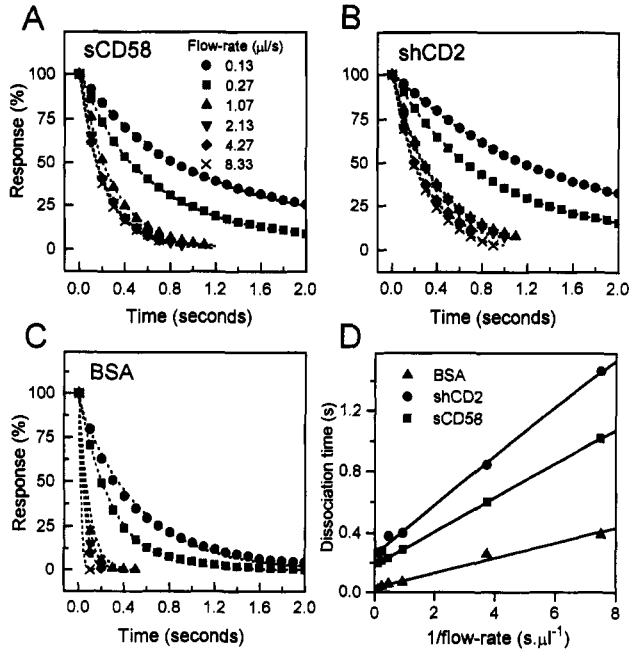

FIGURE 9: Measuring the dissociation rate constants for sCD58 and shCD2 dissociation from immobilized shCD2 and sCD58, respectively. Fall in response following injection of (A) sCD58 (0.25 mg/mL) over 200 μM of thiol-immobilized shCD2, (B) shCD2 (0.14 mg/mL) over 100 μM thiol-immobilized sCD58; or (C) BSA (at 10 mg/mL) over 100 μM of thiol-immobilized shCD2. The samples were injected at the indicated flow-rate for 3.8 s (at flow-rate 8.33 μL/s) or 7.5 s (at flow-rates 0.13–4.27 μL/s). The maximal equilibrium sCD58, shCD2, and BSA responses were 670, 520, and 1400 RUs, respectively. The same samples injected though a control flow-cell gave responses of 60, 55, and 1400 RUs. The apparent dissociation times were obtained by fitting monoexponential decay curves (dotted lines) to the data. (D) Plot of the apparent dissociation time against 1/flow-rate for BSA (triangles), shCD2 (circles), and sCD58 (squares) using data from A, B, and C. The extrapolated apparent dissociation time at 1/flow-rate = 0 was determined by linear regression analysis.

$$T_{wash} = T_c + \text{flow-cell volume/flow-rate}$$

where T_c is the washing time at infinite flow-rate. In subsequent experiments the washing time was minimized by measuring the dissociation time at increasing flow-rates. Such an analysis of sCD58 dissociating from thiol-immobilized shCD2 (Figure 9A) and, in the reverse orientation, shCD2 dissociating from thiol-immobilized sCD58 (Figure 9B) clearly shows that the apparent dissociation time decreases as the flow-rate is increased. This dissociation time approaches a minimum at very high flow-rates (Figures 9A and B) which is still significantly longer than the washing time, as determined with BSA (Figure 9C). The apparent dissociation time with the washing time minimized was estimated by plotting the observed dissociation time against the flow-rate and then extrapolating the plot to infinite flow-rate (Figure 9D, y-intercepts). The dissociation times obtained for sCD58 and shCD2 were 0.19 and 0.26 s, respectively (Figure 9D), which correspond to dissociation rate constants (k_{off}) of 5.3 and 3.9 s^{-1}.

