Affinity and Kinetic Analysis of L-selectin (CD62L) Binding to Glycosylation-dependent Cell-adhesion Molecule-1*

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The selectin family of cell adhesion molecules mediates the tethering and rolling of leukocytes on blood vessel endothelium. It has been postulated that the molecular basis of this highly dynamic adhesion is the low affinity and rapid kinetics of selectin interactions. However, affinity and kinetic analyses of monomeric selectins binding their natural ligands have not previously been reported. Leukocyte selectin (L-selectin, CD62L) binds preferentially to O-linked carbohydrates present on a small number of mucin-like glycoproteins, such as glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), expressed in high endothelial venules. GlyCAM-1 is a soluble secreted protein which, following binding to CD62L, stimulates β₂-integrin-mediated adhesion of lymphocytes. Using surface plasmon resonance, we show that a soluble monomeric form of CD62L binds preferentially to O-linked carbohydrates present on a small number of mucin-like glycoproteins, such as glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), expressed in high endothelial venules. GlyCAM-1 is a soluble secreted protein which, following binding to CD62L, stimulates β₂-integrin-mediated adhesion of lymphocytes.

The extravasation of leukocytes into tissues is a multistep process initiated by the tethering and subsequent rolling of leukocytes along endothelial surfaces (1, 2). The selectin family of cell adhesion molecules (CD62L (L-selectin), CD62E (E-selectin), and CD62P (P-selectin)) plays a particularly important role in these highly dynamic leukocyte-endothelial interactions (3–6). CD62L is expressed constitutively on leukocytes whereas CD62E and CD62P are expressed on endothelial cells activated by inflammatory mediators (3–6). Selectins are type I transmembrane proteins with membrane-distal Ca²⁺-dependent (C-type) lectin domains (3–6). They can bind a diverse group of oligosaccharides (7), but their physiological ligands appear to be a small group of glycoproteins, most of which are mucins (3, 4, 6–8). Although selectins bind predominantly to carbohydrate structures present on these glycoprotein ligands, recent data indicate that the protein backbone of P-selectin glycoprotein ligand-1 (PSGL-1,1 CD162) contributes to the binding of CD62P (reviewed in Ref. 8), and possibly CD62L (9).

It has been postulated that selectins are able to mediate tethering and rolling on vascular endothelium because they bind their ligands with very fast association and dissociation rate constants (10). However, the affinity and kinetics of selectin interactions with their physiological ligands remain poorly characterized. Selectins have been shown to bind synthetic oligosaccharides related to siaIyalted and/or sulfated Lewisα (Leα, galactose β1→3)fucose α1→3(N-acetyl)glucosamine) or its stereoisomer Lewisβ (Leβ, galactose β1→3fucose α1→4(N-acetyl)glucosamine) with very low affinities (KD 0.1–5 μM (11–19)). It is possible that these studies underestimated the affinities because: (i) with few exceptions (13, 18, 19), they were based on inhibition by synthetic oligosaccharides of multivalent selectin-ligand interactions, and (ii) these oligosaccharides may differ in structure from physiological selectin ligands (7). Indeed soluble, recombinant forms of CD62P (20) and CD62E (21) have been reported to bind leukocytes with much higher affinities (KD ≤ 1 μM). However, the accuracy of the latter affinity measurements is also in doubt because the CD62E used was oligomeric (as assessed by size exclusion chromatography (21)), and the CD62P may have been contaminated by small amounts of multivalent CD62P (4).

CD62L has been shown to bind particularly well to O-glycans present on certain glycoforms of the mucins glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1 (22), CD34 (23), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1 (24)). GlyCAM-1, the best characterized of these CD62L ligands, is a soluble protein (25) secreted by endothelial cells (26) which is present in mouse serum at concentrations of ~1.5 μg/ml (27). Although lymphocytes and neutrophils can tether and roll on surfaces coated with GlyCAM-1, it is not yet clear whether GlyCAM-1 functions as an adhesion molecule in vivo. Indeed, it has been proposed that the binding of soluble GlyCAM-1 to leukocyte CD62L inhibits CD62L-mediated adhesion (25, 28). Furthermore, CD62L is capable of transducing signals when cross-linked (29–31), and the binding of soluble GlyCAM-1 to lymphocyte CD62L has been shown to stimulate β₂-integrin-mediated adhesion (31).

In the present study we expressed a soluble, monomeric form of rat CD62L and used surface plasmon resonance to measure

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1 The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; CD62L-CD4, the extracellular portion of rat CD62L fused to domains 3 and 4 of rat CD4; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; HEV, high endothelial venules; PNA, peripheral node addressin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis.

