Signaling lymphocytic activation molecule (SLAM, CDw150) is homophilic but self-associates with very low affinity

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Running title: SLAM self-associates with very low affinity
Summary

SLAM (signaling lymphocytic activating molecule, CDw150) is a glycoprotein that belongs to the CD2 subset of the immunoglobulin superfamily (IgSF) and is expressed on the surface of activated T- and B-cells. It has been proposed that SLAM is homophilic and required for bi-directional signaling during T- and B-cell activation. Previous work has suggested that the affinity of SLAM self-association might be unusually high, undermining the concept that protein interactions mediating transient cell-cell contacts, such as those involving leukocytes, have to be weak in order that such contacts are readily reversible. Using surface plasmon resonance (SPR)-based methods and analytical ultracentrifugation (AUC), we confirm that SLAM is homophilic. However, we also establish a new theoretical treatment of SPR-derived homophilic binding data which indicates that SLAM-SLAM interactions (solution $K_d \sim 200 \mu M$) are in fact considerably weaker than most other well-characterized protein-protein interactions at the cell surface (solution $K_d \sim 0.4-20 \mu M$), a conclusion that is supported by the AUC analysis. Whilst further analysis of the AUC data implies that SLAM could form “head to head” dimers spanning adjacent cells, the very low affinity raises important questions regarding the physiological role and/or properties of such interactions.

(193 words)
Introduction

Transient, low affinity interactions between the proteins present on the surfaces of leukocytes or other cell types are critical for the normal functioning of the immune system (1). Among the best characterised interactions of this type are those mediated by the CD2 subset of the immunoglobulin superfamily (IgSF)\(^1\) (2,3). Proteins in this subset are characterized by the presence of paired membrane-distal V-set and membrane-proximal C2-set IgSF domains with distinctive disulphide bond arrangements (4), and the subset now includes CD2, CD58, CD48, 2B4, SLAM (CDw150), CD84 and Ly-9.

It is postulated that the CD2 subset arose via successive duplications of a common ancestral gene originally encoding a homophilic cell adhesion molecule (5). In addition to sequence and structural relationships, this proposal is supported by the fact that many of the genes encoding these molecules are clustered at two duplicated loci in humans and mice and that most, if not all, of the interactions of these proteins are restricted to one or more members of the CD2 subset (6,7). Substantial overlap in the ligand binding specificities and signaling properties of these molecules further underscores their close evolutionary history. For example, murine CD2 and CD48 both bind 2B4 (6), the cytoplasmic domains of 2B4 and SLAM each bind an adaptor signaling protein known as SLAM-associated protein (SAP) (8,9) and ligand or antibody-mediated crosslinking of human CD2 (reviewed in (10)) or SLAM (11) induces T cell proliferation. Finally, rat and human soluble (s) CD2 crystals are dominated by homophilic “head-to-head” crystal lattice contacts proposed (12,13), and subsequently shown (14), to mimic the topology of natural ligand interactions. Previously, however, there has been no clear evidence that any homophilic interactions of potential physiological significance occur among the existing members of this subset of the IgSF.

SLAM was initially identified with a monoclonal antibody (mAb) that activated T cells and bound a previously uncharacterised activation antigen (11). It is expressed by CD45RO+ T-cells, immature
thymocytes, and a proportion of B-cells, and is rapidly upregulated upon the activation of T cells, B cells and dendritic cells (11,15,16) but not NK cells (17). It has been shown in mice that highly polarized Th1 but not Th2 cells express high levels of SLAM (17). Cross-linking studies with mAbs have suggested that SLAM is a receptor that influences T-cell (18,19) and B-cell responses to antigen (16,20). Activated human T cells and B cells express mRNA species encoding three forms of SLAM: a membrane-bound protein encoded by the longest open reading-frame and containing three consensus cytoplasmic SH2 domain-binding sequences, a variant membrane-anchored form with a truncated cytoplasmic domain and a soluble, secreted form of SLAM (11,15). One or more of the SH2 domain-binding [TIYXX(V/I)] sequences, which are also present in the cytoplasmic domains of 2B4, CD84 and Ly-9 (21), bind the SH2 domain of SAP (8,21,22). It is proposed that signal transduction by SLAM is regulated via a novel mechanism in which SAP competes with an SH2 domain-containing phosphatase (8,20) for the TIYXX(V/I) sequences (8). Defects in the SAP gene are responsible for an X-linked, lymphoproliferative syndrome characterised by enhanced susceptibility to primary Epstein-Barr virus infections in humans (8,23,24).

The prevailing view of the function of SLAM is that it is homophilic and that through bi-directional signalling it regulates T- and B-cell responses (8). Preliminary data implying that the affinity might be as high as 0.1 nM (15) argue against a role in T-cell-B-cell interactions, however, since all of the well-characterized cell-cell interactions have much lower affinities, presumably to ensure that such interactions are readily reversible (1). To resolve this issue, we have characterised the self-association of soluble (s) forms of SLAM using SPR-based methods and analytical ultracentrifugation. This analysis, for which a new theoretical framework for characterizing homophilic protein-protein interactions using SPR-based methods had to be established, indicates that SLAM self-associates with an affinity much lower than was previously anticipated.
Experimental procedures

Monoclonal antibodies

MAbs used were: SLAM mAbs IPO3 (16) (Kamiya Biomedical Company, Seattle, US), and A12 (11) (kindly provided by DNAX, Palo Alto, Ca); rat CD4 mAb, OX68; and rat CD2 mAb, OX34. The OX mAbs are referenced in the European Collection of Animal Cell Cultures (www.camr.org.uk/ecacc.htm). IgG FITC goat anti-mouse was supplied by Serotec (Kidlington, U.K.).

Expression of recombinant proteins

Chimeric protein. Vectors for expressing a chimeric form of SLAM (sSLAMCD4) consisting of the entire extracellular region fused with domains 3 and 4 of rat CD4 and containing a C-terminal biotinylation sequence were constructed as described (6,25) from human SLAM cDNA ((11) kindly provided by DNAX, Palo Alto, Ca). The join at the Sal I (g tcg acc) junction of SLAM with CD4 was SLAM: DPSST (the residues containing the Sal I site are underlined).

sSLAM. The extracellular region of SLAM (sSLAM) was amplified by polymerase chain reaction (PCR) from cDNA prepared from MT-2 cells (HTLV-1 - transformed human T cells). The 5’ primer was complementary to the SLAM leader sequence (MDPKGLLS; (11)), added an Xba I site, and inserted, immediately upstream of the initiation codon, the 25 bases that precede the rat CD4 initiation codon (26). The 3’ primer was complementary to the membrane proximal SPWPGCRTDPSETK-encoding sequence and added nucleotides encoding a short linker (amino acids GGG), the BirA biotinylation signal sequence (amino acids LNDIFEAQKIEW; (27)), a tag consisting of six histidines, a stop codon and another Xba I site. The PCR fragment was subcloned into the glutamine synthetase-based gene expression vector, pEE14 (28). All constructs were checked by dideoxy sequencing.

