Brief Definitive Report

Amino Acid Residues Required for Binding of Lymphocyte Function-associated Antigen 3 (CD58) to its Counter-Receptor CD2

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Summary

Efficient activation and regulation of the cellular immune response requires engagement of T cell accessory molecules as well as the antigen-specific T cell receptor. The lymphocyte function-associated antigen (LFA) 3 (CD58)/CD2 accessory pathway, one of the first discovered, has been extensively characterized in terms of structure and function of the CD2 molecule, which is present on all T lymphocytes and natural killer cells of the human immune system. The binding site of human CD2 for LFA-3 has been localized to two epitopes on one face of the first immunoglobulin (Ig)-like domain of this two-domain, Ig superfamily molecule. Human LFA-3 is genetically linked and is 21% identical in amino acid sequence to CD2, suggesting that this adhesive pair may have evolved from a single ancestral molecule. We have aligned the amino acid sequences of LFA-3 and CD2 and mutagenized selected amino acids in the first domain of LFA-3 that are analogous to those implicated in the binding site of CD2. The data show that K30 and K34, in the predicted C-C′ loop, and D84, in the predicted F-G loop of LFA-3, are involved in binding to CD2, suggesting that two complementary sites on one face of the first domain of each molecule bind to each other.

Human LFA-3 (CD58) is a widely distributed cell surface molecule capable of adhesion and signaling to T cells via its receptor, CD2 (1). LFA-3 is one of a number of "T cell accessory molecules," so called because LFA-3 engagement of CD2 on responder cells costimulates T cell proliferation induced by anti-T cell receptor (TCR/CD3) antibody or antigen. The actions of accessory molecules such as LFA-3 are hypothesized to be required for the efficient generation of optimal antigen-specific responses. The timing and intensity of the antigen-specific and accessory signals is thought to determine the character of the T cell response, which can range from loss of ability to respond to antigen (anergy) to up-regulation of immune functions and proliferation (activation).

LFA-3 and its counter-receptor CD2 belong to the Ig superfamily, as shown by nuclear magnetic resonance (NMR) and x-ray crystallography (2–4). Because LFA-3 and CD2 exhibit 21% amino acid homology and are closely linked genetically, it is considered likely that they share a common ancestral molecule. Interestingly, in mouse and rat, an LFA-3 homologue has not been identified. In these species (but not in humans), CD48, also structurally related to CD2 and LFA-3, is a ligand for CD2 (5). CD2, CD48, and LFA-3 are all located on human chromosome 1 (6).

CD2 has been extensively studied at a molecular and functional level. Three functional epitopes on the human CD2 molecule have been described and are recognized by mAbs $T11_1$, $T11_2$, and $T11_3$ (7). Pairs of mAbs such as $T11_2$ and $T11_3$ induce T cell proliferation. Similarly LFA-3 in conjunction with certain CD2-specific mAbs costimulates T cell proliferation. $T11_1$, which maps to the first (most NH_2-terminal) Ig domain of CD2, can block adhesion to LFA-3. An elegant study by Peterson and Seed (8), using saturation mutagenesis of human CD2 extracellular domains followed by expression and a double selection for epitope maintenance and loss of binding to LFA-3, localized the $T11_1$- and $T11_2$-binding epitopes to two sets of highly charged residues within the first domain. These CD2 epitopes corresponded closely to sets of residues involved in binding LFA-3, which they termed regions I and II. An NMR-derived structure for rat CD2 (2) further localized these sites three dimensionally to one face of the CD2 molecule. These results have since been confirmed and refined by a number of other studies (9–12). Rat and human CD2 are 40% identical in amino acid sequence, allowing reasonably confident prediction of the human CD2 structure, which was recently confirmed by NMR studies (4).

To focus on areas of LFA-3 likely to be important in binding CD2, we hypothesized that the simplest scenario for evolu-

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tion of CD2 and LFA-3 from an ancestral molecule would be conservation of the active residues of the ancestral binding site. We aligned the amino acid sequence of the first domain of LFA-3 with that of CD2 to identify amino acid residues analogous to the active residues of CD2. We then mutagenized these residues, using plasmids encoding LFA-3/IgG1 soluble fusion proteins as parental constructs. The mutants were assayed for binding to CD2 and maintenance of LFA-3 epitopes. We now report that a subset of the targeted residues are indeed important for binding of LFA-3 to CD2.