Although this technique minimizes the washing time it does not exclude the possibility that there is rebinding of the dissociated ligand to the matrix. This is a well-recognized problem on the BIAcore that will cause an underestimation of the k_{off} (Felder et al., 1993; Panayotou et al., 1993). The presence of rebinding during the dissociation phase is suggested by the fact that the slopes of the dissociation time versus 1/flow-rate plots (Figure 9D) are steeper for shCD2 (0.16) and sCD58 (0.11) than for BSA (0.057) and that the slopes of these plots decrease when the concentration of the immobilized ligand is decreased (not shown). These slopes are steeper because, when there is rebinding during the washing phase, increasing the flow-rate not only decreases the washing time but also decreases the rebinding time. Further evidence for the presence of rebinding was obtained by examining the effect of varying the concentration of immobilized shCD2 [imm-shCD2] on the apparent dissociation time of sCD58 (Figure 10A). A decrease in [imm-shCD2] induced by partial blocking with the region 2 CD2 mAb (Figure 3B) led to a faster dissociation of sCD58 (Figure 10A) while having no effect on the BSA dissociation (Figure 10B). This difference was observed at both high and low flow-rates (Figure 10) and even when extrapolating to infinite flow-rate (not shown) which indicates that rebinding is not eliminated entirely by increasing the flow-rate. Rebinding can be decreased by (1) injection of a low molecular weight soluble competitor ligand during the dissociation phase (Felder et al., 1993; Panayotou et al., 1993), (2) measuring the initial rate of dissociation after complete saturation of the immobilized ligand, and (3) lowering the total [immobilized ligand]. However the low affinity and extremely rapid dissociation rate of the human shCD2–sCD58 interaction made all these methods impractical. The presence of rebinding indicates that the actual k_{off} is probably faster than 3.9–5.3 s^{-1}. Taking the lower limit for the k_{off} to be 4 s^{-1} and the K_d to be 9 μM the k_{on} can be calculated to be *at least* 400 000 M^{-1} s^{-1} which is typical of association rate constants between mAbs and IgSF molecules (Mason & Williams, 1980; Davis et al., 1992).

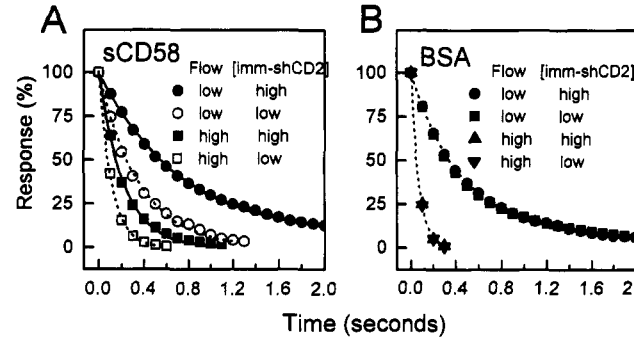

FIGURE 10: Evidence for the presence of rebinding during the dissociation phase. The effect of varying [imm-shCD2] on the dissociation rate of (A) sCD58 and (B) BSA. In (A) the fall in response is shown following the injection of sCD58 (0.3 mg/mL) for 7.5 s at a flow-rate of 0.27 (low, circles) or 2.13 (high, squares) μL/s over immobilized shCD2 with [imm-shCD2] at 57 (low, open) or 260 (high, closed) μM. [imm-shCD2] was decreased by partial blocking with a region 2 CD2 mAb (see Figure 3). The maximal equilibrium sCD58 response was 600 and 170 RU at high and low [imm-shCD2]. The same sample injected through a control flow-cell gave a response of 45 RU. (B) The fall in response of BSA (10 mg/mL) was examined as in A except that flow-rates of 0.13 (low) or 1.07 (high) μL/min were used. The maximal equilibrium BSA response was 1200 and 1260 RU at high and low free [imm-shCD2]. The curves in A and B are monoexponential decay curves fitted directly to the data.

DISCUSSION

Affinity and Kinetics of the CD2–CD58 Interaction. In the present study we have shown that a monomeric soluble form of human CD2 binds to monomeric soluble CD58 with a very low affinity (K_d 9–22 μM) and that the interaction has an extremely fast dissociation rate constant ($k_{off} \geq 4$ s^{-1}) and a fairly fast association rate constant ($k_{on} \geq 400\,000$ M^{-1} s^{-1}). Several precautions were taken to ensure that these values