2 O. Dvir, F. Shimron, M. S. Singer, S. D. Rosen, and R. Alon, unpublished data.
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The monovalent affinity and kinetics of its interaction with GlyCAM-1 was artificially increased ($K_d = 140 \mu M$) when proceeding from high to low CD62L-GlyCAM-1 concentrations and decreased ($K_d = 90 \mu M$) when proceeding from low to high concentrations (data not shown). Therefore the binding ($CD62L_{bound}$) at each CD62L-GlyCAM-1 concentration was adjusted ($CD62L_{adjusted}$) for the level of GlyCAM-1-GlyCAM interaction on the surface immediately preceding that injection, using the formula:

$$CD62L_{adjusted} = \frac{CD62L_{bound} \times GlyCAM_{total}}{GlyCAM_{innao}}$$  (Eq. 1)

where $GlyCAM_{innao}$ is the level of immobilized GlyCAM-1 immediately preceding the first CD62L-GlyCAM-1 injection. When this adjustment is made the same $K_d$ values are obtained irrespective of the order of CD62L-GlyCAM-1 injections (Fig. 3D).

RESULTS

Expression and Analysis of Monomeric CD62L-GLyCAM-1—The extracellular portion of rat CD62L was expressed in Chinese hamster ovary-K1 cells as a fusion protein with domains 3 and 4 of CD4 (CD62L-CD4, Fig. 1A) and purified on an anti-CD4 mAb affinity column (Fig. 1B). The CD62L-CD4 fusion protein migrated at $\sim 76$ kDa in SDS-PAGE (Fig. 1B) under reducing conditions, consistent with the calculated protein molecular mass of 52 kDa plus utilization of several of the 7 potential N-glycosylation sites (Fig. 1A). CD62L-CD4 migrated slightly faster under nonreducing condition ($\sim 70$ kDa, Fig. 1B), demonstrating that it does not form intermolecular disulfide bonds, and consistent with the presence of intramolecular disulfides.

Several lines of evidence suggest that the CD62L-CD4 is correctly folded. First, it bound to 3 previously described CD62L mAbs (HRL1, HRL2, and HRL3) (Fig. 2), two of which (HRL1 and HRL3) block binding of CD62L to its natural ligands (32, 33). Second, CD62L-CD4 was used to raise a new mAb (OX85, see “Experimental Procedures”). OX85, in addition to binding CD62L-CD4 (Fig. 2), binds lymphocyte populations known to express CD62L (34, 35) and to a well characterized (33) chimeric protein comprising rat CD62L fused to the Fc portion of human IgG1 (CD62L Ig, data not shown). Third, CD62L-CD4 coated fluorescent microspheres bind selectively to high-endothelial venules (HEV) in lymph node vessels (35). Fourth, CD62L-CD4 binds both mouse regulatory T cell addressin (PNAD, purified using the MECA-79 mAb) (35) and mouse GlyCAM-1 (see below). And finally, as expected for interactions involving C-type lectins, binding of CD62L-CD4 to HEV, PNAD, and GlyCAM-1 was inhibited by EDTA (see Ref. 35 and Table 1).

Accurate affinity measurements require knowledge of the
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Expression and purification of monomeric CD62L-CD4.

A, a schematic depiction of the domain structure of the CD62L-CD4 fusion protein. CL, E, and C refer to C-type lectin, epidermal growth factor, and complement control protein superfamily domains, respectively (67). V and C2 refer to V-set and C2-set immunoglobulin superfamily domains (67). Predicted N-linked glycosylation sites are represented by filled circles. B, top: CD62L-CD4 (3 μg) was analyzed by SDS-PAGE on a 12% acrylamide gel under reducing (β-mercaptoethanol) and nonreducing conditions. Bottom: protein A-Sepharose beads coated either with OX85 or a control mAb (W3/25) were incubated with CD62L-CD4, pelleted, and the supernatants analyzed for the presence of CD62L-CD4 by reducing SDS-PAGE on 12% acrylamide. C, purification of CD62L-CD4 by size-exclusion chromatography. CD62L-CD4 (3 mg in 0.5 ml) was run on a Superdex S200 HR10/30 column (Pharmacia) at 0.5 ml/min in Hepes-buffered saline. The calibration markers shown (Sigma) were alcohol dehydrogenase (M, 150,000) and bovine serum albumin (M, 66,000). The indicated fractions (*) were combined, concentrated 10–20-fold, and used within 48 h with storage at 4°C.

