Mutational Analysis of the CD6 Binding Site in Activated Leukocyte Cell Adhesion Molecule

John E. Skonier,[‡] Michael A. Bowen,[‡] John Emswiler,[‡] Alejandro Aruffo,[‡,§] and Jürgen Bajorath*[,‡,§]

Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121, and Department of Biological Structure, University of Washington, Seattle, Washington 98195

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ABSTRACT: The interaction between CD6 and its ligand activated leukocyte cell adhesion molecule (ALCAM) mediates adhesion of thymocytes to thymic epithelial cells. The extracellular region of ALCAM includes five Ig-like domains, and its N-terminal V-like domain specifically binds to the membrane-proximal scavenger receptor cysteine-rich domain of CD6. Previously, six ALCAM residues were identified by alanine scanning mutagenesis to contribute to the interaction with CD6. All of these residues mapped to the predicted A′GFCC′C″ face of ALCAM's N-terminal domain. Here we describe the results of experiments designed to further study the CD6 binding site. Other mutagenesis experiments at four previously studied sites were carried out to better understand their importance for the interaction with CD6, and different receptor binding assays were employed to compare the contribution of these and other ALCAM residues to the CD6−ligand interaction. A total of ten new ALCAM mutants were prepared, and three additional residues were identified as critical for CD6 binding. These studies have enabled us to classify ALCAM residues according to their importance for binding and to describe the CD6 binding site in some detail.

The human CD6 ligand, termed ALCAM[1] (Bowen et al., 1995), is expressed on activated T and B cells, monocytes, and TE cells (Wee et al., 1994; Bowen et al., 1995; Patel et al., 1995), whereas CD6 (Aruffo et al., 1991) is expressed by thymocytes and mature T cells (Reinherz et al., 1982; Morimoto et al., 1988). CD6−ALCAM interactions mediate thymocyte−TE cell adhesion and have been implicated in regulating T cell functions (Vollger et al., 1987; Bowen et al., 1995). Both proteins are also expressed in the brain. ALCAM is highly homologous to the chicken neural adhesion molecule BEN (Pourquie et al., 1992), Thus, CD6−ALCAM interactions may play a role in both the immune and nervous system. However, the precise nature and magnitude of effects triggered by CD6−ALCAM interactions are not yet understood.

CD6 is a member of the SRCRSF (Resnick et al., 1994). Its extracellular region includes three SRCR domains. In contrast, ALCAM belongs to the IgSF (Williams & Barclay, 1988). Like BEN, ALCAM's extracellular region includes five Ig domains (Bowen et al., 1995), and its N-terminal domain displays IgSF V-set (Williams & Barclay, 1988) characteristics (Bajorath et al., 1995). The CD6−ALCAM interaction is the only cell surface protein interaction studied to date which involves members of the SRCRSF and IgSF.

As soluble Ig fusion proteins, ALCAM's N-terminal domain and the membrane-proximal domain of CD6 specifically bind to each other in ELISA (Whitney et al., 1995; Bowen et al., 1996). The stoichiometry of the ALCAM−CD6 interaction in solution is 1:1 (Bowen et al., 1996). Alanine scanning mutagenesis (Wells, 1991) was carried out to identify residues in ALCAM's binding domain important for binding to CD6 (Skonier et al., 1996). Thirty-one ALCAM mutant proteins were generated in a systematic screen of the predicted β-sheet and loop regions of ALCAM's N-terminal domain, and their binding to CD6 was characterized by ELISA and surface plasmon resonance (BIAcore) (Jönsson et al., 1995). In these studies, six ALCAM residues were identified whose mutation affected CD6 binding (Skonier et al., 1996).

The six residues which affected CD6 binding at varying levels mapped, with no exception, to the predicted A′GFCC′C″ face of ALCAM's binding domain, whereas mutants with wild-type-like binding were scattered throughout the domain. Residues forming the A′GFCC′C″ β-sheet of IgSF V-(like) binding domains in other cell surface proteins have also been implicated in interactions with diverse ligands including IgSF members (Arulanandam et al., 1993; van der Merwe et al., 1995; Peach et al., 1995), integrins (Lee et al., 1995), and carbohydrates (van der Merwe et al., 1996; Vinson et al., 1996).

