The Amino-terminal Immunoglobulin-like Domain of Activated Leukocyte Cell Adhesion Molecule Binds Specifically to the Membrane-proximal Scavenger Receptor Cysteine-rich Domain of CD6 with a 1:1 Stoichiometry*

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‡The abbreviations used are: IgSF, immunoglobulin supergene family; ALCAM, activated leukocyte cell adhesion molecule; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; Rg, recombinant globulin; SRCR, scavenger receptor cysteine-rich; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography.

Activated leukocyte cell adhesion molecule (ALCAM) was recently identified as a ligand for CD6, a signaling receptor expressed on T cells, a subset of B cells, and some cells in the brain. Receptor-ligand binding assays, antibody blocking experiments, and examination of the tissue distribution of these two cell surface proteins suggest that CD6-ALCAM interactions play an important role in mediating the binding of thymocytes to thymic epithelial cells and of T cells to activated leukocytes. Presently, the details of CD6-ALCAM interactions and of signaling through CD6 are unknown. A series of truncated human ALCAM and CD6 immunoglobulin fusion proteins were produced and tested in different binding assays to analyze ALCAM-CD6 interactions in more detail. In this study, we report that the amino-terminal Ig-like domain of human ALCAM specifically binds to the third membrane-proximal scavenger receptor cysteine-rich (SRCR) domain of human CD6. Using thrombin-cleaved Ig fusion proteins containing single or multiple ALCAM or CD6 domains, we were able to determine that the stoichiometry of the interaction between the amino-terminal ALCAM domains and the membrane-proximal CD6 SRCR domain is 1:1. These results provide the first example of an Ig-like domain mediating an interaction with an SRCR domain.

Ig supergene family (IgSF) members have been shown to interact with a wide variety of other molecules, including integrins, cytokines, and other IgSF members. Many of these interactions are mediated through protein-protein contacts, although a subset of these proteins, known as sialoadhesins, recognize sialic acid (1). Recently, we have reported on a novel interaction between an IgSF member, activated leukocyte cell adhesion molecule (ALCAM), and a member of the scavenger receptor cysteine-rich (SRCR) family of proteins, CD6 (2). Soluble recombinant proteins consisting of the extracellular domains of either ALCAM or CD6 fused to human IgG1 constant domains were shown to specifically bind to COS cell transfectants expressing CD6 or ALCAM, respectively.

ALCAM is a type I membrane protein whose extracellular domain is composed of five Ig-like domains: two amino-terminal V set Ig domains followed by three domains of the C2 set, a hydrophobic transmembrane domain, and a short cytoplasmic anchor sequence (2). ALCAM is also known as SC-1/JDM-GRASP/BEN in the chicken (3–5) and as KG-CAM in the rat (6). The chicken counterpart of ALCAM is a neural adhesion molecule capable of supporting neurite outgrowth (4, 5). Data from the chicken indicate that ALCAM is capable of homophilic interactions (4, 5), and the possibility of such interactions has also been suggested on the basis of molecular modeling (7). We have previously reported that COS cells that expressed CD6 were able to bind to ALCAM positive thymic epithelial cells, which suggested that CD6 and ALCAM binding can mediate adhesive interactions between thymocytes and thymic epithelial cells (2).

CD6, also a type I membrane protein (8), is expressed by thymocytes, T cells, a subset of B cells, and in the brain (9–14). Within the SRCR family (15), CD6 is most closely related to CD5. Both molecules have a similar extracellular domain organization (8), consisting of three SRCR domains, and display a similar pattern of cellular expression. Antibody cross-linking studies have implicated CD6 (and CD5) in T cell activation. For example, anti-CD6 mAb can induce activation of T cells in conjunction with phorbol 12-myristate 13-acetate, anti-CD2, and anti-CD3 (10, 13) and in some cases are mitogenic in the presence of adherent cells (13). Additionally, CD6 becomes hyperphosphorylated on Ser and Thr residues (16–19) and phosphorylated on Tyr residues (19) following T cell activation. Presently it is unknown how CD6 transduces signals or which kinases are responsible for its phosphorylation.