Chimeric proteins were expressed by transiently transfecting 293T cells with 40 µg plasmid
DNA/5 x10^6 cells/175 cm² flask using calcium phosphate as described (6). CHO-K1 cells were transfected with the pEE14 construct (28,29) and methionine sulfoximine-resistant clones were screened for expression by Western-blotting of the tissue culture supernatant with an anti-penta-His antibody (Qiagen GmbH). The best clone was grown to confluence in bulk culture before switching to medium supplemented with 2 mM sodium butyrate, as described (29).

Protein purification, His tag removal and biotinylation

sSLAM was purified from spent tissue culture supernatant by affinity chromatography using Ni-NTA resin (Qiagen GmbH) followed by size exclusion chromatography on a Superdex 200 HR 10/30 column. When required, the histidine tag at the carboxy terminus was removed by incubating 2.5 mg of sSLAM in 1.5 ml Hepes-saline buffer (10 mM Hepes pH 7.4, 150 mM NaCl) with 1.2 U of carboxypeptidase A conjugated to agarose beads (Sigma Chemicals, Poole, UK) for 16h at 30°C with agitation. Removal of the carboxy terminal histidines was at least 80% efficient according to amino acid analysis. The extinction coefficient of the de-tagged protein was determined by amino acid analysis to be 1.03 cm²/mg. Prior to BIAcore™ analysis, sSLAM was passed through a Superdex 75 gel filtration column to remove aggregated protein (Pharmacia, Biotech Ltd.). For immobilisation in BIAcore™ experiments, purified sSLAM or sSLAMCD4 in concentrated tissue culture supernatant were biotinylated by incubation with recombinant BirA enzyme (obtained from Avidity, Denver, CO, USA or as a kind gift from Dr C.A. O’Callaghan) in 10 mM Tris-HCl, pH 8, 7.5 mM MgCl₂, 5mM NaCl, 5mM ATP and 1 mM biotin, overnight (6,30). The protein was then buffer-exchanged into HBS to remove free biotin prior to use.

General SPR methods

All binding experiments were carried out on a BIAcore 2000 instrument (BIAcore AB, St Albans, UK) using HBS buffer, 25 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant P20 supplied by the manufacturer. Streptavidin (Pierce, Rockford, USA) was coupled at 0.2 mg/ml in 10
mM sodium acetate pH 5 to research grade CM5 chips (BIAcore AB) using amine coupling kits (BIAcore AB) and an activation time of 5 min, resulting in immobilization levels of ~ 3000-4000 response units (RU). Equilibrium binding analysis was undertaken as described (7): increasing and decreasing concentrations of sSLAM (5 µl injections at a flow rate of 20 µl/min) were passed over proteins immobilized at high, intermediate, and low levels. For kinetic analysis, dissociation rates were measured as described (7). Experiments were performed at 25°C unless indicated. Affinity and kinetic data were analysed using the curve fitting tools of Origin v.5.0 (MicroCal Software Inc, Northampton, MA).

A theoretical framework for SPR-based analysis of homophilic interactions

Assuming that all of the molecules of immobilised sSLAMB are equally available to take part in homophilic interactions, the relevant equations at equilibrium are:

(a) For interactions between molecules in solution,

\[
[A_s,A_s] = \frac{1}{K_{d1}} *[A_s]^2
\]

(b) For interactions between immobilized molecules,

\[
[A_f,A_f] = \frac{1}{K_{d2}} *[A_f]^2
\]

(c) For interactions between soluble and immobilized molecules

\[
[A_s,A_f] = \frac{1}{K_{d3}} *[A_s]*[A_f]
\]

where \([A_s]\) and \([A_f]\) are the concentrations of monomeric soluble and monomeric immobilised molecules respectively and \([A_s,A_s], [A_f,A_f]\) and \([A_s,A_f]\) are the concentrations of the three species of dimeric complexes. The corresponding dissociation constants are designated \(K_{d1}, K_{d2}\) and \(K_{d3}\). In keeping with the general interpretation of BIAcore™ data, in what follows \(K_{d3}\) will be assumed to be
equal to \(K_{d1}\). The relationship between \(K_{d1}\) and \(K_{d2}\) will be considered when the experimental data are discussed.

In calculating \([A_s]\) and \([A_s,A_s]\) from equation (1) use is made of the fact that, at equilibrium, the concentration of these molecular species is the same in the chip as it is in the solution passing through it. Putting \([A_s] = [A_{so}] - 2[A_s,A_s]\), where \([A_{so}]\) is the total concentration of molecules in the soluble phase, equation (1) can be solved to give,

\[
[A_s] = \frac{K_{d1}}{4} \left[1 + \frac{8[A_{so}]}{K_{d1}}\right]^{\frac{1}{2}} - 1
\]

(4)

Similarly, equations (2) and (3) yield, after some rearrangement,

\[
[A_r] = \frac{K_{d2}}{4} \left[1 + \frac{[A_s]}{K_{d1}}\right] \left[1 + \frac{8[A_{fo}]}{K_{d2} \left[1 + \frac{[A_s]}{K_{d1}}\right]}\right]^{\frac{1}{2}} - 1
\]

(5)

where \([A_{fo}]\) is the total concentration of immobilised molecules on the chip and \([A_s]\) is given by equation (4).

Equations (4) and (5), which give \([A_s]\) and \([A_r]\) in terms of the two concentrations \([A_{so}], [A_{fo}]\) and the two dissociation constants, \(K_{d1}\) and \(K_{d2}\), are used, in equation (6) below, to give theoretical values for the response units obtained from the BIAcore\textsuperscript{TM}. Equation (6) is

\[
R.U. = P \frac{1}{K_{d1}} \frac{1}{[A_s][A_r]}
\]

(6)

where \(P\) is the number of response units/M of dimeric molecules formed by association between molecules in solution and those immobilised on the chip. For a molecule with an \(M_r\) of 30K the value
of P is taken to be 3 RU/µM. Titration of the sSLAM signal in the reference flow cell (see Results; Figure 2B) allowed accurate determination of P and level of sSLAMb immobilized (i.e. [A₀]).

With these substitutions, the full binding equation becomes

\[
R.U. = \frac{3}{4} \times \left[ \left( 1 + \frac{8[A_{so}]}{K_{d1}} \right)^{\frac{1}{2}} - 1 \right] \times \frac{K_{d2}}{4} \times \left[ 1 + \frac{[A_{s}]}{K_{d1}} \right] \times \left[ 1 + \frac{8[A_{f_0}]}{K_{d2} \times \left[ 1 + \frac{[A_{s}]}{K_{d1}} \right]^{2}} \right]^{\frac{1}{2}} - 1
\]

(7)

To derive \( K_{d1} \) and \( K_{d2} \) from the experimental data involves choosing values of these parameters that best fit the observations. This choice is facilitated by the fact that at low values of \([A_{so}]\) the soluble molecule is virtually all in monomeric form, that is \([A_{s}]\) approximately equals \([A_{so}]\). Further, the value of \([A_{f}]\) is given by,

\[
[A_{f}] \approx \frac{K_{d2}}{4} \times \left[ \left( 1 + \frac{8[A_{f_0}]}{K_{d2}} \right)^{\frac{1}{2}} - 1 \right]
\]