Herein we describe a refined and extended mutagenesis analysis of ALCAM's CD6 binding site. Previously identified mutant proteins with partially reduced binding to CD6

* Address correspondence to this author at BMS-PRI, 3005 First Ave., Seattle, WA 98121. Tel: (206) 728-3612. Fax: (206) 727-3602.
[‡] Bristol-Myers Squibb Pharmaceutical Research Institute.
[§] University of Washington.
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[1] Abbreviations: ALCAM, activated leukocyte cell adhesion molecule; CDR, complementarity determining region; CD6D3S, membrane proximal extracellular domain of CD6 including the stalk region; D1, N-terminal extracellular domain of ALCAM; D2, second extracellular domain of ALCAM; D3, third extracellular domain of ALCAM; ELISA, enzyme-linked immunosorbent assay; Fv, variable fragment; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; IgG, immunoglobulin G; IgSF, immunoglobulin superfamily; mAb, monoclonal antibody; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SRCR domain, scavenger receptor cysteine rich-domain; SRCRSF, scavenger receptor cysteine-rich protein superfamily; TE, thymic epithelium; V-domain, immunoglobulin variable domain.

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have been studied in different receptor−ligand binding assays, and other mutagenesis experiments were carried out at previously studied sites. Additional experiments have identified three other ALCAM residues as critical for the interaction with CD6. These studies have confirmed the CD6 binding site and have allowed us to classify ALCAM residues according to increasing importance for CD6 binding. With the aid of a molecular model, ALCAM residues which contribute to the interaction have been mapped, providing a first, albeit approximate, view of an SRCRSF recognition site.

MATERIALS AND METHODS

ALCAM and CD6 Ig Fusion Proteins. Ig fusion proteins including ALCAM's first (D1-Ig), first and second (D1-D2-Ig), and first to third (D1-D2-D3-Ig) domains and full-length ALCAM (including five Ig-like domains) were constructed by PCR as described (Bowen et al., 1996). D1-Ig, D1-D2-D3-Ig, and ALCAM mutants expressed as D1-D2-D3-Ig were constructed using human IgG constant regions. D1-D2-Ig and mutants expressed as D1-D2-Ig were constructed using murine IgG constant regions. Soluble fusion proteins of human CD6 (CD6-Ig) and the third extracellular domain of CD6 (CD6D3S-Ig) were constructed and expressed in COS as described (Bowen et al., 1996).

Construction and Expression of ALCAM Mutant Proteins. The desired mutations and additional mutations for diagnostic restriction enzyme sites were introduced by overlap extension PCR into cDNAs encoding two (D1-D2) and/or three N-terminal ALCAM domains (D1-D3). The respective PCR fragments were cloned into the expression vector CDM7B$^-$ which includes the hinge and constant regions of human IgG (Hollenbaugh et al., 1995). Each mutation was verified by restriction enzyme analysis and cDNA sequencing. Transient expression in COS cells as described (Bajorath et al., 1995b) yielded supernatants including the mutant Ig fusion proteins.

Binding of Mutant Proteins to ALCAM Anti-D1 mAb by ELISA. A 96-well ELISA plate (Immulon 2, Dynatech, Chantilly, VA) was coated with anti-D1 mAb (Bowen et al., 1996), blocked, and incubated with serial dilutions of COS cell supernatants of wild-type or mutant proteins as described (Skonier et al., 1996). The wells were incubated with HRP-conjugated donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA) and developed with chromogenic substrate TMB (Genetic Systems, Redmond, WA). Absorbency was measured at dual wavelength (450 and 630 nm).