CD62L-CD4 eluted at molecular mass r 66,000 (Fig. 1B). Using globular, unglycosylated proteins as calibration markers, CD62L-CD4 eluted at molecular mass ~140,000 (Fig. 1C), which is higher than the molecular mass measured by SDS-PAGE (~76 kDa, Fig. 1B). However, asymmetric glycosylated proteins such as CD62L-CD4 typically elute much earlier in size exclusion chromatography than predicted by their Mr. For example, the asymmetric, glycosylated proteins sCD2, sCD80, and sCD48-CD4 (Mr ~ 30,000, 35,000, and 50,000 on SDS-PAGE), which are known to be monomeric in solution, elute at Mr ~ 52,000, 63,000, and 84,000 on the same column (45). Taken together, these data suggest that CD62L-CD4 exists as a monomer in solution. The monomeric peak of CD62L-CD4 (Fig. 1C) was used for affinity and kinetic measurements which were performed within 48 h of size exclusion chromatography to minimize the accumulation of multivalent aggregates (46).

Affinity of CD62L-CD4 Binding to GlyCAM-1—GlyCAM-1 purified from mouse serum was immobilized on the sensor surface indirectly using the rabbit polyclonal antibody CAMO2, which was raised against a peptide from the middle (non-mucin) region of GlyCAM-1 (see “Experimental Procedures”). When GlyCAM-1 is injected over a sensor surface to which CAMO2 had been covalently coupled, there is an increase in response, which indicates binding (Fig. 3A). Following the injection, while the GlyCAM-1 remains bound, a range of CD62L-CD4 concentrations are then injected briefly over this surface (Fig. 3A) and simultaneously injected over a control sensor surface with only CAMO2 (not shown). An expanded view of the response during injection of three concentrations of CD62L-CD4 over GlyCAM-1 reveals that the response attains equilibrium within seconds of the start of each injection and returns to baseline within seconds of the end of the injection (Fig. 3B). Because the BIACore detects changes in refractive index, the high protein concentrations injected (up to 26 mg/ml or 0.5 mM) give a large background signal. This is evident when the response trace from the control surface is overlaid (Fig. 3B). The difference between the response seen with injection over the GlyCAM-1 surface compared with the response seen with injection over the control surface represents the actual binding of CD62L-CD4 to GlyCAM-1 (Fig. 3, B and C). Measured in this way, no binding is seen when CD62L-CD4 is injected in the presence of EDTA, or when the control CD4 chimera sCD48-
CD4 (43) is injected, indicating that the binding involves the CD62L portion of CD62L-CD4 (Table I). Direct fitting of a standard Langmuir binding isotherm to the data indicates that the binding is saturable, with a $K_D$ of 105 μM (Fig. 3C, inset). A Scatchard plot of the same data is linear and also gives a $K_D$ value of 105 μM (Fig. 3D, closed circles). Provided that binding is adjusted to compensate for the slow dissociation of GlyCAM-1 from the surface (see “Experimental Procedures”), the same $K_D$ is obtained when the order of CD62L-CD4 injections is reversed (Fig. 3D, open circles). These affinity measurements were highly reproducible (Table II). Interestingly, CD62L-CD4 bound with the same affinity at 25 and 37 °C (Table II), consistent with a small enthalpic and a large entropic contribution to the binding energy over this temperature range. All subsequent measurements were performed at 25 °C.

Recent evidence suggests that the binding of CD62L to PSGL-1 may involve the protein backbone as well as O-linked carbohydrates (9). A polyclonal antibody directed at an N-terminal PSGL-1 peptide inhibits CD62L binding (9). This raises the question as to whether the immobilization of GlyCAM-1 via CAMO2 (which was raised to a peptide from the carboxyl terminus of GlyCAM-1) (22) somehow diminishes CD62L-CD4 binding. To address this we studied CD62L-CD4 binding to GlyCAM-1 immobilized via the antibody CAMO5 (anti-peptide 3 antibody in Ref. 22), which was raised against a peptide from the carboxyl terminus of GlyCAM-1. CD62L-CD4 bound with the same affinity to CAMO5- and CAMO2-immobilized GlyCAM-1 (Table II), arguing strongly against any effect of GlyCAM-1 immobilization on CD62L-CD4 binding.

**Kinetics of CD62L-CD4 Binding to GlyCAM-1**—Following the injection of CD62L-CD4, the response dropped with a half-time of ~0.07 s (Fig. 4), which is similar to the time it takes to wash the sample out of the flow-cell at the flow-rate used (100 μl/min) (47). This is confirmed by the observation that the background response (when CD62L-CD4 is injected through a control flow-cell) falls at the same rate (Fig. 4). Thus the rate at which the response falls represents the washing time rather than the intrinsic dissociation rate constant. Although the washing time can be decreased further by increasing the flow-rate (up to a maximum of 500 μl/min) (47), it would still not be possible to measure directly the dissociation rate constant because data cannot be collected on the current BIACore at intervals shorter than 0.1 s. Nevertheless, it is possible to conclude from the available data that CD62L-CD4 dissociates at GlyCAM-1 with a $k_{off}$ of at least 10 s$^{-1}$. Direct measurement of the association rate constant was not possible because equilibrium was reached within 1 s (Fig. 3B). However, with the $k_{off} \geq 10$ s$^{-1}$ and the $K_D = 100$ μM the association rate constant ($k_{on}$) can be calculated to be 1*10$^{10}$ M$^{-1}$ s$^{-1}$.