(8)

It follows that, at values of \([A_{so}]\) much lower than the \( K_{d} \), the fraction \([A_{so}]_{\text{bound}}/[A_{so}]_{\text{free}}\) (defined as \( g \)), is given by

\[
g = \frac{[A_{so}] - [A_{s}]}{[A_{f}]} \approx \frac{K_{d2}}{4 \times K_{d1}} \times \left[ \left( 1 + \frac{8[A_{f_0}]}{K_{d2}} \right)^{\frac{1}{2}} - 1 \right] = \frac{\text{RU}}{3 \times [A_{so}]}
\]

(9)

Equation (9) may be rearranged to give,

\[
K_{d2} = 2 \times K_{d1} \times g \times \left[ \frac{(K_{d1} \times g)}{([A_{f_0}] - K_{d1} \times g)} \right]
\]

(10)
This expression may be used to express $K_{d2}$ in terms of $g$ and $K_{d1}$ in equation (7). The result is,

$$R.U. = \frac{3}{8} \left[ \left( 1 + \frac{8[A_{s0}]}{K_{d1}} \right)^{\frac{1}{2}} \right]^{\frac{1}{2}} \left[ \left( \frac{K_{d1}g}{([A_{f0}] - K_{d1}g)} \right) \right]^{\frac{1}{2}} \left[ 1 + \frac{[A_{s1}]}{K_{d1}} \right]^{\frac{1}{2}} \left( \frac{4[A_{f0}]}{([A_{f0}] - K_{d1}g) \left( \left( \frac{K_{d1}g}{([A_{f0}] - K_{d1}g)} \right) \right]^{\frac{1}{2}} \right) - 1$$

(11)

If the value of $g$, for a given value of $[A_{f0}]$, is determined experimentally, equation (11) may be used to calculate response units for various values of $[A_{s0}]$, for any chosen value of $K_{d1}$. In practice a curve-fitting computer program is used to determine the value of $K_{d1}$ that best fits the observed dependence of RU on $[A_{s0}]$.

Alternatively, if $g$ is measured for two or more different values of $[A_{f0}]$, equation (10) may be used to calculate $K_{d1}$ and $K_{d2}$. Thus, if $g_a$ and $g_b$ are two values of $g$, obtained by using two different concentrations, $[A_{f0}]_a$ and $[A_{f0}]_b$ of immobilised molecules on the chip, then it follows from equation (10) that,

$$K_{d1} = \frac{g_b^2[A_{f0}]_a - g_a^2[A_{f0}]_b}{g_a^2g_b^2(g_b - g_a)}$$

(12)

Alternatively, if $K_{d1}$ is assumed to be equal to $K_{d2}$ then, again from equation (10),

$$K_{d1} = K_{d2} = \frac{[A_{f0}]}{g * (2 * g + 1)}$$

(13)

Analysis of the experimental results, for sSLAM, indicates that they are compatible with the two dissociation constants being equal. Consequently, equation (13) can be used to calculate $K_{d1}$ from the RU values obtained at low concentrations of sSLAM passed through the BIAcore™ chip. More generally, if $K_{d1}$ and $K_{d2}$ are not assumed to be equal equation (11) must be used to evaluate $K_{d1}$. 
Calculation of the effective concentration of the immobilised phase

To set up the immobilised phase ($A_f$), sSLAMb was generated by biotinylating the COOH terminus of sSLAM in order to anchor it to streptavidin covalently attached to the sensor surface. Streptavidin has four biotin-binding sites, but considerations of steric hindrance strongly suggest that at most three of these remain accessible to biotin after immobilising the streptavidin to the chip. Consequently, the immobilised sSLAMb probably exists in monomeric, dimeric and trimeric forms. The presence of the bivalent and trivalent forms introduces a complication in calculating equilibrium constants for the immobilised sSLAMb molecules in that these multimers may be expected to make more stable homophilic complexes between themselves than does the monomeric form of sSLAMb. In order to take this complexity into account we assume that only the monomeric form of immobilised sSLAMb is available to react with the soluble sSLAM passing through the chip.

If $[B]$ is the total molar concentration of sSLAMb on the chip and $[Av]$ is the concentration of streptavidin then, assuming that three biotin binding sites remain accessible on each streptavidin molecule, the proportion of the total immobilised sSLAMb that exists as a monomer is given by,

$$\frac{[\text{SLAM}_{\text{monomer}}]}{[\text{SLAM}_{\text{total}}]} = \left(1 - \frac{[B]}{3 \times [Av]}\right)^2$$

(14)

The application of equation (10) to the experimental data given in the Results indicates that, at [sSLAMb$_{\text{total}}$] values of 82.7 µM, 186 µM and 438 µM the fraction of the total immobilised sSLAMb that exists as a monomer is, respectively, 0.91, 0.80 and 0.56. The corresponding values for the effective concentrations of sSLAMb are 75 µM, 148 µM and 246 µM. A correction of this magnitude is clearly essential since, without it, the application of equation (12) to the experimental data obtained with 82.7 µM, and 438 µM sSLAMb immobilised, predicts a negative value for $K_d_1$. In contrast, the application of equation (12) to the 82.7 µM and 186 µM results gives a positive value for the same dissociation constant. Similarly, uncorrected values of $[A_{f0}]$ substituted in equation (9)
give very disparate values for the ratio of $K_d_1 / K_d_2$ when the observed values of $g$ are used to calculate it.

**An alternative binding model**

In the preceding analysis it was assumed that those molecules of immobilised sSLAMb, that were not involved in the multimeric homophilic interactions described above, were available to react both with each other and equally with sSLAM passing through the chip. This assumption would be invalid if the multimeric interactions imposed some conformational restraint on the gel matrix that impeded homophilic interactions between molecules of immobilised sSLAMb but not between immobilised and soluble forms of the molecule. To analyze this model, $K_d_2$ was taken to be essentially infinite. In this case the binding equation reduces to:

$$R, U = 3 * \left[ \left(1 + \frac{8[A_{f_o}]}{K_d_1} \right)^{1/2} - 1 \right] * \left[ \frac{[A_{f_o}]}{3 + \left(1 + \frac{8[A_{f_o}]}{K_d_1} \right)^{1/2}} \right]$$

The term $[A_{f_o}]$ is equal to $g.K_d_1$, where $g$ is, as before, the observed ratio of bound to free sSLAM at low concentrations of sSLAM. With this equality substituted into equation (15) a comparison can be made between the experimental data and the prediction of this alternative model, without an explicit value of $[A_{f_o}]$ being required. Application of this model to the experimental data demonstrated that it was invalid since the values of $K_d_1$ that it predicted yielded corresponding values of $[A_{f_o}]$ that were actually larger than those used in the experiments (see Results).