Mutant Protein Concentrations by ELISA. Assays with mutant proteins were carried out using COS cell culture supernatants. The concentrations of mutant proteins including D1-D3 were normalized relative to wild type using an anti-D3 mAb (Bowen et al., 1996) following the anti-D1 mAb ELISA protocol described above. For mutant proteins including D1-D2, ELISA plates were coated with goat anti-murine IgG2a (Southern Biotechnology, Birmingham, AL) at 5 μg/mL and, following blocking and washing steps, incubated with HRP-conjugated goat anti-murine IgG (Biosource International, Camarillo, CA) and developed as described above.

Determination of Approximate Oligomerization States. The apparent molecular masses of ALCAM and CD6 fusion proteins were determined by SDS−PAGE under nonreducing conditions. Since the fusion proteins are glycosylated, determined molecular masses are only approximate. Fusion proteins in solution were analyzed by size exclusion chromatography on a Waters Co. 7.8 × 300 mm 300SW HPLC column. Samples were chromatographed at a flow rate of 0.35 mL/min in 10 mM Tris-HCl, pH 7.5, 10 mM potassium phosphate, and 0.15 mM sodium chloride. Proteins ranging from RNase (13.7 kDa) to ferritin (440 kDa) were used as molecular mass standards. The approximate oligomerization state of each protein was determined by dividing molecular masses of complexes obtained via size exclusion chromatography by molecular masses of monomeric fusion proteins as determined by SDS−PAGE.

ELISA Assays for Binding of Mutant Proteins to CD6. A high avidity ELISA with CD6-Ig immobilized on the plate was carried out as described (Skonier et al., 1996). In a lower avidity ELISA assay, plates were coated with CD6D3S-Ig at 1 μg/mL. After the blocking and washing steps, serial dilutions of COS cell supernatants containing D1-D2-Ig mutant proteins ($1-10^{-5}$ μg/mL) were added and assay was detected as described (Skonier et al., 1996).

Binding of ALCAM Fusion and Mutant Proteins by BIAcore Analysis. CD6 binding of ALCAM D1-Ig, D1-D2-Ig, and D1-D2-D3-Ig and mutants expressed as D1-D2-D3-Ig was studied by BIAcore (Pharmacia, Uppsala, Sweden) analysis (Jönsson et al., 1991). Amine coupling chemistry (Karlsson et al., 1991) was used to immobilize CD6-Ig on the CM5 sensor chip. Filtered cell culture supernatants were injected, and HEPES-buffered saline was used as the running buffer. The dissociation of bound protein was measured. The data were fit to an exponential function to obtain dissociation (off) rates.

Mapping of ALCAM Residues. The positions of residues whose mutation affected or, alternatively, did not affect CD6 binding were mapped on a three-dimensional model (Brookhaven Protein Data Bank code "1kjc") of ALCAM's N-terminal domain (Bajorath et al., 1995). The computer graphics analysis was carried out using InsightII (MSI, San Diego, CA).

RESULTS AND DISCUSSION

Mutagenesis Strategy. For the initial alanine scanning mutagenesis of the ALCAM binding domain (Skonier et al., 1996), residues outside IgSF consensus positions (Williams & Barclay, 1988) were selected which, on the basis of IgSF topological sequence comparison (Bajorath et al., 1995; Williams & Barclay, 1988), mapped to loop regions or exposed positions on the two β-sheet faces (ABED, A′G-FCC′C″) of the first Ig V-domain. Mutation of six residues was found to partially reduce (87Met, 90Thr, 91Glu) or abolish (26Phe, 40Phe, 43Phe) CD6 binding (Table 1). The residues involved in receptor binding were mapped to the predicted A′GFCC′C″ face of the binding domain, while 25 mutants with wild-type-like binding to CD6 were found in all regions of the domain. Mutation of only one residue (53Tyr) affected both CD6 binding and the binding to anti-D1 mAb, thus indicating a significant structural perturbation. We have extended these studies by replacing residues which were found to be critical for CD6 binding with residues other than alanine and have subjected additional residues in the binding site region to mutagenesis. For mutants with partially reduced CD6 binding, additional binding assays were carried out.