We have recently reported that human ALCAM is able to bind to the membrane-proximal SRCR domain of murine CD6 (20). Because the interaction of ALCAM-CD6 was the first example of an interaction between an IgSF member and an SRCR family member, we wanted to determine if the binding site on ALCAM for CD6 could be localized to single or multiple Ig-like domains. In this report we present the results of experiments that localize the major binding site to a single domain and determine the stoichiometry of ALCAM-CD6 binding. First, a series of ALCAM and CD6 Ig fusion proteins containing different combinations of extracellular domains were tested for their ability to bind to COS cell transfectants expressing CD6 or ALCAM, respectively. Second, fusion proteins were tested by enzyme-linked immunosorbent assay (ELISA) for binding activity to immobilized fusion proteins. In addition, monoclonal antibodies that recognize specific domains of both proteins were used in blocking studies to provide additional evidence for
which domains mediate interactions. Lastly, soluble proteins without Ig tails were used to define the stoichiometry of ALCAM-CD6 interactions. Results from these binding studies demonstrate that the terminal V domain of ALCAM specifically interacts with the membrane-proximal SRCR domain of CD6 with a 1:1 binding stoichiometry. ALCAM Ig-like domains not involved in receptor binding are thought to trigger oligomerization, which may provide for high avidity interactions in this receptor-ligand system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cell Lines, and Monoclonal Antibodies**

Cos cells, anti-CD6 producing hybridomas T12 and 12.1, and the T cell lymphoma cell line HPB-ALL were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, penicillin, and streptomycin. Normal human epidermal keratinocytes were obtained from Clonetics Corp. (San Diego, CA) and grown according to the manufacturer's instructions with the provided medium. CHO-CD6 cells were stably transfected with a vector encoding CD6–15 (8) and were used for cell binding and immunochemical analyses. UMC6D was from Ancell (Bayport, MN); and MBG6, F10–20-5-11, 12.15, and 24G4 were obtained from the Third Human Leucocyte Differentiation Antigens Workshop.

**Production of ALCAM and CD6 Fusion Proteins**

Complementary DNA fragments encoding individual or groups of domains of ALCAM and CD6 were produced by polymerase chain reaction methodology with oligonucleotides containing the appropriate restriction sites to mediate fusion with the thrombin-human IgG1 cassette (Rg fusion proteins) or the thrombin-mouse IgG2a cassette as already described (21, 22). The ALCAM fusion proteins contained the following amino acid sequences: CD6 Rg, Met1–Leu334; ALCAM VV Rg, Met1–Glu247; ALCAM V Rg, Met1–Leu144. ALCAM V2CCC Rg contained residues Pro346–Ala526, and the amino-terminal secretory sequence was derived from human CDS cDNA. The following CD6 fusion proteins, which also used the CD5 amino-terminal secretory sequence, contained the following amino acids according to the published sequence (8): CD6 Rg/mIgG2a, Asp25–Arg297; CD6D1–2 Rg, Asp25–Ala271; CD6D2–S Rg, Glu158–Arg297; CD6D2 Rg, Glu158–Ala271; CD6D3–S Rg, Ser260–Arg297; CD6D1–3 Rg was constructed as described previously (23) and contained Met1–Ser260. The production of CDS Rg was as described (21).

All proteins were produced by transient transfection of COS cells and purified by protein A-Sepharose chromatography. Protein concentrations were determined using a Bradford dye binding procedure (BioRad) against a mouse IgG protein standard. To analyze the fusion proteins, transiently transfected COS cells were pulsed with 35S trans-label (Amersham Corp.), and purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

**Screening of Hybridoma Supernatants and Purification of mAb**

Hybridoma supernatants were screened using an antigen capture-based ELISA that employed a horseradish peroxidase-conjugated goat anti-mouse IgG second step reagent (Zymed Laboratories, South San Francisco, CA) for the detection of bound mAb. Briefly, 50 ng of fusion protein diluted in 50 mM sodium bicarbonate, pH 9.6, was adsorbed overnight at room temperature to wells of Immulon 1 microtiter plates (Dynatech Laboratories). The wells were washed three times with phosphate-buffered saline (PBS) and then various concentrations of the truncated ALCAM Rg fusion proteins were added to the wells. Binding was detected with horse radish peroxidase-conjugated donkey anti-human IgG as above.