**GlyCAM-1 binds with High Affinity to Immobilized CD62L**—Since GlyCAM-1 is a soluble protein which interacts with membrane-tethered CD62L, we analyzed the binding of soluble GlyCAM-1 to immobilized CD62L-CD4 (Fig. 5). GlyCAM-1 binding is detectable as its concentration is increased above the mean serum level (~1.5 μg/ml, ~30 nm) (Fig. 5). Furthermore, the bound GlyCAM-1 dissociates slowly with $k_{off}$ values ≤0.001 s$^{-1}$ (Fig. 5). These data strongly suggest that the GlyCAM-1 is binding multivalently to the immobilized CD62L. It is also notable that GlyCAM-1 binds with a similar avidity to mouse and rat CD62L (Fig. 5).

**DISCUSSION**

Accuracy of the Measurements—Accurate affinity measurements require that the recombinant CD62L-CD4 chimeric protein possesses the same ligand binding properties as native rat CD62L. We believe this to be the case for the following reasons. First, the chimera contains almost the entire extracellular portion of CD62L. Second, all four CD62L mAbs tested bound CD62L-CD4. Third, CD62L-CD4 bound selectively to HEV in lymph node sections (35). Fourth, the binding of CD62L-CD4 to GlyCAM-1, porcine PNAd, and lymph node HEV was inhibited by EDTA (35), and finally, GlyCAM-1 bound with a similar avidity to CD62L-CD4 as it bound to other well characterized and independently made CD62L proteins such as rat (33) and mouse (36) CD62L Ig (Fig. 5).

The affinity we obtained for the CD62L-CD4-GlyCAM-1 interaction could represent an underestimate if only a small proportion of the soluble CD62L-CD4 is correctly folded and able to bind GlyCAM-1. Our demonstration that ≥60% of the CD62L-CD4 retains mAb binding activity suggest that this possibility is very unlikely. One caveat is that we measured the affinity and kinetics of rat CD62L binding to mouse GlyCAM-1. However, because mouse GlyCAM-1 binds with a similar avidity to mouse and rat CD62L Ig (Fig. 5), we believe we are justified in assuming that mouse and rat CD62L bind mouse GlyCAM-1 with similar properties. This is not unexpected considering the high degree of conservation between mouse and rat CD62L (93% identity between C-type lectin domains (48)).

While migration of CD62L-CD4 on size exclusion chromatography was consistent with an asymmetric monomer, we could not rule out the possibility that it existed as a dimer. If CD62L-CD4 does indeed exist as a dimer, and binds divalently, our measurements would represent an underestimate of the $K_D$ and the $k_{off}$. However, this would not alter the main conclusions of this study, which are that CD62L-CD4 binds to a physiological glycoprotein ligand with an exceptionally low affinity and fast kinetics, that this affinity is in agreement with the affinity measurements obtained for CD62L binding to sulfated forms of sialyl Lewis x, and that GlyCAM-1 is likely to bind multivalently to cell surface CD62L.

**Comparison with Previous Studies on CD62L**—To our knowledge this is the first affinity and kinetic analysis carried out in a cell-free system of the interaction between a selectin molecule and a defined physiological glycoprotein ligand (Table III). CD62L binding to GlyCAM-1 involves O-glycans which carry sialic acid, sulfate, and fucose groups (49–51), consistent with the involvement of sulfated and sialylated derivatives of Le$^a$ or its stereoisomer Le$^b$. The affinity of CD62L for synthetic forms

### Table I

| Sample injected | Response (RU) | Binding$^d$ |
|-----------------|--------------|-------------|
| CD62L-CD4 (1 mg/ml) | 241 ± 1 | GlyCAM-1 surface |
| CD62L-CD4 (1 mg/ml) + 20 mM EDTA | 1166 ± 3 | GlyCAM-1 surface |
| sCD48-CD4 (3 mg/ml) | 443 ± 2 | GlyCAM-1 surface |

$^a$ The samples were injected over the indicated surfaces for 5 s at a flow rate of 60 μl/min. The mean and range are shown of responses seen when the same sample is injected over two identical surfaces.

$^b$ CAMO2 immobilized (~11,000 RU).

$^c$ GlyCAM (~450 RU) immobilized via CAMO2.