Table 1: Summary of Initial Mutagenesis Results[a]

ALCAM mutant	anti-D1	CD6 ELISA	CD6 BIAcore
26Phe/Ala	+	−	−
40Phe/Ala	+	−	−
43Phe/Ala	+	−	−
53Phe/Ala	−	−	−
87Met/Ala	+	+	+/−
90Thr/Ala	+	+	+/−
91Glu/Ala	+	+/−	+/−

[a] "Anti-D1" reports the binding of ALCAM anti-D1 mAb to mutant protein as comparable to wild-type ALCAM (+) or significantly reduced (−). "CD6 ELISA" reports the binding of D1-D2-D3-Ig mutant proteins to immobilized CD6-Ig by (high avidity) ELISA as comparable to wild type (+), up to 5-fold reduced (+/−), or at least 100-fold reduced (−). "CD6 BIAcore" shows kinetic off-rates of D1-D2-D3-Ig mutant protein binding to CD6 as comparable to wild type (+), ∼10-fold faster (+/−), or undetectable (−). Data are taken from Skonier et al. (1996).

Table 2: Approximate Oligomerization States of ALCAM Ig Fusion Proteins[a]

Ig fusion	∼MW/monomer, kDa	/complex, kDa	# monomers
D1-D2-D3	160	703	4−5
D1-D2	130	314	2−3
D1	90	129	1−2

[a] "MW" indicates the approximate molecular mass of the Ig fusion protein as determined by SDS−PAGE, whereas "/complex" reports the MW obtained by HPLC; "# monomers" gives the calculated number of monomers per complex.

Mutants with Partially Reduced CD6 Binding. For three mutant proteins, 87Met/Ala, 90Thr/Ala, and 91Glu/Ala, reduction in binding was only detectable by BIAcore analysis but not by ELISA (for example, 87Met/Ala means that wild-type residue 87Met was changed to Ala). BIAcore analysis was more sensitive to detect reductions in binding which were measured as faster kinetic off-rates. One explanation for the above observation is that the ELISA assay interaction between immobilized CD6-Ig and ALCAM D1-D2-D3-Ig proteins represents a high avidity assay which does not allow the detection of moderate reductions in binding. To test this possibility, we designed a lower avidity ELISA assay. Size exclusion chromatography revealed that the Ig fusion proteins form different oligomeric states in solution (Table 2). Similar to full-length ALCAM, D1-D2-D3-Ig consists of 4−5 monomers, whereas D1-D2-Ig and D1-Ig consist of 2−3 and 1−2 monomers, respectively. CD6-Ig and CD6D3S-Ig each consist of ∼3 monomers (not shown). Thus, the binding of ALCAM D1-D2-Ig and, in particular, D1-Ig to immobilized CD6-Ig or CD6D3S-Ig should provide a lower avidity interaction than detected in the previously used ELISA. Although D1-Ig binds to CD6 by high avidity ELISA (Bowen et al., 1996), the kinetic off-rate of binding was too fast to measure accurately on the BIAcore (Table 3). On the other hand, the kinetic off-rates for ALCAM D1-D2-Ig and D1-D2-D3-Ig were measured reliably (Table 3). For this reason, we have expressed the 87Met/Ala, 90Thr/Ala, and 91Glu/Ala mutants as ALCAM D1-D2-Ig proteins and tested their binding to CD6D3S-Ig by ELISA. Figure 1 shows a comparison of the binding of these ALCAM mutant proteins to CD6 in the high and low avidity ELISA assays. In the high avidity assay, only the binding of 91Glu/Ala, but not 90Thr/Ala and 87Met/Ala, is partially reduced. In the low avidity ELISA assay, the binding of both 90Thr/Ala and 91Glu/Ala ALCAM mutants to CD6 is significantly reduced, whereas the binding of 87Met/Ala to CD6 remains essentially unaffected. By BIAcore, the binding of all three mutants was reduced with ∼10-fold faster off-rates than wild-type ALCAM.