Materials and Methods

LFA-3 Domain 1 Modeling. To construct a three-dimensional homology model of domain 1 of LFA-3, a procedure similar to that described by Greer (13) was used with the aid of QUANTA software (Molecular Simulations, Inc., Sunnyvale, CA). The LFA-3 amino acid sequence was aligned with that of human and rat CD2, and the alignment was refined manually so as to maximize the sequence similarity matches of the presumed β-strand segments. Coordinates for those segments were obtained from the rat CD2 structure (3). The loops corresponding to insertions or deletions were built either by fragment search in the Brookhaven Protein Data Bank (Brookhaven, NY), or, in the case of those with one or two residues, were manually built and subjected to energy minimization with CHARMm to obtain the minimal energy conformations (14). The whole model also was subjected to several cycles of energy minimization.

Mutagenesis and Expression of LFA-3/Ig Plasmids. LFA-3/Ig fusion constructs, previously described (12), were used as parental constructs for the mutagenesis. Constructs encoding soluble LFA-3/Ig fusion protein composed of LFA-3 domain 1 (previously described as LFA-3TIP, here called "D1/Ig"), or domain 1 + 2 (previously described as LFA-3FLIgG1, here called "D1+2/Ig") fused to the human IgG1 H chain hinge, CH2 and CH3 regions, were subcloned into the transient expression plasmid CDM8 (15). Targeted mutants were made as described previously (16, 17). Sequence across the mutagenized region was determined for at least one isolate of each mutant, and plasmids were electroporated into COS7 cells for expression as described previously (18). Supernatants containing secreted LFA-3/Ig were harvested 48–72 h after electroporation, and the quantity of fusion protein was normalized by ELISA by use of goat anti-human IgG serum (Jackson ImmunoResearch Labs, Inc., West Grove, PA) coated on Maxisorb plates (Nunc, Roskilde, Denmark) to capture, and HRP-conjugated goat anti-human IgG (Pierce Chemical Co., Rockford, IL) serum to detect, titered antigen, by use of standard procedures.

Adhesion Assay and Flow Cytometry. Binding of mutant LFA-3/Ig protein to CD2 was detected by "staining" CD2-expressing Jurkat cells with LFA-3/Ig–containing supernatants that had been quantitatively normalized by dilution. Bound LFA-3/Ig was detected with PE-conjugated goat anti-human IgGFc Fabs (Jackson ImmunoResearch Labs, Inc.), followed by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) of 10,000 cells.

Binding to LFA-3–specific mAbs was assayed by ELISA. Supernatants were quantitatively normalized by dilution, incubated in 96-well plates coated with anti-human IgG serum to capture LFA-3/Ig, and washed. LFA-3–specific mAbs previously described (12, 19) were added at a maximum concentration of 10 μg/ml, titered by serial twofold dilution, and detected with horse radish peroxidase–goat–anti–mouse IgG. The minimum dilution of mAb that gave maximal signal was used to calculate the percent binding to each mAb of each mutant, compared with the parental control supernatant. Polyclonal guinea pig serum also used in binding studies was made by immunizing with Freund's adjuvant plus recombinant soluble (rs) LFA-3 (20).

Results

Design of Mutant Constructs. Previously, it was shown that the first Ig-like domain of LFA-3 is sufficient for binding to CD2 (12). The alignment shown in Fig. 1 was used to predict residues of LFA-3 analogous to binding residues in CD2, which we hypothesized might participate in mutually complementary binding regions. Priority was given to region I LFA-3 residues (which, according to our hypothesis, would bind to region II of CD2) because we have previously shown that LFA-3/CD2 binding is most sensitive to mutations within CD2 region II (12), and because $T11_1$ mAbs (to region II of CD2) block adhesion to LFA-3, while $T11_2$ mAbs do not.

Construction, Expression, and Assay of Mutant Constructs. Mutant constructs were made by use of gapped-heteroduplex oligonucleotide-directed mutagenesis. After transfection into COS7 cells and collection of supernatants containing secreted soluble fusion protein, expression was assessed by ELISA by