**Analytical ultracentrifugation**

(i) Data collection

Samples to be analyzed by sedimentation velocity were contained in double sector centrepieces (pathlength 3 or 12 mm depending on protein concentration) and spun at 40,000 rpm in a Beckman
Optima XL-A analytical ultracentrifuge at 37°C. The sedimentation of the protein boundary was observed using a variety of incident wavelengths of light, chosen to give an interpretable series of boundary traces for the experimental protein concentration. At high concentrations a schlieren signal was observed in the absorbance scans. This has been reported once before when it was shown that it is a function of the precise configuration of the AUC monochromator (31) which varies between instruments. This was controlled for by collecting data at different wavelengths and resulted in an additional peak in the g(s*) profile at low s* at wavelengths where a schlieren signal was elicited. The schlieren contribution was allowed for at this stage.

(ii) Data analysis

Data were analyzed using the time derivative (g(s*)t) method calculated with the program dc/dt (32) wherein

\[ g(s^*) = \left( \frac{d}{dt} \left[ \frac{c(r,t)}{c_0} \right] \right) \left( \frac{\omega^2 t^2}{\ln(r/m/r)} \right) \left( \frac{r}{r_m} \right)^2 \]  

and g(s*)t (in arbitrary units) is the distribution of apparent sedimentation coefficients (s*) at the boundary calculated from the time derivative of a series of boundary traces, c is concentration (in absorbance units), r is radial distance from the centre of the rotor (in cm), t is the time since the start of the experiment (in seconds), c0 is the concentration at time 0, \( \omega \) is the rotor velocity (in radians/second), and rm is the radius at the meniscus. The s* values determined by fitting the g(s*)t profiles were plotted against protein concentration, extrapolated to zero concentration and corrected for the effects of buffer density and viscosity and the temperature at which the experiment was performed to yield values of \( s_{20,w}^0 \), the sedimentation coefficient in water at 293 K at infinite dilution. This value was used to calculate the frictional ratio \( ff_0 \) displayed by the two species using the equation (33),
\[
\frac{f}{f_0} = \frac{M \left( 1 - \bar{\nu} \rho_0 \right)}{N_A 6\pi \eta_0 s_{20,w}^0 \left( \frac{3\bar{\nu}M}{4\pi N_A} \right)^{\frac{1}{3}}}
\]  

wherein \( M \) is the mass of the species (in g/mol), \( \bar{\nu} \) its partial specific volume (in ml/g), \( \rho_0 \) is the density of water, \( N_A \) is Avogadro’s number and \( \eta_0 \) is the viscosity of water (in poise). The frictional ratio is the ratio between the experimental frictional coefficient and that of a sphere with the same mass. This was corrected for a range of hydration factors to obtain the experimental Perrin function describing the axial ratio of the species \( (34) \),

\[
P_{\text{exp}} = \frac{f}{f_0} \left[ 1 + \left( \frac{\delta_{\text{app}}}{\bar{\nu} \rho_0} \right) \right]^{\frac{1}{3}}
\]  

wherein \( P_{\text{exp}} \) is the Perrin function and \( \delta_{\text{app}} \) is the apparent hydration (in g H\(_2\)O/g protein).

Experimental values for the sedimentation coefficient in water at 293 K (see Results) were also corrected for hydration using a similar relationship

\[
s_0 = s_{20,w}^0 \left[ 1 + \left( \frac{\delta_{\text{app}}}{\bar{\nu} \rho_0} \right) \right]^{\frac{1}{3}}
\]  

where \( s_0 \) is the anhydrous sedimentation coefficient. All curve fitting was carried out using the non-linear least-squares curve-fitting package, ProFit (Quantum Software, CA).

(iii) sSLAM modelling

A model for sSLAM was based on the structure of rat sCD2 \( (12) \) with 8 octaglycyl chains modelled in random orientations at the positions which align with the glycosylation sequons seen in the SLAM protein sequence. The octasaccharides modelled at each position represent the average N-linked carbohydrate structure added by CHO cells \( (35) \). This model was converted to hydrodynamic beads using the program \textit{AtoB} \( (36) \) and hydrodynamic parameters calculated using the program \textit{SOLPRO} \( (37) \). A dimeric model was based on the complex between CD2 and CD58 \( (14) \) by aligning domain 1 of the monomeric model with the CD2 and CD58 domains present in the complex structure using the
program *SHP* (38). The hydrodynamic parameters of this model were calculated in the same way as for the monomer.

**RESULTS**

*sSLAM self-associates*

Two soluble, recombinant forms of human SLAM were prepared for this study, one consisting of the extracellular region of SLAM including a COOH-terminal histidine tag (sSLAM) and another, chimeric form consisting of the extracellular region fused to domains three and four of rat CD4 (sSLAMCD4). Additional sequence was incorporated at the COOH-termini so that each protein could be biotinylated using the BirA enzyme (the biotinylated forms are called sSLAMb and sSLAMCD4b, respectively). Both sSLAMb and sSLAMCD4b immobilized on sensor surfaces via streptavidin bound strongly to two SLAM mAbs, A12 (11) and IPO-3 (16), which compete for identical or overlapping binding sites on the molecule (Figure 1A and B; data not shown). Assuming bivalent binding of the antibodies, the responses observed indicate that at least 70% of the immobilised SLAM was antigenically active. This, together with the high levels of expression of the two proteins, indicates that both proteins were correctly folded. On gel filtration, sSLAM eluted anomalously early at all concentrations (eluting at the same volume as a globular protein of 100 kD; data not shown). In our experience (7), such behaviour is characteristic of heavily glycosylated asymmetric proteins (sSLAM has 8 potential N-glycosylation sites) and, overall, the gel filtration data were consistent with sSLAM being monomeric at low protein concentrations.

Changes in response observed when sSLAM protein was passed over immobilised sSLAMb, or a control protein, human 2B4CD4 (6), are shown in Figure 1C. The control response is due to the high concentration of the protein injected into the flow cell; the difference in response between the two flow cells represents specific binding (self-association). Similar levels of binding were also observed when sSLAM was passed over immobilized sSLAMCD4b (data not shown). Binding was observed
following removal of the histidine tag from sSLAM with carboxypeptidase A indicating that the responses were not due to artefactual histidine tag-mediated interactions (data not shown). sSLAM did not bind to the immobilized control proteins CD4 or human sCD84CD4 [(39); M.H.B. and Pablo Engel, unpublished]. Moreover, no binding was observed when an irrelevant histidine-tagged protein was injected over immobilised sSLAMb (data not shown). To further confirm the specificity of binding, saturating levels of SLAM mAbs were passed over immobilized sSLAMb prior to injection of sSLAM. Both SLAM mAbs completely blocked binding (see for example, Figure 1D) indicating that binding is specific and that the antibody epitopes overlap the ligand binding site of SLAM.

sSLAM injection induced the dissociation of the mAb A12 from the surface (Figure 1D). This is most likely to be a consequence of injected sSLAM preventing the rebinding of bivalently bound mAb that had partially dissociated.