Table 3: Dissociation Rates of ALCAM Fusion Proteins by BIAcore Analysis[a]

Ig fusion	response units	*k*-off, s^{-1}
D1-D2-D3	136	$\sim 10^{-4}$
D1-D2	250	$\sim 10^{-4}$
D1	64	$(\sim 10^{-2})$

[a] Binding levels to the CD6-coupled chip are given in response units. "*k*-off" gives the dissociation rates. Kinetic off-rates of binding are concentration independent. The off-rate of D1-Ig is too fast to measure accurately.

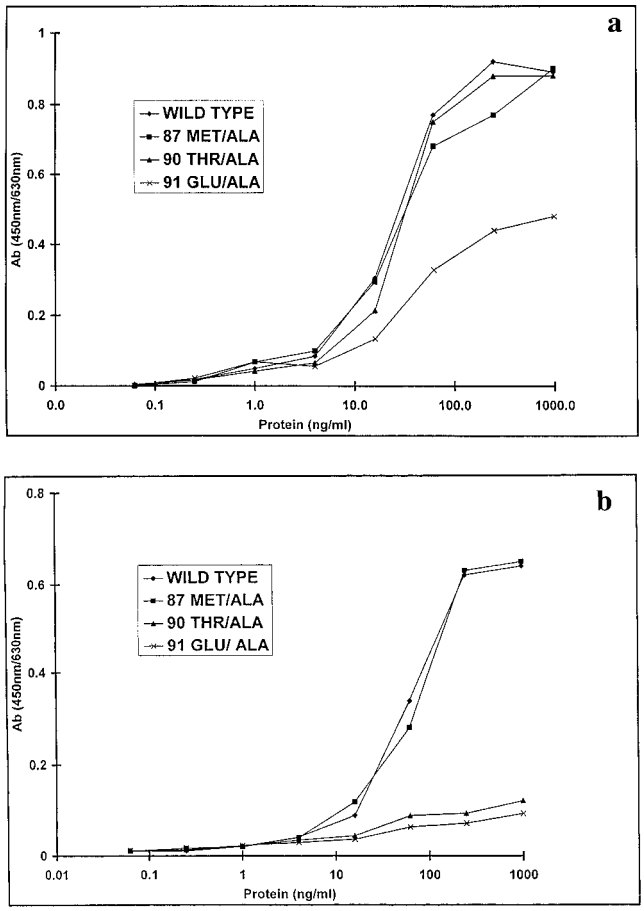

FIGURE 1: Binding of ALCAM mutant proteins to CD6 in two different ELISA assays. Binding experiments are shown for mutant proteins with partially reduced binding to CD6. In (a), ALCAM mutants were expressed as ALCAM D1-D2-D3-Ig, and their binding to immobilized CD6-Ig was tested (higher avidity). In (b), mutants were expressed as ALCAM D1-D2-Ig, and binding to immobilized CD6D3S-Ig was assayed (lower avidity).

Alternative Residue Replacements. The binding of three Phe/Ala mutants at positions 26, 40, and 43 of ALCAM was significantly reduced or undetectable in both ELISA and BIAcore assays (Table 1). These mutant proteins bound like wild type to anti-ALCAM D1 mAb which requires the presence of an overall correctly folded D1 (Bowen et al., 1996). Taken together, the data suggested that the three phenylalanine residues are directly involved in ALCAM−CD6 binding. However, the possibility remained that these three large hydrophobic residues are more important for local conformational integrity than binding, for example, by

Table 4: Reassessment of Mutagenesis Sites[a]

ALCAM mutant	anti-D1	CD6 ELISA	CD6 BIAcore
26Phe/Glu	+	−	−
40Phe/Glu	+	−	−
43Phe/Glu	+	−	−
43Phe/Tyr	+	−	−
53Tyr/Phe	+	+	+

[a] "Anti-D1" reports the binding of ALCAM anti-D1 mAb to mutant protein at wild-type levels (+). "CD6 ELISA" reports the binding of D1-D2-D3-Ig mutant proteins to immobilized CD6-Ig by ELISA as comparable to wild type (+) or at least 100-fold reduced (−). "CD6 BIAcore" shows kinetic off-rates of mutant protein binding to CD6 as comparable to wild type (+) or undetectable (−).