$^d$ Response with injection over GlyCAM-1 surface minus response with injection over control surface.
It is noteworthy that the IC50 values for 650% (IC50) multivalent CD62L-ligand interactions (Table III) concentrations of soluble oligosaccharide required to inhibit by of these oligosaccharides has been estimated by measuring the maxima of 513 and 477 response units, respectively. The kinetics of CD62L interactions have been studied indirectly by analysis, in laminar flow, of transient leukocyte binding events (tethers) to planar surfaces coated with PNAd, a heterogenous mixture of CD62L ligands including CD34 (55). Since flow subjects these leukocytes to a shear force which increases the koff, the koff in the absence of an applied force ("intrinsic" koff) was estimated by extrapolating to zero flow rate (55). Using this approach, Abon and colleagues (55) showed that these tethers are mediated by one or a few CD62L/PNAd bonds and detach with an intrinsic koff of ~7 s^-1, which agrees well with the solution koff for the CD62L/GlyCAM-1 interaction obtained in the present study (~10 s^-1).

Comparison with CD62E and CD62P—Attempts have been made to measure the affinity of both CD62P and CD62E for of these oligosaccharides has been estimated by measuring the concentrations of soluble oligosaccharide required to inhibit by 50% (IC50) multivalent CD62L-ligand interactions (Table III) (12–14, 19, 52). It is noteworthy that the IC50 values for 6-sulfo-sLeX and 6-sulfo-sLeX (Table III), two major capping groups present in GlyCAM-1 O-linked oligosaccharides (53, 54), are only slightly higher (250–800 μM) than the Kd measured in the present study for CD62L binding to GlyCAM-1 (~250 μM). Because these inhibition studies relied on inhibition of multivalent interactions by monomeric oligosaccharides (12–14, 19, 52), the IC50 values obtained are likely to underestimate the actual affinity. Thus, our results are consistent with the main CD62L ligands carried by GlyCAM-1 being 6-sulfo-sLeX and 6-sulfo-sLeX (14, 52–54), or the branched and extended O-glycans in which these capping structures occur (54).

### Table II

| GlyCAM-1 immobilized via | Temperature °C | Kd μM |
|-------------------------|----------------|-------|
| CAMO2                   | 25             | 108 ± 4 (n = 8) |
| CAMO2                   | 37             | 100 ± 6 (n = 2) |
| CAMO5                   | 25             | 102 ± 24 (n = 2) |

Approximately 12,000 RU (range 11,000 to 12,800 RU) of CAMO2 or 8,300 RU (range 8,250–8,450 RU) of CAMO5 were covalently coupled to sensor surfaces (see "Experimental Procedures") and 350–460 RU of GlyCAM-1 were bound to these surfaces via these Abs.

The affinity was measured by equilibrium binding as described in the legend to Fig. 3.

Mean ± S.D. of eight determinations using two different preparations of CD62L-CD4.

Mean ± range of two determinations.

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![Image](60x372 to 296x729)

**FIG. 3.** Measuring the affinity of CD62L-CD4 binding to immobilized GlyCAM-1 on the BIAcore. A, GlyCAM-1 (~15 μg/ml) was immobilized by injecting it at 1 μl/min for 15 min (long bar) over a sensor surface to which ~12,800 response units of the anti-GlyCAM-1 antibody CAMO2 had been covalently coupled. The flow rate was then increased to 60 μl/min and a range of decreasing CD62L-CD4 concentrations (492 μM and six 2-fold dilutions thereof) were injected for 5 s each over the immobilized GlyCAM-1. Using the BIAcore 2000 in multichannel mode, the same CD62L-CD4 samples were injected through a control flow cell (FC) with only CAMO2 (~11,400 response units) on the sensor surface to measure the background response. For clarity, a scale is used which does not show the large responses to the two highest concentrations of CD62L-CD4 (+, see C). The subsequent 3 injections (enclosed in box) are shown in B in an expanded scale. B, three concentrations of CD62L-CD4 were injected (short bars) over surfaces with (solid line) or without (dotted line) GlyCAM-1 immobilized. C, the equilibrium responses measured during injection of CD62L-CD4 in control flow cell (squares) and GlyCAM-1 flow cell (triangles) are plotted. The difference between the responses in the control and GlyCAM-1 flow cells represents actual binding (circles). C, inset, CD62L-CD4 binding after adjustment for dissociation of GlyCAM-1 during the experiment (see "Experimental Procedures"). The initial level of GlyCAM-1 immobilized was ~460 response units. The line represents a nonlinear fit of the Langmuir binding isotherm to the data and gives a Kd of 105 μM and a binding maximum of 512 response units. D, a Scatchard plot is shown of the binding data in C (filled circles). Also shown is a Scatchard plot of the binding observed when the order of CD62L-CD4 injections was reversed (open circles). Linear regression fits to these data gave Kd values of 105 and 106 μM, respectively, and binding maxima of 513 and 477 response units, respectively.