Affinity of sSLAM self-association

The injection of a range of concentrations of sSLAM over immobilized sSLAMb at 37°C (Figure 2A) clearly indicated that binding was saturable (Figure 2B and C). The determination of homophilic affinity constants using SPR differs from that for heterophilic interactions in that there are three, rather than just one, interactions to consider. The observed responses (measured in RU) reflect only the binding of the molecules in solution to those immobilised in the flow cell, whereas interactions between immobilised molecules and between molecules in the solution phase will also occur, both of which act to reduce the observed response at a given concentration of injected sSLAM. A theoretical model was therefore developed that takes these additional interactions into account, and the equations derived from this model were used to calculate the sSLAM-sSLAM affinity constant from the binding data.

At low concentrations of soluble reactant in the flow cell, the RU observed are directly proportional to the concentration, \([A_{SO}]\), of the reactant used (Figure 3A). This is true for all three values of the
concentration $[A_{f0}]$ of the immobilised component on the chip. A theoretical derivation of the constant of proportionality, $g$, (the ratio $[A_{so}]_{\text{bound}}/[A_{so}]_{\text{free}}$), between RU and $[A_{so}]$ is derived in the Experimental Procedures (see equation (9)). The values of $g$, obtained from Figure 3A are as follows: $g = 0.248$; $g = 0.388$; $g = 0.547$ for the low, intermediate and high values of $[A_{f0}]$ used in the experiments. If pairs of these values of $g$ are substituted into equation (12) we obtain three values for $K_{d1}$ and $K_{d2}$. They are: $K_{d1} = 162$ $\mu$M, $181$ $\mu$M and $217$ $\mu$M and $K_{d2} = 92$ $\mu$M, $134$ $\mu$M and $220$ $\mu$M, respectively. The closer concordance for the three values of $K_{d1}$, compared to $K_{d2}$, reflects the greater sensitivity of the latter parameter to small variations in the value of $K_{d1}$. In calculating these values for the dissociation constants, the values of $[A_{f0}]$ were corrected to allow for multimer formation as described (see equation (14)).

It is apparent from the values of $K_{d1}$ and $K_{d2}$ calculated above that, within experimental accuracy, the two equilibrium constants are very similar. If, however, the assumption is made that $K_{d1} = K_{d2}$ then equation (13) can be used to calculate $K_{d1}$ from the observed values of $g$ given above. The results are $203$ $\mu$M, $216$ $\mu$M and $211$ $\mu$M, respectively for $75$ $\mu$M, $148$ $\mu$M and $246$ $\mu$M effective concentrations of immobilised sSLAMb in the flow cells. A comparison of these results with those obtained in the previous section, in which no assumption was made about the equality of the two equilibrium constants, indicates that in both instances the value of $K_{d1}$ is approximately $200$ $\mu$M.

When the concentration, $[A_{so}]$, of sSLAM in the soluble phase, is not negligible compared to $K_{d1}$, the variation of the observed response units with concentration is no longer linear. In this case the full equilibrium relations, described by equations (7) and (11), can be used to analyse the experimental data. The procedure for using equation (7) was as follows. The linear part of the response curve was used to determine $g$. With these values of $g$, equation (10) was used to calculate the values of $K_{d2}$ for different, chosen values of $K_{d1}$. These pairs of values of $K_{d1}$ and $K_{d2}$ were then substituted into
equation (7) and the results compared with observation. An example is illustrated in Figure 3B, where the data obtained for \([A_{f0}]\) equal to 148\(\mu\)M is compared with the kinetic theory. With this value of \([A_{f0}]\) the observed value of \(g\) is 0.388, and equation (10) of the Experimental Procedures yields, after some rearrangement,

\[
K_{d2} = \frac{K_{d1}^2}{(1.29) \times (383 - K_{d1})}
\]

(20)

The significance of this relationship is that, for all values of \(K_{d1}\) and \(K_{d2}\) that satisfy it, the value of \(g\), the gradient, at the origin, of \([A_{so}]\)bound versus \([A_{so}]\)free, remains constant. Using this relationship, the calculated values of \(K_{d2}\) in \(\mu\)M, for the corresponding different assumed values of \(K_{d1}\) (shown in parentheses), are as follows: 0.0096 (2.16), 0.999 (21.6), 27 (100), 216 (216), 840 (300), 36109 (383).

It is evident from Figure 3B that the three lowest chosen values of \(K_{d1}\) do not fit the data, but for values of about 200 \(\mu\)M and above, all values agree to within experimental error. Note that, from the above relationship between \(K_{d1}\), \(K_{d2}\) and \(g\), the maximum value of \(K_{d1}\) occurs when \(K_{d2}\) is infinite (i.e. the immobilised sSLAMb does not self-associate). This maximum is 383 \(\mu\)M and it follows that the experimental data imply that \(K_{d1}\) lies in the range 200-383 \(\mu\)M. Figure 3C illustrates the result of applying equation (11) to the experimental data and using a computer-based best fit to evaluate \(K_{d1}\). The values of the equilibrium constant obtained in this way are \(K_{d1} = 181, 282, \) and 160 \(\mu\)M for, respectively, 75, 148 and 246 \(\mu\)M of accessible immobilised sSLAMb. These results, which are compatible with a value of \(K_{d1} \approx 200 \mu\)M, suggest that the upper limit of 383 \(\mu\)M discussed above is likely to be an over estimate. In summary, the mean of the results obtained by the analyses described above (160, 162, 181, 181, 203, 211, 216, 217 and 282 \(\mu\)M) is \(K_d = 201 \pm 37 \mu\)M. While this statistical analysis is of limited value since it depends on \(K_d\) values derived from the data using three
different methods of analysis, the broad level of agreement observed between them supports the conclusion that the $K_d$ value is $\sim 200 \mu M$.

An alternative model was considered in the Experimental Procedures where self-association of monomeric immobilised sSLAMb was assumed not to occur, and hence $K_d2$ is effectively infinite. When equation (15) is used to simulate the experimental data where departure from linearity is observed (7 data points), the calculated values of $K_d1$ are 333 $\mu M$, 534 $\mu M$ and 532 $\mu M$, respectively, for the three concentrations of immobilised sSLAMb. As described, the values of $K_d1$ derived in this way can be used to *calculate* the corresponding values of $[A_{f0}]$. The results are 130 $\mu M$, 207 $\mu M$, and 182 $\mu M$. The first two of these calculated values exceed the measured raw data values of $[A_{f0}]$ which were 82 $\mu M$ and 186 $\mu M$, respectively, and the last is significantly less than the measured value of 438 $\mu M$. It is evident from these results that the alternative model does not fit the observations and that it can therefore be disregarded.

*sSLAM homodimers dissociate rapidly*

The low affinity of the interaction seen using the BIAcore™ was confirmed by estimating the rate constant for dissociation of sSLAM from sSLAMb (Figure 4) as this parameter is unaffected by interactions in the solution phase or between molecules immobilised in the flow cell. Even at 25°C most (>80%) of the sSLAM dissociated from sSLAMb so rapidly that only lower limits (>15 s$^{-1}$) could be given for the $k_{off}$, consistent with the very weak binding observed at equilibrium (Figure 2). A minor component of the protein dissociated much more slowly ($k_{off} < 1$ s$^{-1}$). Whilst mass transport limitations and rebinding can lead to biphasic binding kinetics, these effects are unlikely to account for two dissociation phases with such markedly different rate constants. The slow component of binding can probably be accounting for by small amounts of aggregated protein likely to have appeared after gel filtration. The $k_{on}$, calculated from a $K_d1$ of 200 $\mu M$ and a dissociation rate of
15 s
sup-1
, is 7.5 x 10
sup-4
 M
sup-1
s
sup-1
.