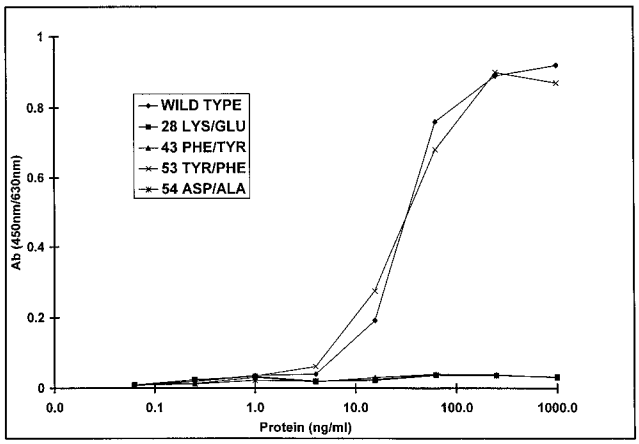

FIGURE 2: Binding of ALCAM mutant proteins by ELISA. Representative binding experiments are shown for mutants with wild-type or abolished binding to CD6. Mutants were expressed as ALCAM D1-D2-D3-Ig, and their binding to immobilized CD6-Ig was tested.

participating in packing interactions. If so, Phe/Ala mutations may only cause relatively small structural perturbations sufficient to compromise CD6 binding but not anti-ALCAM D1 mAb binding. Therefore, more drastic Phe/Glu mutations were prepared at these positions which would be expected to disrupt the domain structure if the phenylalanine residues were involved in packing interactions. Such large-magnitude structural perturbations are easily detected by mAb binding. On the other hand, if these residues are solvent exposed and critical for CD6 binding, the replacement of phenylalanine residues by charged residues should be tolerated structurally but abolish the ALCAM−CD6 interaction. The results of these experiments are summarized in Table 4. Each of the Phe/Glu mutations at positions 26, 40, and 43 bound anti-D1 mAb like wild-type ALCAM, but the binding to CD6 was essentially undetectable in both the high avidity ELISA and BIAcore assays. This supports the conclusion that 26Phe, 40Phe, and 43Phe are CD6 contact residues. The conservative mutation 43Phe/Tyr was also prepared (Table 4). Representative ELISA and BIAcore binding experiments are shown in Figures 2 and 3, respectively. As expected, 43Phe/Tyr bound anti-D1 mAb like wild-type ALCAM. However, even this conservative mutation was sufficient to abolish CD6 binding. This demonstrates that the presence of a phenylalanine at position 43 in ALCAM is critical for CD6 binding.

Only one previously generated ALCAM mutant, 53Tyr/Ala (Table 1), did not bind to either anti-D1 mAb or CD6, which indicated a substantial structural perturbation caused by the amino acid substitution. Therefore, the conservative