**ALCAM Rg and CD6 Rg Binding to COS Transfectants**

COS cells transiently transfected with ALCAM (2) or CD6 (8) cDNA were stained by indirect immunofluorescence and analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA). Three days after transfection, COS cells were detached with phosphatebuffered saline/10 mM EDTA and washed in staining solution (Dulbecco's modified Eagle's medium/1% bovine serum albumin/0.1% sodium azide). ALCAM expressing cells (5 × 105) were treated for 1 h on ice with 25 µg/ml of the CD6 Rg fusion proteins (except for CD6D1–3 Rg, where 50 µg/ml was used) and washed twice with staining solution. Binding was detected with fluorescein isothiocyanate-conjugated goat anti-mIgG (BioSource International, Camarillo, CA). CD6 expressing COS cells were treated with 20 µg/ml of the ALCAM fusion proteins for 1 h, and binding was detected as above.

**Production of Anti-human ALCAM Monoclonal Antibodies**

A 6–8 week old female BALB/c mouse (Tacoma, Germantown, NY) was immunized with 50 µg of purified ALCAM Rg administered intraperitoneally as an emulsion in Ribi adjuvant (R-730, Ribi ImmunoChem Research, Inc., Hamilton, MT). Two identical immunizations were performed on day 19 and 29. On day 63, 50 µg of protein in phosphate-buffered saline was administered intravenously. Three days later, spleen and lymph nodes cells were fused with X63-Ag8.653 myeloma cells (24) according to a published protocol (25). The cell suspension was seeded into 96-well culture plates in the presence of hybridoma growth medium (Isco's modified Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% D-glucose, 10% D-glutamine, 10% Ionomycin (Sigma), 10% D-mannose, 10% D-thymidine).

**Results**

**ALCAM Ig-like Domains**

The truncated ALCAM fusion protein binding to CD6—immunol 1 plates were coated overnight with 60 ng/well of CD6 mIgG diluted in 50 mM sodium bicarbonate as above. Plates were blocked with nitrogen diluted and then various concentrations of the truncated ALCAM Rg fusion proteins were added to the wells. Binding was detected with horse radish peroxidase-conjugated donkey anti-human IgG as above.

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Those supernatants that reacted with ALCAM Rg but not a similarly constructed irrelevant Rg fusion protein were further evaluated by indirect immunofluorescence for their ability to bind to the ALCAM expressing T cell lymphoma cell line HPB-ALL. Cells were analyzed by flow cytometry on a FACScan. Those hybridomas that produced mAb that recognized HPB-ALL cells were then cloned by limiting dilution.

For the production of mAb containing supernatants, hybridomas were grown in medium containing Ultra low IgG fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD). mAb were purified by affinity chromatography with GammaBind Plus Sepharose (Pharmacia Biotech Inc.).

**Determination of the Domain Specificity of Anti-CD6 and Anti-ALCAM mAb**

Immumol 1 plates were coated with CD6 Rg (60 ng/well), ALCAM Rg, or truncation mutant fusion proteins overnight as explained above. The plates were blocked as above, and then either cell supernatants or purified mAb specific for CD6 or ALCAM were allowed to bind to the immobilized fusion proteins. Binding of the mAb was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (BioSource International) and substrate as detailed above.
Gel Filtration Analysis of Soluble Proteins and Protein Sequencing

Molecular weights of purified proteins and complexes were analyzed by gel filtration chromatography on a Waters Co. 7.8 x 300-mm 300SW high performance liquid chromatography column. Samples were chromatographed at a flow rate of 0.35 ml/min in 10 mM Tris-Cl, pH 7.5, 10 mM potassium phosphate, 0.15 M NaCl. Protein standards used to determine the relative molecular masses of soluble receptors and their complexes were as follows: blue dextran, 2 x 10^6 Da; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa; and RNase, 13.7 kDa.

Protein complexes were analyzed by incubating the purified proteins together for 30 min at room temperature prior to analysis. Approximately 3 μg of each protein was used for the analysis in a final volume of 25 μl. The samples were chromatographed using phosphate-buffered saline buffer with 10 mM Tris-Cl, pH 7.5, and a flow rate of 0.35 ml/min. A fraction of the material from the leading edge was collected and immobilized to a polyvinylidene difluoride membrane for amino-terminal sequencing. Automated sequence analysis was performed in a pulsed liquid protein sequencer as described previously (26).