AUC confirms sSLAM self-association

The conclusions of the SPR-based analysis were verified independently and extended using sedimentation velocity AUC methods. Figure 5A shows a series of absorbance scans collected at regular intervals during a sedimentation velocity run. Changes in the location and shape of the boundary between the protein-depleted solvent (HBS) and sample remaining in solution as the experiment proceeds can be analysed via the time-derivative of pairs of such scans to yield a function, \( g(s^*) \) (equation 16, Experimental Procedures), giving the distribution of sedimentation coefficients of the species present at the boundary (Figure 5B, bottom panel (32)). A single, ideal monodisperse species will display a normal (Gaussian) distribution of \( s^* \) arising from the symmetrical nature of its boundary. Systematically varying residuals (Figure 5B, top panel) indicate that the distribution does not fit a single Gaussian peak whereas a very good fit was observed for a two-species model (Figure 5B, middle panel). Higher-order models also gave poor fits (data not shown). The distribution midpoints represent the sedimentation coefficient of each species which, for the data shown in Figure 5B, were 5.48 S (Svedbergs) and 6.2 S. The effects of buffer density and viscosity due to buffer salts and the experimental temperature were corrected for using the program SEDNTERP (40). To account for the diffusion-limiting effects of the protein concentrations used, the sedimentation coefficients were measured at a series of concentrations (Figure 5C). Extrapolation to infinite dilution yielded an \( s_{20,w}^0 \) of 3.8 ± 0.1 S for the smaller species and 4.7 ± 0.2 S for the larger species.

For interacting species, the ratios of complexed to uncomplexed forms at equilibrium are highly concentration dependent close to the \( K_d \). Since the Gaussian fits describe the population of species in the sample, the areas of the fitted peaks reflect the proportion of each species measured by their absorbance. A plot of the molar ratios (R), derived from these absorbance ratios, versus total sSLAM concentration, confirmed the concentration dependence of the formation of the two species observed at the boundary (Figure 6). This result implies that the two species represent the two states of an
interacting system, in this case monomeric and homodimeric forms of sSLAM, and eliminates factors such as oligosaccharide heterogeneity as their source, since in such cases $R$ would be invariant.

Comparison with a theoretical derivation of $R$ in terms of total sSLAM concentration for a given range of affinities indicates that the $K_d$ for the interaction is high, within the range of $100 \mu M – 1 \text{mM}$ (Figure 6), in good agreement with the SPR analysis.

The observed sedimenting boundary for a system in monomer-dimer equilibrium is the sum of the two partial boundaries belonging to the monomer and dimer, and the shapes of the partial boundaries will determine their relative separation. Previous simulations have suggested that a rapid equilibrium characterized by a fast off rate ($k_{\text{off}} > 10^{-2} \text{s}^{-1}$) will tend to prevent resolution of a dimeric species from the monomer (41) due to the merging of their partial boundaries. The question therefore arises as to why we observe the two species given that the SPR analysis indicates that sSLAM dissociates very rapidly ($k_{\text{off}} > 15 \text{s}^{-1}$). Boundary sharpening due to restricted diffusion has previously been shown to be an effect of both molecular elongation and solute concentration (42,43). Sharpening the partial sedimentation boundaries of two species will tend to resolve them whereas broadening will tend to merge the species. The elongated nature of the sSLAM dimer giving a low diffusion coefficient and high Perrin function (see below) means that, countering the fast dissociation, the dimer partial boundary will be sharpened with respect to that of the monomer (41). The boundary of the somewhat less asymmetric monomer (see below) will be similarly affected, albeit to a correspondingly smaller extent. In addition, both partial boundaries will be sharpened due to the high concentration of sSLAM present (42). We suggest that the sum of these effects explains why we observe monomeric and dimeric peaks in $g(s^*)$, despite the rapid equilibrium. We were unable to fit sedimentation equilibrium data from parallel experiments due to the complex nature of the boundary shape, presumably due to non-ideality at the very high sSLAM concentrations necessary to observe self-association (data not shown).
**Topology of sSLAM homodimers**

The sedimentation coefficient of a molecule is a function of its mass and shape. The experimental Perrin function, \( P_{\text{exp}} \), is a size-independent parameter describing the degree of elongation of the species. The values of \( s_{20,w}^0 \) calculated above were used to determine \( P_{\text{exp}} \) for the monomeric and homodimeric species of sSLAM at a range of possible hydration levels using equations (17) and (18) (Table I). At each level of hydration, \( P_{\text{exp}} \) for the dimer is larger than that of the monomer. These values were compared with the sedimentation coefficients and calculated Perrin functions, \( P_{\text{calc}} \), determined for models of the monomeric and homodimeric forms of glycosylated sSLAM, based on the crystal structures of human sCD2 [(13); Figure 7A] and the head-to-head CD2 domain 1-CD58 domain 1 complex [(14); Figure 7B], as described in the Experimental Procedures. The calculated \( s \) and \( P_{\text{calc}} \) values for these models are 4.07 S and 1.47 for the monomer, respectively, and 5.08 S and 1.60 for the dimer, in reasonable agreement with the experimental values (Table I). Our modeling is likely to be inexact in that it does not allow for any inherent flexibility such as that of the carbohydrate moieties. Nevertheless, the analysis strongly suggests that the dimer has an end-to-end rather than side-to-side geometry since, for homodimers with side-to-side geometry, \( P_{\text{exp}} \) would remain essentially unchanged and the sedimentation coefficient would near double. The calculated \( s \) and \( P_{\text{calc}} \) values for the monomeric and side-to-side homodimeric forms of sB7-1 (44), for example, are 2.58 S and 1.27, and 4.08 S and 1.28, respectively.

**Discussion**

We have shown that the extracellular region of SLAM is homophilic and that it self-associates with very low affinity. The SPR-based methods implemented in the BIAcore™ are ideally suited to the detection of weak protein interactions because binding can be followed in real time and very small volumes of reactants are required for analysis. The work of Wyer et al. has shown that immobilisation of large amounts of highly “active” protein can facilitate the detection of very low affinity interactions by increasing the signal-to-noise ratio (44). Following this lead, we readily
detected the very weak binding of sSLAM to biotinylated sSLAM immobilised via streptavidin.

Although sSLAM-sSLAM binding could be demonstrated relatively easily, the quantitative analysis of homophilic interactions using SPR methods presents special problems due to self-association that occurs within the solute fraction and between immobilised molecules. An initial difficulty involved estimating the proportion of immobilised sSLAMb available for interactions with sSLAM in solution. For injections at a single concentration, sSLAM binding does not increase with increasing levels of immobilised sSLAMb at the rate predicted by equation (5), indicating that some of the immobilised protein is unavailable for binding. Since streptavidin was used to immobilise sSLAMb, it is likely that stable, multivalent homotypic interactions reduce the concentration of immobilised sSLAMb available to react with the soluble protein. To allow for the possibility that the immobilised sSLAMb that was able to bind sSLAM from solution could also self-associate, an equilibrium constant Kd₂ was incorporated into the theoretical analysis. The experimental data indicated that Kd₂ is indeed finite, confirming that the available immobilised sSLAMb does self-associate.