![Image](60x372 to 296x729)

**FIG. 4.** Kinetics of CD62L-CD4 dissociating from GlyCAM-1. CD62L-CD4 (40 μM) was injected for 3 s over GlyCAM-1 (~460 response units bound via CAMO2) or a control surface (CAMO2 alone) at a flow rate of 100 μl/min. The dissociation of CD62L-CD4 (after subtraction of the background response) is shown (circles) normalized as a percentage of the maximum amount of CD62L-CD4 bound (90 response units). Also shown is the fall in response following injection over the control surface (squares) normalized as a percentage of the maximum response (178 response units). A nonlinear fit of an exponential decay curve to the CD62L-CD4 dissociation data (circles) gives a koff of 10 s^-1 (solid line). FC, flow cell.
physiological ligands present on leukocytes (20, 21). Radiolabeled soluble recombinant monomeric CD62P has been reported to bind neutrophils and HL60 cells with an affinity \( K_d \) of 70 nm at least 3 orders of magnitude higher than the reported affinity we report for CD62L binding GlyCAM-1 (20). However, Ushiyama et al. (20) did not exclude the possibility that the CD62P preparation contained small amounts of multimeric material (4), and so it is possible that they overestimated the affinity (43, 46, 56). Similarly, a soluble recombinant form of CD62E inhibited the binding of HL60 cells to immobilized CD62E with an \( IC_{50} \) of \(-1 \mu\)m (21). However, size exclusion chromatography showed that the CD62E existed as a multimer in solution, suggesting that this study may also have overestimated the true affinity (21).

There are many published measurements of the affinity of CD62E and CD62P binding to sulfated and/or sialylated derivatives of Le\(^a\) or Le\(^a\) (Table III). The best of these oligosaccharide ligands have been reported to bind CD62E and CD62P with affinities of \( K_d \sim 10^7 \) and \(-220 \mu\)m, respectively (Table III).

Because of the discrepancy between the high affinities reported for CD62E and CD62P binding to cells and their low affinity for these ubiquitous oligosaccharides (Table III), it has been suggested that these selectins might bind to carbohydrate (7) and, perhaps, protein structures restricted to these physiological ligands. There is evidence that CD62E binds preferentially to tetraantenary \( N \)-linked carbohydrates with an unusual sialylated di-Le\(^a\) on the one arm (57). This is consistent with the finding that the binding of CD62E to E-selectin ligand-1, a major glycoprotein ligand purified from myeloid cells, requires sialylated, fucosylated \( N \)-linked carbohydrates (58–60). Optimal binding of CD62P to its ligand PSGL-1 (CD162) requires sulfation of tyrosine groups near the \( NH_2 \) terminus of PSGL-1, in addition to sialylated and fucosylated O-linked oligosaccharides (8).

The kinetics of CD62P- and CD62E-ligand interaction have been studied indirectly by analysis of transient leukocyte tetherers to CD62E and CD62P immobilized on planar surfaces (55, 61). The intrinsic \( k_{on} \) for CD62P- and CD62E-mediated tethers was \(-1 s^{-1} \) and \(-0.7 s^{-1} \), respectively (55, 61). These values are \(-10\)-fold slower than the intrinsic \( k_{on} \) of CD62L-mediated tethers (55) and also \(-10\)-fold slower than the \( k_{on} \) reported in the present study for the CD62L-GlyCAM-1 interaction (Table III). Taken together, these data suggest that CD62E and CD62P interact with their respective physiological ligands with higher affinities and slower dissociation rate constants than CD62L (Table III). These differences may contribute to the slower kinetics of CD62L versus CD62P-CD62E-mediated leukocyte tethering and rolling (see below).

**Implications for Adhesion**—Since GlyCAM-1 is a soluble secreted molecule (25, 26), it could be argued that affinity and kinetic data for the CD62L/GlyCAM-1 interaction do not have direct implications for understanding leukocyte-endothelium interactions. However, it has recently been shown that lymphocytes and neutrophils can tether and roll on surfaces coated with GlyCAM-1. Furthermore, it seems likely that the carbohydrate structures on GlyCAM-1, MadCAM-1, and CD34 to which CD62L binds are very similar, if not identical. First, the CD62L-binding glycoforms of all three of these mucin-like molecules are expressed by the same cell type, namely high-endothelial cells (22–24). Second, the O-glycans on both CD34 and GlyCAM-1 contain sialic acid, sialic acid, and fucose (49). Finally, the binding of CD62L to both CD34 and GlyCAM-1 has been shown to require sialylation and sulfation (49–51, 62).