were accurate. Firstly, recombinant shCD2 and sCD58 were shown to bind to large panels of monoclonal antibodies (Table 1), providing strong evidence that these molecules are correctly folded. This has been confirmed in the case of shCD2 since the crystal structure of this protein has been solved (Bodian et al., 1994). Secondly, the affinity and kinetic measurements were performed at physiological temperature, pH, ionic strength, [Mg^{2+}], and [Ca^{2+}]. Thirdly, the shCD2 preparation was shown to be at least 80% active by mAb binding studies. Fourthly, multimeric binding was excluded. Finally, similar results were obtained when the affinity and kinetic determinations were performed in either orientation (shCD2 or sCD58 immobilized) and using either amine or thiol coupling. Thiol coupling would modify only the COOH-terminal IgSF domains (see Results), and it is known that the CD58 and CD2 binding sites are on the NH_2-terminal IgSF domains of CD2 (Peterson & Seed, 1987; Richardson et al., 1988; Somoza et al., 1993) and CD58 (Miller et al., 1993), respectively. There was a 2.4-fold difference between the human CD2–CD58 affinity determined in the opposite orientations (22 and 9 μM with sCD58 and shCD2 in solution, respectively), and sCD58 also appeared to have slightly faster dissociation rate than shCD2 (Figure 9D). However, these differences were small and may be the result of experimental errors in estimating of the concentrations of soluble and immobilized shCD2 and sCD58.

In a previous analysis (van der Merwe et al., 1993a) of the interaction of rat CD2 with rat CD48 we obtained an accurate affinity (K_d 60–90 μM) but the dissociation rate was so fast that we could not distinguish between actual dissociation time and the time taken to wash the flow-cell. In the present study we were able to clearly distinguish between washing and dissociation and thus obtain more accurate kinetic data. This was possible because (i) the human CD2–human CD58 interaction has a slightly higher affinity than the rat CD2–CD48 interaction; (ii) the time-resolution of the BIAcore was improved to allow data collection at 0.1-s intervals; and (iii) we used a new approach to minimize the washing time which involved extrapolating to infinite flow-rate. Despite these improvements our estimates of the k_{off} and the k_{on} are only lower limits because we were unable to eliminate rebinding during the dissociation phase.

Several previous studies have estimated the avidity of the human CD2 and CD58 interaction using multimeric forms of these molecules (Dustin et al., 1987; Selvaraj et al., 1987) or by inhibition of a multimeric interaction with a monomeric form of CD58 (Dustin et al., 1989). However, only one previous study has attempted to measure rigorously the actual monomeric affinity of the interaction (Sayre et al., 1989). Sayre and co-workers obtained a 23–55-fold higher affinity (K_d 0.4 μM) than we did by equilibrium saturation binding analysis using a ^{125}I-labeled soluble CD2 construct similar to the one used in the present study (Sayre et al., 1989). The discrepancy between the two results may result in part from the difference in the temperature at which the determinations were made [37 °C in the present study and 4 °C in the study by Sayre et al (1989)]. More importantly, real-time analysis on the BIAcore enabled us to measure equilibrium binding levels directly without disturbing the equilibrium and to exclude rigorously the presence of any multimeric binding (van der Merwe & Barclay, 1994). This is important because our results indicate that shCD2, like other soluble IgSF proteins (van der Merwe et al., 1993a), does form multimeric aggregates which can contribute to binding even when present at levels ($\leq$ 2%) too low to detect by gel-filtration or analytical ultracentrifugation. Our results show that equilibrium is attained in the CD2–CD58 interaction within seconds but in the study of Sayre et al. (1989) binding was allowed to equilibrate for 1 h, which would have allowed adequate time for low concentrations of multimeric shCD2 to bind to high levels. When the bound ^{125}I-shCD2 was subsequently separated from the free ^{125}I-shCD2 by washing the extremely rapid dissociation of monomeric shCD2 from sCD58 ($k_{off} \geq 4$ s^{-1}) would have resulted in most monomeric ^{125}I-shCD2 dissociating during the separation step so that the ^{125}I-shCD2 which remained bound would have been mostly multimeric. We have shown that the presence of even small amounts of multimeric, aggregated rat sCD48-CD4 can lead to a measured affinity for rat sCD2 which is 50-fold higher than the actual affinity (Davis et al., 1993).