Taking these factors into account, the theoretical analysis of the experimental data yielded several estimates of the Kₐ for homophilic sSLAM interactions in solution. It is a feature of the analysis that small errors in the experimental data will give rise to significant differences in the calculated values of Kd₁ and Kd₂. It was therefore difficult to define Kd₁ more precisely than that it lay within the range of 200-383 µM. According to curve fitting using equation (11), however, the upper limit of 383 µM can be regarded as an overestimate. Overall, the broad level of agreement observed between the SPR-based estimates supports the conclusion that the Kₐ is approximately 200 µM, in good agreement with the AUC analysis. The experimental data are also consistent with Kd₁ and Kd₂ being approximately equal. If such equality can be confirmed in the BIAcore™ analysis of other homophilic interactions, the calculation of Kd₁ will reduce to the simple application of equation (13).
The geometry of homophilic protein interactions at the cell surface varies between parallel “side-to-side” interactions such as those observed for B7-1 (45), antiparallel side-to-side interactions as observed in the hemolin structure (46) and proposed for NCAM (47), L1 (48), and PECAM (49,50), and “head-to-head” docking as seen in P0 (51). The AUC analysis argues against the possibility that SLAM forms dimers with parallel or antiparallel side-to-side geometry. The sSLAM homodimer shown in Figure 7B is modelled on the complex that human CD2 and CD58 are likely to form at the cell surface. In this complex, CD2 and CD58 each dock at structurally equivalent sites centred on the AGFCC'C'' β-sheets of domain 1 at the “top” of the molecule, resulting in the docked molecules spanning a distance of ~150 Å between apposing cells (14,52). The close concordance between the measured hydrodynamic properties of the sSLAM homodimer and those calculated for the dimeric model based on the CD2/CD58 complex, strongly implies that sSLAM homodimers would form molecular contacts between T- and B-cells with the same topology.

In view of the very low affinity of sSLAM interactions measured here, however, it is far from certain that such contacts would form in vivo. Most protein interactions at the leukocyte cell surface have solution (or “three-dimensional”) dissociation constants in the range of 0.4-15 µM (1,3). Previously, the key exceptions to this rule were murine CD2-CD48 interactions (K_d = 75 µM), and CD8αα [K_d = 200-300 µM (44)] and CD4 (K_d > 2 mM, unpublished data) coreceptor interactions with MHC class I and II, respectively. The more relevant parameter, however, is the “two-dimensional” affinity which allows comparisons of interactions between proteins confined within adjacent lipid bilayers (53,54). For murine CD2-CD48 interactions, the two-dimensional affinity barely supports the formation of molecular contacts between cells and model planar membranes when the proteins are at physiological densities (54). It therefore cannot necessarily be concluded that the extracellular domains of proteins with solution dissociation constants higher than 75 µM will spontaneously interact at physiological densities. For CD8 it has been argued that additional interactions within the T cell might stabilize its ternary interaction with the T cell receptor and MHC class I (44). SLAM appears to belong to this
second class of cell surface molecules whose ligand interactions are of unusually low affinity. It remains to be established whether or not the low affinity of SLAM self-association is sufficient for homophilic recognition at the cell surface, whether it requires very high levels of SLAM expression or simultaneous interactions with other molecules, or whether additional, higher affinity SLAM ligands remain to be discovered. At this stage it is perhaps only possible to conclude that monomeric, secreted SLAM is unlikely to function as a soluble, homophilic growth factor (15).

The analysis of other homophilic interactions suggests that, overall, they tend to be very weak. For example, AUC analyses have indicated that the homophilic interactions of the brain protein, P0 (51), and of the first two domains of NCAM (47), each have a millimolar $K_d$. The affinities of these interactions are comparable to that of the apparently non-physiological homophilic interaction of CD2 molecules (55). The very low affinity measured here for sSLAM self-association is certainly at odds with previous work indicating that sSLAM is active at concentrations $\leq 10 \mu g/ml$ and that the solution affinity of sSLAM might be as high as $\sim 0.1 \text{ nM}$ (15). It has been reported that a soluble form of human CD8αα is also active at much lower concentrations than would be expected to give substantial levels of binding of the soluble protein (56). We are unable to account for these observations beyond noting that protein aggregation occurring during the course of the experiments could, in principle, explain the apparent biological properties of these protein preparations.

In conclusion, we have characterised the first homophilic interaction within the CD2 subset of the IgSF. Whilst it is generally believed that all IgSF proteins probably derive from a single homophilic precursor (57), contemporary proteins with this property appear to be rare. An emerging structural feature of the weakly interacting proteins found at the cell surface is that their binding sites are characterized by relatively poor surface complementarity (52). Such proteins might, as a consequence, be particularly tolerant to variation in ligand binding face composition, allowing rapid sequence divergence and the generation of new specificities following gene duplication, leading to
the early loss or dilution of primordial homophilic interactions. Such a process might account for the
large number of functionally overlapping and structurally related molecules populating the cell
surface, as exemplified by the CD2 subset of the IgSF.

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**Footnote**

1 Abbreviations: AUC, analytical ultracentrifugation; CD4, domains 3 and 4 of rat CD4; CHO, Chinese hamster ovary; HBS, Hepes buffered saline; h, human; IgSF, immunoglobulin superfamily; mAb, monoclonal antibody; RU, response unit(s); SLAM, signaling lymphocytic activating molecule; sSLAM, soluble SLAM; sSLAMb, biotinylated sSLAM; SPR, surface plasmon resonance; S, Svedbergs.
Figure Legends

Figure 1. sSLAM self-associates
SLAM mAbs A12 (100 µg/ml) (A) and IPO-3 (50 µg/ml) (B) were injected (solid bars) through flow cells with sSLAMCD4b immobilized at 2418 RU or 1130 RU, respectively, to confirm that the immobilized protein was correctly folded. sSLAM (76 µM) was also injected over immobilized sSLAMb (2280 RU) or a control protein, 2B4CD4b (2244 RU), before (C) and after (D) saturation binding of A12 mAb (injected at 100 µg/ml). In (D) the decreased response is likely to be the result of sSLAM preventing rebinding of the mAb to immobilized sSLAMb.

Figure 2. Affinity of sSLAM self-association
(A) sSLAM was injected at the indicated concentrations through flow cells with immobilized sSLAMb (1273 RU) or as a negative control, 2B4CD4b (1285 RU), at 37°C. (B) The responses at equilibrium (A) in the flow cells in which sSLAMb (circles), and/or the control protein (squares), were immobilized, and the difference between the responses (giving specific binding, C), are each plotted against the sSLAM concentration. Removal of the oligohistidine tag with carboxypeptidase A had no effect on the affinity measurements. The linear fit used to determine P (equation 6 of Experimental Procedures) is shown in (B).