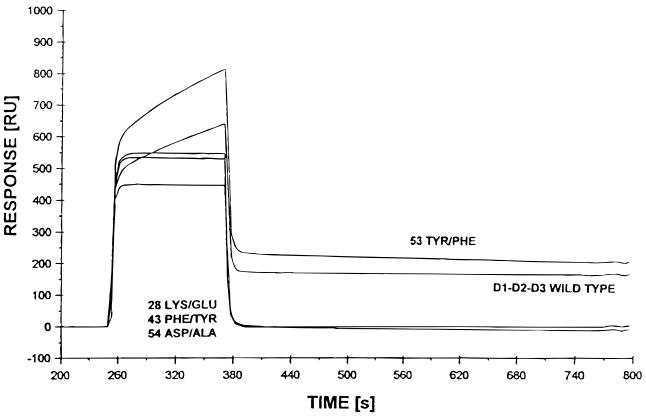

FIGURE 3: Dissociation rates of ALCAM mutant proteins by BIAcore analysis. Binding experiments are shown for the same mutant proteins as in Figure 2. Binding levels are given in response units. Abrupt changes in response at the beginning and end of the injections are due to refractive index differences between the cell culture supernatant and the running buffer. Binding was measured following these changes. Mutant proteins 28Lys/Glu, 43Phe/Tyr, and 54Asp/Ala do not bind CD6 (i.e., the effective response units are close to zero). In contrast, 53Tyr/Phe binds comparably to wild type. The binding levels/dissociation rates for wild type and 53Tyr/Phe are $170/1.3 \times 10^{-4}$ and $226/3.3 \times 10^{-4}$.

Table 5: Other ALCAM Residues Critical for CD6 Binding[a]

ALCAM mutant	anti-D1	CD6 ELISA	CD6 BIAcore
28Lys/Ala		not expressed	
28Lys/Glu	+	−	−
48Lys/Ala	+	−	−
54Asp/Ala	+	−	−
85Val/Ala	+	+	+

[a] "Anti-D1" reports the binding of ALCAM anti-D1 mAb to mutant protein at wild-type levels (+). "CD6 ELISA" reports the binding of D1-D2-D3-Ig mutant proteins to immobilized CD6-Ig by ELISA as comparable to wild type (+) or at least 100-fold reduced (−). "CD6 BIAcore" shows kinetic off-rates of mutant protein binding to CD6 as comparable to wild type (+) or undetectable (−).

mutant 53Tyr/Phe was prepared and tested (Table 4, Figures 2 and 3). In contrast to 53Tyr/Ala, the 53Tyr/Phe mutant protein bound both anti-D1 mAb and CD6 like wild-type ALCAM. These findings are consistent with the presence of a structural defect as a consequence of the 53Tyr/Ala mutation, which does not occur when this residue is conservatively mutated. This suggests that this residue is not directly involved in the ALCAM−CD6 interaction.

Identification of Other Residues Critical for the ALCAM−CD6 Interaction. Considering the location of the six residues important for CD6 binding in the ALCAM IgSF V-domain, four other residues, 28Lys, 48Lys, 54Asp, and 85Val, were subjected to mutagenesis. The results are summarized in Table 5, and representative binding experiments are shown in Figures 2 and 3. The 28Lys/Ala mutation was not expressed in sufficient quantities and was not characterized. In contrast, a charge reversal mutant at this position, 28Lys/Glu, was well expressed. This mutant protein bound anti-ALCAM D1 mAb like wild type but did not bind to CD6. Equivalent results were obtained for the 48Lys/Ala and 54Asp/Ala mutants which identified 28Lys, 48Lys, and 54Asp as critical for the ALCAM−CD6 interaction. In contrast, mutant protein 85Val/Ala bound anti-D1 mAb and CD6 like wild-type ALCAM. Thus, a total of nine residues were confirmed to be important for the ALCAM−CD6 interaction. The results of three different binding assays