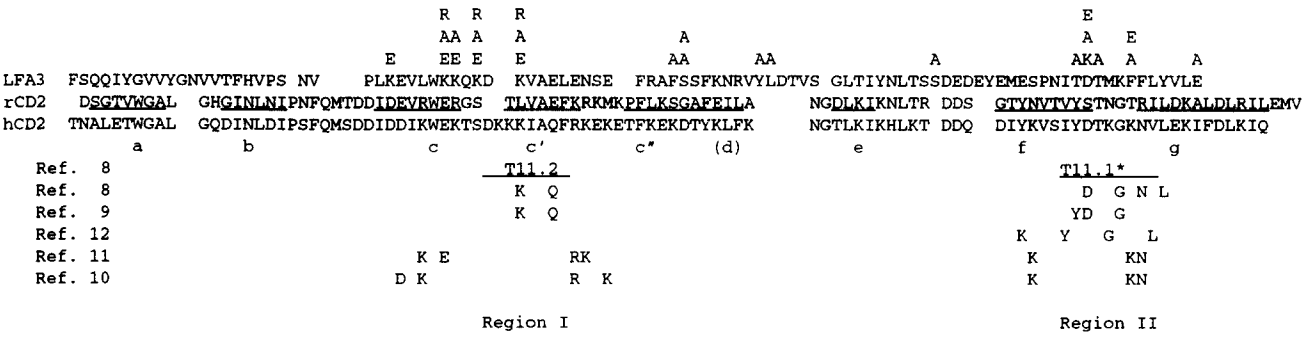

Figure 1. Alignment of LFA-3 with human (*h*) and rat (*r*) CD2 amino acid sequences. β strands, determined from NMR and x-ray crystallographic analysis of rat CD2 as described in the text, are underlined and lettered *a*, *b*, *c*, *c'*, etc. Human CD2 epitopes, which are required for binding of blocking mAb $T11_1$ (*Region II*) and signaling mAb $T11_2$ (*Region I*) are indicated. Residues of CD2 implicated in binding to LFA-3 in other publications are indicated below the alignment (8–12). Residues mutagenized for this study are indicated above the alignment. Some were mutagenized in combination, whereas others were performed singly, as indicated in Fig. 2.

Table 1. *Binding of Monoclonal Antibodies and Jurkat Cells to LFA-3/Ig Domain 1 Mutants*