Since selectins seem to have evolved to mediate highly dynamic leukocyte-endothelial interactions such as tethering and rolling, there has been speculation as to what properties of selectins facilitate these interactions. One suggestion has been that selectins are effective because they bind their carbohydrate ligands with exceptionally fast association and dissociation rate constants (10). Consistent with this hypothesis, we show that the \( k_{on} \) and \( k_{off} \) values for the CD62L/GlyCAM-1 interaction are \(\geq 10^5 \) m\(^{-1}\) s\(^{-1}\) and \(\geq 10 \) s\(^{-1}\), respectively. However, kinetic studies of other cell-cell recognition molecules, which are not known to mediate tethering and/or rolling, have revealed that rapid binding kinetics may be a general feature of the molecular interactions mediating cell-cell recognition (43, 45, 47). For example, the ligand/receptor pairs CD2/CD58 (47) and CD28/CD80 (45) have \( k_{on} \) values of \(\geq 4 \times 10^5 \) \( m^{-1} \) \( s^{-1}\) and \( k_{off} \) values of \(\geq 4 \times 10^5 \) s\(^{-1}\). This suggests that fast binding constants, although perhaps necessary, are not sufficient for tethering and rolling. It should be emphasized, however, that fast association rates can be achieved both by fast association rate constants \( (k_{on}) \) and by high surface densi-
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### Table III

Summary of published affinity and kinetic data for selectin interactions

| Selectin | Ligand | Assay | $K_d$ | $k_{on}$ | $k_{off}$ | Ref. |
|----------|--------|-------|------|--------|----------|------|
| CD62L$^a$ | GlyCAM-1 | Direct binding | 108 | $\geq 100,000$ | $\geq 10$ | This study |
| sLe$^a$ | Direct binding | 3,900 | 280,000 | 1080 | 19 |
| sLe$^a$ | Inhibition of multivalent CD62L binding immobilized sLe$^a$-glycolipids | 2,300 | | | 12 |
| 6'-Sulfo-sLe$^a$ | Inhibition of CD62L Ig binding immobilized sLe$^a$ | 250 | | | 52 |
| 6'-Sulfo-sLe$^a$ | Inhibition of CD62L Ig binding PNAd | 800 | | | 14 |
| 3',6-Sulfo-sLe$^a$ | Inhibition of CD62L Ig binding GlyCAM-1 | 2,000 | | | 15 |
| PNAd | Transient leukocyte tethers to immobilized PNAd under flow | | | | 55 |

| CD62E | sLe$^a$ | Direct binding | 107 | | | 18 |
| sLe$^a$ | Direct binding | 720 | 230,000 | 164 | 19 |
| sLe$^a$, sLe$^a$ | Inhibition of multivalent CD62E binding immobilized sLe$^a$-glycolipids | 100 | | | 12 |
| sLe$^a$, sLe$^a$ | Inhibition of CD62E Ig binding immobilized bovine serum albumin-sLe$^a$ | 220,750 | | | 16 |
| sLe$^a$ | Inhibition of CD62E Ig binding immobilized sLe$^a$ | 160 | | | 52 |
| Leucocytes | Inhibition by oligomeric CD62E of cells binding immobilized CD62E | 1 | | | 21 |
| Leucocytes | Transient leukocyte tethers to immobilized CD62E under flow | | | | 0.7$^d$ |

| CD62P | sLe$^a$ | Direct binding | 7800 | 67,000 | | 19 |
| sLe$^a$ | Direct binding | 1300 | 522 | | 12 |
| 6'-Sulfo-sLe$^a$ | Inhibition of CD62P Ig binding immobilized sLe$^a$ | 220 | | | 52 |
| Leucocytes (PSGL-1$^a$) | Direct binding of CD62P (multimeric aggregates (47)) | 0.07 | | | 20 |
| Leucocytes (PSGL-1$^a$) | Transient leukocyte tethers to immobilized CD62P under flow | | | | 0.9$^d$ |

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$^a$ Abbreviations: Sia, sialic acid; sLe$^a$, Sia$_2$-3galactose$_{1-2}$-3(fucose$_1$-4)-N-acetylglucosamine; sLe$^a$, Sia$_2$-3galactose$_{1-2}$-3(fucose$_1$-4)/(N-acetyl)glucosamine; SE, sulfate ester; 6-sulfo-sLe$^a$, Sia$_2$-3galactose$_{1-2}$-3(fucose$_1$-4)/(N-acetyl)glucosamine; 6'-sulfo-sLe$^a$, Sia$_2$-3(SE-6)galactose$_{1-2}$-3(fucose$_1$-4)/(N-acetyl)glucosamine; 3',6-sulfo-sLe$^a$, (SE-3)galactose$_{1-2}$-3(fucose$_1$-4)/(N-acetyl)glucosamine.