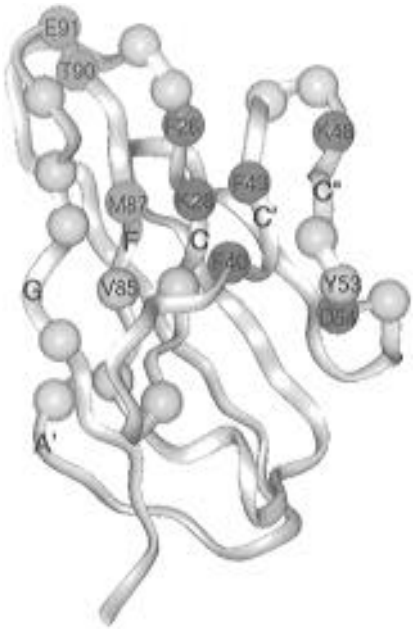

FIGURE 4: Outline of the CD6 binding site in ALCAM. Residues targeted by mutagenesis were mapped on a molecular model of the binding domain. The view is face-on the A′GFCC′C″ β-sheet which contains the CD6 binding site. The CDR analogous region loops are at the top. The α-carbon positions of residues are shown as spheres and color-coded according to their importance for binding (gold, unimportant; blue, important but not essential; magenta, critical). ALCAM residues which, when mutated, do not affect binding (Skonier et al., 1996) are only shown if located on the A′GFCC′C″ face. Residues analyzed in this study are labeled. For clarity, the single-letter code for amino acids was used here (e.g., 85Val in the text is V85 in this figure). The representation was produced using InsightII (MSI, San Diego, CA).

suggest that three ALCAM residues (87Met, 90Thr, 91Glu) support binding but are not essential, whereas six residues (26Phe, 40Phe, 43Phe, 28Lys, 48Lys, 54Asp) are critical for CD6 binding.

It should be noted that a mutagenesis-based classification of residues as more or less important for binding is to some extent dependent on the particular mutations generated. For example, the mutation 85Val/Ala, which does not affect CD6 binding, is relatively subtle and may be tolerated even if 85Val is involved in CD6−ALCAM contacts. The result shows, however, that a contact mediated by 85Val, if it exists, is not critical for the interaction. This is in contrast to, for example, 43Phe where even the most conservative mutation dramatically reduces binding. More drastic mutations of 85Val (e.g., 85Val/Arg) may have compromised the interaction indirectly. In our analysis, we have mainly focused on the identification of ALCAM residues which form important contacts to CD6. Drastic mutations were only generated to assess conclusions drawn from alanine scanning mutagenesis or, alternatively, if mutations to alanine were not expressed.

Mapping of Proposed Binding Residues and Description of the CD6 Binding Site in ALCAM. Residues targeted by mutagenesis were mapped on a molecular model of the ALCAM binding domain (Bajorath et al., 1995) which was generated on the basis of the CD8 X-ray structure (Leahy et al., 1992) (Figure 4). The model-based analysis of residue positions is approximate in nature. However, several conclusions can be drawn. All nine residues important for binding map to exposed positions. The ALCAM−CD6 interaction is centered on a cluster of three hydrophobic (26Phe, 40Phe, 43Phe) and three charged (28Lys, 48Lys, 54Asp) residues, all of which are critical for binding. These spatially adjacent residues map to the C- (26Phe, 28Lys), C′- (40Phe, 43Phe), and the C″- (48Lys, 54Asp) strands and form a coherent binding surface. ALCAM residues which are not important for CD6 binding surround this region. Residues which support CD6 binding are found in the F-strand (87Met) and the F−G loop (90Thr, 91Glu), adjacent to the C-strand. In contrast, no binding residues were identified in the A′- and G-strands. Therefore, the surface formed by the C-, C′-, and C″-strands appears to be the center of the CD6 binding site in ALCAM. The C′−C″ region is variable in many Ig V-like domains and therefore well suited to participate in the formation of IgSF binding sites for structurally distinct ligands. This is exemplified by the analysis of the IgSF−SRCRSF interaction between ALCAM and CD6.

Conclusions. Mutagenesis experiments and receptor−ligand binding assays have identified nine residues in ALCAM which are important for the interaction with CD6. The contributions of these residues differ. Most important are three phenylalanine and three charged ALCAM residues which, when mapped on a three-dimensional model of the N-terminal Ig V-domain, cluster in a region formed by parts of the C-, C′-, and C″-strands. The A′GFCC′C″ face of V-(like) domains of members of the IgSF has been shown to contain the binding site for diverse molecules including IgSF proteins, integrins, carbohydrates, and a member of the SRCRSF.

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