Amino acids changed	Mutant #	Jurkat cell CD2	GP ser	7A6*	TS2/9*	1A3*	1A2*	1C4	PAK-1
K24E	M83	72.95 ± 14.95	91.69	64.97 ± 22.43	4.03 ± 1.00	75.93 ± 19.52	89.97 ± 14.79	31.02 ± 7.56	57.08 ± 11.34
K24E	FLM83	235.15 ± 158.15	92.62	77.45 ± 19.40	80.46 ± 3.71	84.33 ± 29.11	98.26 ± 12.93	92.74 ± 14.60	114.28 ± 27.44
K29KQKDK/RKQRDR	M109	0.00 ± 0.00	121.53	21.16 ± 16.29	11.47 ± 6.99	5.95 ± 4.22	59.77 ± 5.88	59.36 ± 15.10	59.18 ± 6.65
K29KQKDK/RKQRDR	FLM109	0.00 ± 0.00	107.27	83.24 ± 15.30	95.43 ± 5.30	19.09 ± 10.58	112.69 ± 4.29	134.09 ± 42.78	115.74 ± 19.65
K30A	M94	15.05 ± 6.05	120.13	77.60 ± 16.92	69.23 ± 3.14	84.93 ± 17.14	76.60 ± 7.78	61.90 ± 10.33	56.06 ± 6.58
K30A	FLM94	0.00 ± 0.00	97.09	113.40 ± 29.55	101.56 ± 7.16	115.71 ± 30.66	120.11 ± 21.02	145.89 ± 66.93	104.10 ± 10.85
K34A	M84	0.00 ± 0.00	107.67	30.89 ± 12.60	39.91 ± 8.62	40.30 ± 12.38	85.34 ± 11.56	50.34 ± 13.55	50.16 ± 7.96
K34A	FLM84	0.00 ± 0.00	86.80	88.40 ± 15.59	3.37 ± 2.26	5.15 ± 4.51	96.29 ± 10.57	122.71 ± 53.15	113.85 ± 23.35
S47A	FLM116	107.33 ± 26.48	128.33	112.19 ± 13.93	101.92 ± 15.12	103.72 ± 14.43	107.27 ± 8.83	140.71 ± 50.01	121.11 ± 25.14
S69A	FLM117	182.40 ± 5.90	134.01	116.55 ± 16.00	101.34 ± 12.29	103.50 ± 18.22	109.84 ± 12.56	135.70 ± 57.92	123.80 ± 24.92
D84K	M76	14.20 ± 8.20	85.20	59.83 ± 19.08	66.38 ± 14.95	75.61 ± 13.60	79.50 ± 8.43	53.87 ± 9.09	57.08 ± 10.04
T83DT/ADA	M96	174.95 ± 100.95	115.89	87.36 ± 25.05	78.64 ± 9.83	105.28 ± 13.05	96.57 ± 8.74	107.34 ± 2.80	104.96 ± 13.27
K87E	M110	107.75 ± 7.75	121.14	111.75 ± 17.85	105.19 ± 4.45	112.54 ± 18.16	111.28 ± 10.37	116.09 ± 8.82	129.95 ± 13.97
L93A	FLM118	100.54 ± 1.57	119.18	117.50 ± 14.92	105.51 ± 6.63	103.80 ± 14.02	109.29 ± 9.57	137.96 ± 48.95	119.06 ± 20.54
Epitope loss mutants									
K29KQK/EKQE	M73	0.00 ± 0.00	133.99	2.03 ± 1.26	4.09 ± 3.72	1.97 ± 1.40	0.88 ± 0.27	1.74 ± 0.55	0.55 ± 0.78
K29KQK/AKQA	M93	0.00 ± 0.00	134.80	15.94 ± 6.32	19.31 ± 3.16	25.41 ± 7.55	17.40 ± 2.99	11.49 ± 2.09	10.04 ± 1.34
K30E	M74	0.00 ± 0.00	138.23	0.88 ± 0.92	2.03 ± 2.09	1.50 ± 2.12	0.11 ± 0.16	1.96 ± 1.40	0.94 ± 1.33
K34E	M75	2.10 ± 2.10	137.17	0.63 ± 0.90	1.49 ± 2.10	0.00 ± 0.00	11.16 ± 1.16	2.99 ± 0.45	9.82 ± 11.48
F46S/AA	M85	0.00 ± 0.00	119.35	3.23 ± 4.56	3.67 ± 5.06	0.00 ± 0.00	0.04 ± 0.05	20.30 ± 3.45	39.64 ± 21.12
Y54L/AA	M86	0.00 ± 0.00	127.83	4.14 ± 1.05	13.29 ± 2.22	54.71 ± 15.39	2.34 ± 2.35	0.00 ± 0.00	24.95 ± 19.38
D84A	M95	2.80 ± 2.80	92.07	19.10 ± 16.44	31.08 ± 6.29	39.27 ± 8.07	28.92 ± 1.91	16.48 ± 2.59	19.67 ± 1.15
K87A	M97	11.35 ± 0.65	121.03	41.32 ± 21.82	31.90 ± 12.75	38.49 ± 7.77	27.76 ± 2.17	15.24 ± 0.96	19.24 ± 5.08

Binding of monoclonal antibodies and Jurkat cells to LFA-3/Ig domain 1 mutants. Percent wild-type binding after normalization for expression was determined as described in Materials and Methods and in legend to Fig. 2. Numbers represent mean ± standard deviation of two or three experimental determinations for each mutant.
* mAbs that block adhesion of LFA-3 to CD2.

use of anti-human IgG1 polyclonal serum, which binds to the Ig Fc portion of the molecule, in an area unaffected by the mutagenesis. Ability of mutants to bind to CD2 was determined by FACS® analysis of binding to the T leukemia line Jurkat. Results are presented numerically in Table 1 and in summary form in Fig. 2, where constructs displaying staining or binding at a level >50% of the wild-type level are indicated as +, 10–50% as +/−, and 0–10% as −.

Replacement amino acids for the targeted residues were chosen with the goal of maintaining proper local folding of the protein (see below). The best substitution for a particular amino acid was difficult to predict, resulting in several attempts for many of the mutations in areas of particular interest.

Epitopes of LFA-3 Domain 1. It is important to distinguish mutants that have lost CD2 binding activity due to large-scale perturbations in structure from those that show effects limited to contact residues. To identify mutants exhibiting improper folding of domain 1, we stained with a panel of mAbs representative of every known epitope of LFA-3 within these domains, in addition to a guinea pig polyclonal serum. mAbs that map to domain 1 include blocking (i.e., able to block binding of LFA-3 to CD2) mAbs 7A6, TS2/9, 1A3, and 1A2, and nonblocking mAbs 1C4 and PAK-1. Cross-blocking studies have shown that mAbs 7A6, TS2/9, and 1A3 bind to closely related epitopes, while 1A2, 1C4, and PAK-1 bind to distinct though somewhat overlapping epitopes [(19); Chisholm, P., and C. Williams, unpublished results). All of these epitopes were capable of being disrupted by mutations to linearly discontinuous areas of domain 1 (Table 1 and Fig. 2), indicating that they recognize conformational epitopes and are sensitive to local denaturation caused by improper folding. Most of the mutants that retain these sensitive epitopes bind well to CD2, indicating that the substituted amino acid residues can support both proper folding and ligand binding.