$^b$ Calculated ($k_{on} = k_{off}/K_d$).

$^c$ The selectins CD62L and CD62P have also been reported to interact with a variety of other carbohydrate structures including heparin-like ligands, fucoidan, sulfatides, and dextran sulfate (7). Attempts to measure the affinity of these interactions (e.g., Refs. 17 and 69) have been hampered by the fact that these structures probably bind selectins multivalently. 6'-Sulfo-sLe$^a$ and 6'-sulfo-sLe$^a$ are major capping groups in GlyCAM-1 O-linked oligosaccharides.

$^d$ Intrinsic $k_{off}$ (extrapolated to zero flow (55)).

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ties of one or both interacting molecules. Williams (63) proposed that selectins might achieve fast association rates because their oligosaccharide ligands are presented on mucin-like molecules at very high densities. Subsequently all selectin ligands identified, with the exception of E-selectin ligand-1, have been mucin-like molecules (8). One property clearly important for selectin-mediated tethering and rolling is the localization of selectins (e.g. CD62L) or their ligands (e.g. PSGL-1) to the tips of microvilli on leukocytes (64, 65). Interestingly the α4 integrins, which have recently been shown to be capable of mediating tethering and rolling of leukocytes on endothelium, are also apparently localized to the tips of microvilli (2).

The good agreement between the intrinsic $k_{off}$ of CD62L-mediated leukocyte tethers (55) and the solution $k_{off}$ of the CD62L/GlyCAM-1 interaction (Table III), suggests that the duration of leukocyte tethers is dominated by the $k_{off}$ of the underlying molecular interaction. Furthermore, there is an excellent correlation between the $k_{off}$ of CD62L-, CD62P-, and CD62E-mediated tethers and the velocity of CD62L-, CD62P-, and CD62E-mediated leukocyte rolling (55, 56). Taken together these results are consistent with the hypothesis that the $k_{off}$ of selectin-ligand interactions has a major influence on the duration of leukocyte tethers and the velocity of leukocyte rolling. Analysis of the solution kinetics of CD62E- and CD62P-ligand interactions will provide a critical test of this hypothesis.

One or more CD62L-binding protein(s) present in normal mouse serum can partially inhibit adhesion of lymphocytes to HEV in a Stamper-Woodruff assay (25). Our finding that GlyCAM-1 does indeed bind to CD62L at concentrations just above its mean serum level suggests that it may inhibit CD62L-mediated adhesion in vivo (25, 28), but direct evidence for such a role is lacking.

**Implications for Signaling—**GlyCAM-1 is present in mouse serum at a concentration of ~1.5 μg/ml (30 nM) (27). It has recently been reported that murine GlyCAM-1 at concentrations as low as 2.5 μg/ml can stimulate β$_2$-integrin-mediated adhesion of naive human peripheral blood lymphocytes to ICAM-1 (CD54), and that antibodies to human CD62L block this effect, suggesting that the GlyCAM-1 acts by binding to CD62L (31). Consistent with this, we report here that GlyCAM-1 binds to purified immobilized CD62L at concentrations as low as 1.7 μg/ml (~34 nM). Since monovalent CD62L/GlyCAM-1 interaction has an affinity of 108 μM, this result shows that at these low concentrations GlyCAM-1 must bind multivalently to the immobilized CD62L. It follows that GlyCAM-1 binds to preclustered CD62L and/or that it induces clustering of CD62L when it binds to the cell surface. Taken together with the observation that antibody-induced cross-linking of CD62L activates β$_2$ integrin-mediated adhesion (29, 31), these results suggest that GlyCAM-1 activates lymphocyte adhesion by cross-linking CD62L.

In principle, GlyCAM-1 may bind multivalently either because it self-associates to form multimers or because each GlyCAM-1 molecule carries multiple copies of the CD62L binding carbohydrate structure(s). However, the elution position of purified GlyCAM-1 on size exclusion chromatography (Mr,
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45,000–66,000) agrees with its \( K_d \) determined by SDS-PAGE,\(^3\) arguing strongly against a multimeric form of GlyCAM-1. In

In conclusion, in the first affinity and kinetic study of the interaction between a selectin and a defined physiological ligand, we have shown that CD62L binds to GlyCAM-1 with a very low affinity (\( K_d \text{[108 \mu M]} \)) and very fast kinetics (\( k_{\text{on}} \approx 10^8 \text{s}^{-1} \)). We have also provided evidence that, at concentrations just above the level at which it is present in serum, soluble GlyCAM-1 is able to bind multivalently to immobilized CD62L, suggesting a potential mechanism for signaling through CD62L.

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