The Significance of Rapid Binding Kinetics. Leucocyte interactions with other cells are usually transient and need to be reversed when the cells move apart (Dustin & Springer, 1991). The speed of this detachment can be extremely fast as lymphocytes and neutrophils migrate at speeds of up to 0.2 μm/s (Wilkinson, 1982) and neutrophils roll along vascular endothelium at speeds of up to 50 μm/s (Atherton & Born, 1973). It can be calculated that, in order to allow such rapid detachment, CAM complexes spaced at intervals of 0.1 μm would need to dissociate at rates of 2 s^{-1} to 500 s^{-1}. Clearly this would be facilitated by very rapid dissociation rate constants, as demonstrated for human CD2–CD58 complexes in the present study, and for rat CD2–CD48 complexes in a previous study (van der Merwe et al., 1993a). We are not aware of any other studies of the kinetics of CAM interactions, and so we do not know whether fast dissociation rate constants are typical of CAMs in general. Ushiyama and co-workers (Ushiyama et al., 1993) recently reported that a soluble, monomeric form of human P-selectin binds an unidentified O-linked sialylated oligosaccharide ligand on HL-60 cells with a suprisingly high affinity (K_d 70 nM). Lollo and co-workers (Lollo et al., 1993) used an indirect assay based on inhibition of mAb Fab binding to estimate the affinity of soluble murine intercellular adhesion molecule 1 (ICAM-1) binding to lymphocyte function-associated antigen 1 (LFA-1) on T lymphocytes. They estimated that ICAM-1 bound LFA-1 on resting T lymphocytes with a K_d of 100 μM but they argued that stimulation by phorbol 12-myristate 13-acetate led to the conversion of some of the LFA-1 molecules to a high affinity state ($K_d \sim 0.36$ μM). It is difficult to compare either of these reports to the present study since one study measured the affinity indirectly (Lollo et al., 1993) and neither study rigorously excluded the possibility that small amounts of multimeric aggregates in their monomeric protein preparations contributed to the observed binding (Davis et al., 1993; van der Merwe et al., 1993a). If the relatively high affinity of the P-selectin and LFA-1 interactions are confirmed it would suggest that interactions of these molecules may have slower off-rate constants than CD2 interactions. This raises the question of how interactions mediated by these CAMs could be reversed as quickly as required for migrating or rolling leucocytes. Rapid dissociation of stable, LFA-1-mediated interactions could be effected through down-modulation of the avidity of LFA-1 for its ligands (Dustin & Springer, 1989; Dustin & Springer, 1991; Hynes, 1992). It has been suggested that the rapid dissociation of P-selectin-mediated interactions could result from an increase in the dissociation rate constant when the interaction is subjected to the shearing force of flowing blood (Tözeren & Ley, 1992). However, a recent study found that the rolling velocity of neutrophils on E-selectin

reached a plateau as the shear force was increased, suggesting that the dissociation rate constant of the E-selectin–ligand interaction may not be increased by the application of force (Lawrence & Springer, 1993).

The attachment of leucocytes to endothelium under conditions of flow and the subsequent rolling can be mediated by all selectin CAMs (Lawrence & Springer, 1991; Smith et al., 1991; von Andrian et al., 1991; Berg et al., 1993; Lawrence & Springer, 1993) but not by integrin (LFA-1 and Mac-1)–ICAM-1 interactions or by the CD2–CD58 interaction (Lawrence & Springer, 1991). It has been suggested that this functional difference between CAMs may be the result of the selectin CAMs having faster interaction kinetics (Lawrence & Springer, 1991; Williams, 1991b). Our finding that soluble, monomeric forms of human CD2 and CD58 interact with rapid kinetics suggests that differences in molecular rate constants are unlikely to explain these functional differences. Morever, even if selectins did have very rapid solution rate-constants binding of tethered molecules in-vivo would be rate-limited by slow diffusion within the plane of the membrane (Bell, 1978). An alternative way of increasing the interaction kinetics of CAMs is by increasing the ligand concentration or surface density, and so it is likely to be significant that selectins interact with carbohydrate ligands, through their NH_2-terminal C-type lectin domains (Lasky, 1992). Williams (Williams, 1991b) suggested that exceptionally high ligand concentrations might explain the functional properties of selectins and predicted that carbohydrate ligands of selectins are presented by mucin-like cell-surface glycoproteins since these molecules are able to present large numbers of identical tightly packed O-linked oligosaccharides. This prediction has recently been confirmed by the identification of four mucin-like glycoproteins which present carbohydrate ligands to selectins (Lasky et al., 1992; Baumhueter et al., 1993; Berg et al., 1993; Sako et al., 1993). It is probably also significant that L-selectin is located in an exposed position on the tips of microvilli and membrane ruffles of neutrophils whereas the integrin Mac-1 is found mainly on the cell body (Picker et al., 1991; Erlandson et al., 1993). The combination of a very high ligand concentration and an exposed position on the cell surface may underly the ability of selectins to mediate extremely rapid cell–cell adhesion.