Mutations That Conserve Epitopes But Diminish Binding to CD2. The most informative mutants are those that retain sensitive epitopes but show diminished binding to CD2. LFA-3 region I mutants #109, 94, and 84 implicate the two lysine residues K30 and K34, located in the predicted C-C′ loop of LFA-3, in binding to CD2 (Table 1 and Fig. 2). LFA-3 residue K34 is analogous to the important K48 residue in region I of CD2, shown to be involved in both binding of CD2 to LFA-3 (8) and in the functionally important epitope bound by $T11_2$-like mAbs, which are capable of costimulating T cell proliferation when present with mAb $T11_3$. When LFA-3 K34 was changed to A, binding to CD2 was lost, although a panel of mAbs specific for distinct LFA-3 epitopes still bound, indicating that proper folding elsewhere in the molecule was maintained (Fig. 2). The mutation of K30 to A also diminished binding to CD2 without epitope loss, suggesting that, as in CD2, more than one charged residue on the face of the molecule is involved in formation of the binding site. These informative mutants were also made in D1+2/Ig and showed the same phenotype regarding loss of CD2 binding.

Mutations also were made in the predicted F-G loop of LFA-3, which is structurally analogous to region II (functional epitope $T11_1$) of CD2. The mutation D84 to K resulted reproducibly in partial loss of binding to CD2, although all epitopes were maintained. D84 of LFA-3 is analo-

Amino acids changed:	Mutant #:†	Jurkat cell CD2	GP ser	7A6*	TS2/9*	1A3*	1A2*	1C4	PAK-1
K24E	M83†	+	+	+	-	+	+	+/-	+
K24E	FLM83†	+	+	+	+	+	+	+	+
K29KQKDK/RKQRDR	M109	-	+	+/-	+/-	-	+	+	+
K29KQKDK/RKQRDR	FLM109	-	+	+	+	+/-	+	+	+
K30A	M94	+/-	+	+	+	+	+	+	+
K30A	FLM94	-	+	+	+	+	+	+	+
K34A	M84	-	+	+/-	+/-	+/-	+	+	+
K34A	FLM84	-	+	+	-	-	+	+	+
S47A	FLM116	+	+	+	+	+	+	+	+
S69A	FLM117	+	+	+	+	+	+	+	+
D84K	M76	+/-	+	+	+	+	+	+	+
T83DT/ADA	M96	+	+	+	+	+	+	+	+
K87E	M110	+	+	+	+	+	+	+	+
L93A	FLM118	+	+	+	+	+	+	+	+
Epitope loss mutations:									
K29KQK/EKQE	M73	-	+	-	-	-	-	-	-
K29KQK/AKQA	M93	-	+	+/-	+/-	+/-	+/-	+/-	+/-
K30E	M74	-	+	-	-	-	-	-	-
K34E	M75	-	+	-	-	-	+/-	-	-
F46S/AA	M85	-	+	-	-	-	-	+/-	+/-
Y54L/AA	M86	-	+	-	+/-	+	-	-	+/-
D84A	M95	-	+	+/-	+/-	+/-	+/-	+/-	+/-
K87A	M97	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-

Figure 2. Summary of binding of human LFA-3/Ig mutants to CD2 and mAbs. Expression of each construct was normalized by ELISA with an anti-IgFc mAb, as described in Materials and Methods. (†) Mutant numbers prefixed with "M" were made with D1/Ig as the parental construct, while those prefixed with "FLM" (for "full length mutant") were made with D1+2/Ig as the parental construct. Binding to other mAbs and to Jurkat (CD2-bearing) cells was calculated as a percentage of parental D1/Ig or D1+2/Ig binding, after correction for expression (see Materials and Methods). Mutations significantly reduced in binding to CD2 while maintaining most epitopes are indicated in bold face type. See Table 1 for numerical values. +, 50–100%; ±, 10–50%; −, 0–10% wild-type binding.