Figure 3. Estimation of the binding affinity using responses observed at different levels of sSLAMb immobilization
sSLAM was injected at 37°C over flow cells in which sSLAMb was immobilized at 240 RU (75 µM; triangles), 540 RU (148 µM; circles) and 1273 RU (246 µM; squares), or in which the control protein 2B4CD4b had been immobilized at 1285 RU (not shown). The differences in response observed in the flow cells containing immobilized sSLAMb and 2B4CD4b is plotted against the injected sSLAM concentrations for narrow (A) and wider (B and C) ranges of injection. From (A), the slope of the linear fit, g, was calculated as g = 0.248, g = 0.388 and g = 0.547 for the low, intermediate and high
values of [Afo], respectively. In (B), curve-fitting is shown with various chosen values of Kd1 and calculated Kd2 for [Afo] = 148 µM using equation (7) from the Experimental Procedures. The actual responses obtained with sSLAMb immobilized at 148 µM are shown as triangles. In (C), Kd1 was determined by curve-fitting to binding data for [Afo] = 75 µM (Kd1 = 181 µM), [Afo] = 148 µM (Kd1 = 282 µM) and [Afo] = 246 µM (Kd1 = 160 µM), using equation (11) from the Experimental Procedures.

Figure 4. Measurement of the sSLAM dissociation rate

In (A), sSLAM (48 µM) was injected at 20 µL.min⁻¹ through flow cells with sSLAMb (triangles) or the control protein, 2B4CD4b (squares), both immobilized on the sensor surface at 1000 RU. In (B), individual points in the dissociation phase are shown with an expanded time scale. The response in the control flow cell, which represents the rate of washing of sSLAM from the control flow cell (squares), was subtracted from the response in the flow cell to which sSLAMb was immobilized (triangles). A $k_{off} = 15$ s⁻¹ for sSLAM dissociation from sSLAMb, and a $k_{off} = 20$ s⁻¹ for sSLAM removal from the flow cell containing 2B4CD4b, was calculated by exponential decay curve-fitting (line). To aid comparison, the data are normalized, with the response at the start of the dissociation phase set at 100%.

Figure 5. AUC confirmation of the sSLAM self-association

In (A), absorbance scans at 298 nm for sSLAM at ~3 mg/ml collected at intervals of 15 minutes are shown with the sample moving from left to right. Traces mark the migration of sSLAM along the centrifuge cell from the sample meniscus (A) to the cell bottom (Ω). In (B, lower panel), $g(s^*)_t$ for sSLAM at ~3 mg/ml at 310 K (Ω) is fitted with two Gaussian curves (-- -- -- ), representing species with $s^*$ of 5.48 ± 0.02 S and 6.20 ± 0.02 S (calculated from their midpoints). The residuals (•) for this fit are shown in the middle panel. The residuals of a single species fit (data fitted as a single
Gaussian) are shown in the top panel (○) for comparison. In (C), values for $s^*$ for species 1 (○, fit ———) and species 2 (●, fit - - - -) are plotted against sSLAM concentration. The error bars represent the standard errors on the Gaussian fit midpoints. Extrapolation to zero concentration yields $s^*_{0}$ ($s^*$ at infinite dilution) of $5.42 \pm 0.07 \text{ S}$ and $6.73 \pm 0.20 \text{ S}$ which were then corrected for the effects of solvent and temperature to yield $s^*_{0,298}$ values.

**Figure 6. AUC-based estimation of the affinity of sSLAM self-association**

The observed ratio ($R$) of the areas of the two gaussian distributions seen in the $g(s^*)_i$ profiles of sSLAM, determined using the equations

$$R = \frac{A_{\text{Monomer}}^{298}}{A_{\text{Dimer}}^{298}} = \frac{[\text{Monomer}]}{2[Dimer]} = \frac{[\text{sSLAM}]_{\text{free}}}{[\text{sSLAM}]_{\text{bound}}}$$

is plotted against total sSLAM concentration. Using the additional relationships

$$K_a = \frac{[\text{Dimer}]}{[\text{Monomer}]^2}$$

and

$$S_T = [\text{Monomer}] + 2[Dimer]$$

(wherein $S_T$ is the total sSLAM concentration), $R$ can be derived in terms of $S_T$ and $K_a$:

$$R = \frac{\sqrt{1 + 8K_a S_T} - 1}{4K_a S_T - \sqrt{1 + 8K_a S_T} + 1}.$$  

The broken lines illustrate the expected dependence of $R$ on total sSLAM concentration for given values of $K_a$ (from 10 $\mu$M to 1 mM) calculated using this equation. According to this analysis, the $K_a$ for sSLAM self association is between 100 $\mu$M and 1 mM.
Figure 7. Molecular modeling of the sSLAM structure

In (A), the structure of rat soluble CD2 is shown (left) with modelled N-linked oligossaccharides attached in random orientations at positions likely to correspond to SLAM glycosylation sequons according to sequence alignments (data not shown). The corresponding bead model, constructed using AtoB (36), is shown in the same orientation (right). In (B), a model of the sSLAM homodimer, constructed by superimposing the monomer model (A) on the CD2 and CD58 domains solved in complex, as described in the Experimental Procedures, is shown (left). The corresponding bead model of the sSLAM homodimer is also shown. The bead models were used to calculate the theoretical hydrodynamic parameters for the sSLAM monomer and homodimer (see text). The images were generated using BOBSCRIPT (58,59) and RASTER3D (60).

Table I

Experimental Perrin ratios ($P_{\text{exp}}$) and anhydrous sedimentation coefficients ($s_0$) calculated for the two species observed in the $g(s^*)$ profiles at a range of apparent hydrations ($\delta_{\text{app}}$) (see text).

| Apparent hydration ($\delta_{\text{app}}, \text{ g/g}$) | species 1 ($s_{20,w}^\prime = 3.8$ S) | species 2 ($s_{20,w}^\prime = 4.7$ S) |
|--------------------------------------------------|----------------------------------|----------------------------------|
|                                                  | $P_{\text{exp}}$ | $s_0$ | $P_{\text{exp}}$ | $s_0$ |
| 0.1                                              | 1.322               | 4.0   | 1.708               | 4.9 |
| 0.2                                              | 1.271               | 4.1   | 1.643               | 5.1 |
| 0.3                                              | 1.227               | 4.3   | 1.586               | 5.3 |
| 0.4                                              | 1.189               | 4.4   | 1.536               | 5.5 |
| 0.5                                              | 1.155               | 4.5   | 1.492               | 5.6 |
Figure 1, Mavaddat et al.
Figure 2, Mavaddat et al.
Figure 3, Mavaddat et al.
Figure 4, Mavaddat et al.
Figure 5, Mavaddat et al.

A

Absorbance at 298 nm vs. radius (cm)

B

Residuals

Residuals

g(s*) (a.u.)

C

s* (S) vs. sSLAM concentration (µM)
Figure 6, Mavaddat et al.
Figure 7, Mavaddat et al.
Signaling lymphocytic activation molecule (SLAM, CDw150) is homophilic but self-associates with very low affinity
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