Testing for Binding of Human CD2 to CD48 or CD59. The CD2 ligand in mice (Kato et al., 1992) and rats (van der Merwe et al., 1993b) is CD48, and there have been reports that CD48 is an additional CD2 ligand in humans, but crude estimates suggested that human CD48 has a much lower affinity for human CD2 than CD58 (Arulanandam et al., 1993; Sandrin et al., 1993). We were unable to detect binding of a soluble human CD48 construct to immobilized shCD2 under conditions in which an interaction with an affinity of $K_d \leq 200$ μM would have been readily detected (Figure 5B). We were also unable to detect an interaction when shCD48 was immobilized and shCD2 injected at high concentrations. While it is not possible to exclude a CD48–CD2 interaction our results suggest that, if such an interaction exists, it must have an extremely low affinity ($K_d \geq 0.5$ mM). Nevertheless, it is conceivable that an interaction as weak as this may mediate adhesion when multimeric.

There have been reports that human CD2 interacts with the structurally unrelated molecule CD59 (Deckert et al., 1992; Hahn et al., 1992) but a more recent study found no evidence for an interaction (Arulanandam et al., 1993). We were unable to detect binding of monomeric shCD59 to CD2 even when injected at 1.5 mM. Furthermore, multimeric CD59 (sCD59-Hμ) did not bind under conditions in which the multimeric CD58 (sCD58-Hμ) bound CD2 very well. This is particularly significant because conversion of monomeric CD58 (sCD58) to decameric form (sCD58-Hμ) led to a 400 000-fold decrease in the k_{off} (Figures 6C and 9) which, assuming that the k_{on} is not substantially changed, suggests that decameric CD58 has a ~400 000-fold higher avidity than monomeric CD58. These results are unlikely to be the result of abnormal folding of shCD59 because it binds to all CD59 mAbs tested and we have used this material to determine the tertiary structure of sCD59 (Kieffer et al., 1994). CD59 purified from human urine also did not bind CD2, which eliminates the possibilities that altered glycosylation or the truncation of shCD59 at asparagine-70 prevented the binding of shCD59 to shCD2. Taken together, these results argue that any direct CD2–CD59 interaction must have a $K_d \geq 1$ mM.

Previous studies reporting interactions between CD2 and either CD48 (Arulanandam et al., 1993; Sandrin et al., 1993) or CD59 (Deckert et al., 1992; Hahn et al., 1992) relied on assays in which one (Deckert et al., 1992; Hahn et al., 1992; Arulanandam et al., 1993) or both (Sandrin et al., 1993) of the putative ligands were expressed on cell-surfaces. For example, Hahn et al. (1992) and Deckert et al. (1992) reported binding of purified CD59 to CD2 expressed on cells and Arulanandam et al. (1993) reported binding of a purified multimeric CD2-IgM fusion protein to cells expressing CD48. While we have been unable to obtain evidence for direct interactions between purified CD2 and purified CD48 or CD59 it is possible that these molecules may interact weakly or indirectly as parts of higher-order complexes which include other interacting cell-surface molecules.

In conclusion, we have used surface plasmon resonance technology to do a detailed affinity and kinetic analysis of the human CD2–CD58 interaction and to look for an interaction between human CD2 and two other putative ligands CD48 and CD59. Our results demonstrate that, as found for the rat CD2–CD48 interaction, the human CD2–CD58 interaction has a very low affinity (K_d 9–22 μM), substantially lower than previously reported. No binding was detected of human CD2 to either CD48 or CD59, indicating that any direct physical interactions between these molecules must be exceptionally weak and therefore of doubtful physiological significance. We have used a novel approach to measure extremely fast dissociation rate constants and have determined that CD58 dissociates from CD2 with a $k_{off} \geq 4$ s^{-1}. These findings, taken together with our previous report (van der Merwe et al., 1993a), provide further evidence that reversible cell adhesion is mediated by multiple, very weak interactions with rapid kinetics (van der Merwe & Barclay, 1994). Such interactions are well-suited to mediating cell–cell interactions in the immune system in that they permit high avidity, multimeric interactions between cells while allowing cells to separate easily, without the need to invoke special mechanisms.

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