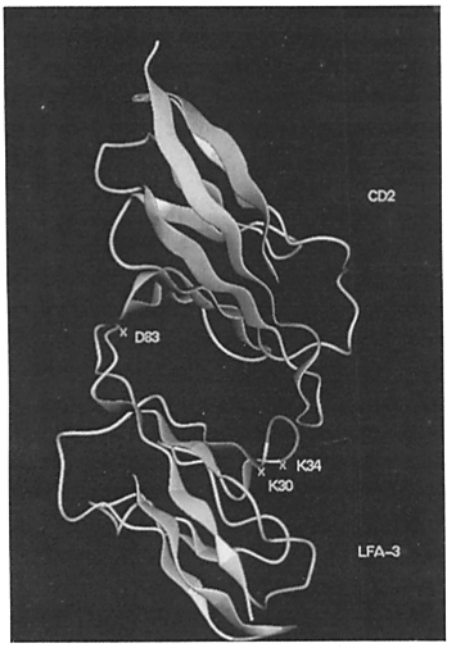

Figure 3. Hypothetical model of domain 1 of LFA-3 showing proposed contacts with CD2. The crystal structure of rat CD2 domain 1 was used as a template for the generation of this three-dimensional model, with QUANTA protein homology modeling software used as described in Materials and Methods. The human CD2 domain 1 three-dimensional structure is very similar to that of the rat by NMR (4) and more recently by x-ray (21) analyses.

gous to D78 of CD2; it is predicted to protrude from the same face of the molecule as the highly charged basic residues of the C-C′ loop, as shown in our hypothetical model (Fig. 3). Other mutations we tested in this area resulted in either unaffected binding to CD2 (T83DT/ADA and K87E) or loss of multiple epitopes, indicating poor folding (D84A and K87A).

In addition to mutations targeted to the predicted regions of binding, we made mutations in areas not expected to be involved in binding. All of these either had no effect on binding to CD2 (K24E, S47A, S69A, L93A) or perturbed folding as well as binding (F46S/AA, Y54L/AA).

Discussion

We have shown that charged residues in the predicted C-C′ and F-G loops of LFA-3 domain 1 are involved in binding of LFA-3 to CD2. According to our alignment of the amino acid sequences of LFA-3, with its genetically linked ligand CD2, these LFA-3 residues are structurally analogous to known binding residues of CD2. The simplest way in which LFA-3 and CD2 molecules on apposing cells could interact is by mutually complementary binding sites (Fig. 3), such as is seen in crystal forms of both rat and, very recently, human CD2 (3, 21).

Because there are many examples of Ig superfamily adhesion molecules that exhibit homophilic (self-) binding (e.g., NCAM, PECAM, CEA, etc.), we and others have hypothesized that CD2 and LFA-3 have evolved after duplication of the gene for a single homophilic ancestral molecule (22, 23). Although it is not possible to test directly an evolutionary hypothesis, our results and the crystal forms mentioned above are indirectly supportive of this view.

The ability of anti-CD2 mAbs or soluble LFA-3 to costimulate T cell proliferation in concert with anti-TCR/CD3 mAb stimulation indicates that binding of LFA-3 to CD2 has a signaling as well as an adhesive function. Molecular dissection of the well-conserved CD2 cytoplasmic domain defined separable portions involved in signaling and ligand avidity regulation, confirming the signaling function (24, 25). The natural affinity of the CD2/LFA-3 interaction is relatively low [$2 \times 10^6\ M^{-1}$ (26)], which would suggest that weak rather than strong adhesion may actually optimize performance of the molecule's accessory role. Although controversial, it has been reported that CD59 also binds CD2, thus potentially strengthening the APC-T cell interaction mediated via CD2 (9, 27). It may be easier than in cases of naturally strong adhesion to find or design a blocking molecule with higher affinity for CD2 than the natural ligand, LFA-3. Such a molecule could block both binding and signaling through the LFA-3/CD2 and potentially the CD59/CD2 pathways, thus performing an immunosuppressive function.

In summary, mutations in amino acids of LFA-3 predicted to be analogous to the binding site of its close relative and coreceptor CD2 can reduce or abolish binding of LFA-3 to this coreceptor. The amino acids comprising these sites are (hypothetically, for LFA-3) part of the C-C′ and F-G loops of the most NH_2-terminal Ig-like domain of each molecule. Knowledge of these binding sites may aid in design of peptides or other small molecules that could block the LFA-3/CD2 pathway in a therapeutic setting, thus providing a new immunosuppressive agent.

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