RESULTS

Mapping of the CD6 Binding Site for ALCAM—To expand upon our results obtained in the murine system (20), we constructed a series of soluble human CD6 domain truncation proteins fused to the constant domains of human IgG1 (Fig. 1, A and B). The extracellular portion of CD6 can be divided into four domains, three SRCR domains (D1–3) and the short membrane-proximal stalk (S) domain fused to the hinge, CH1, and CH2 domains of human IgG1. Truncated fusion proteins contain combinations of the four domains. B, fusion proteins were expressed by transient transfection of COS cells with the chimeric constructs cloned into the pCDM8 expression vector. Cells were pulsed with [35S]Cys/Met translabel overnight, the fusion proteins were adsorbed to protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. C, line drawings representing the domain organization of ALCAM and of the ALCAM fusion proteins used in this study. ALCAM Rg is composed of the entire extracellular region of the protein (V-like domains V1–2, and C-like domains C1–3) fused to human IgG1 domains as above for CD6. Truncated ALCAM fusion proteins contained combinations of the five Ig-like domains. D, ALCAM fusion proteins were labeled with [35S]Cys/Met and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described above for CD6.

Fig. 1. CD6 and ALCAM fusion proteins. A, schematic representation of the domains of CD6 encoded by the chimeric immunoglobulin (Rg) fusion protein genes used in this study. The CD6 Rg fusion protein is composed of the three SRCR domains (D1–3) and the short membrane-proximal stalk (S) domain fused to the hinge, CH1, and CH2 domains of human IgG1. Truncated fusion proteins contain combinations of the four domains. B, fusion proteins were expressed by transient transfection of COS cells with the chimeric constructs cloned into the pCDM8 expression vector. Cells were pulsed with [35S]Cys/Met translabel overnight, the fusion proteins were adsorbed to protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. C, line drawings representing the domain organization of ALCAM and of the ALCAM fusion proteins used in this study. ALCAM Rg is composed of the entire extracellular region of the protein (V-like domains V1–2, and C-like domains C1–3) fused to human IgG1 domains as above for CD6. Truncated ALCAM fusion proteins contained combinations of the five Ig-like domains. D, ALCAM fusion proteins were labeled with [35S]Cys/Met and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described above for CD6.
Third, we tested a panel of anti-CD6 monoclonal antibodies (MBG6, F10–20–5–11, 12.15, 24G4, 12.1, UMCD6, IOT12, G3–6, and T12) for their ability to block the binding of ALCAM Rg to CHO cell lines that had been transfected with CD6 cDNA (CHO-CD6). Preliminary analysis of the domain specificity of the antibodies determined by their ability to bind to the truncated CD6 fusion proteins in an ELISA showed that all the antibodies only recognized fusion proteins that contained the amino-terminal SRCR domain (D1) of CD6 (data not shown).

The antibodies were unable to block the binding of ALCAM Rg to CHO-CD6 cell lines (data not shown), which supports our conclusion that CD6 D1 is not involved in binding to ALCAM. Additional studies in the mouse showed that anti-mCD6 D1 mAb were not effective blockers of CD6-ALCAM interactions, whereas most anti-D3 mAb were strong blockers (20).

Mapping of the ALCAM Binding Site for CD6—To determine which domain(s) of ALCAM participates in the binding to CD6, a series of soluble ALCAM domain truncation proteins were constructed (Fig. 1C). The ALCAM extracellular region, which consists of five Ig-like domains, was truncated by eliminating carboxyl-terminal (membrane-proximal) domains singly or in combination. For example, ALCAM VVCC Rg does not include the carboxyl-terminal C-like domain, whereas ALCAM VVC is missing the two membrane-proximal C-like domains. ALCAM V Rg consists of the two V-like domains, and ALCAM V Vg

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Fig. 2. Binding of CD6 fusion proteins to ALCAM expressing COS cells and to ALCAM. A, COS cells were transiently transfected with full-length ALCAM cDNA in pCDM8, stained by indirect immunofluorescence with the purified CD6 fusion proteins as shown and a fluorescein isothiocyanate labeled second antibody, and analyzed by flow cytometry. Mock transfected COS cells stained with the fusion proteins are shaded, and ALCAM transfected cells are represented by open profiles. B, binding of varying concentrations of CD6 fusion proteins to ALCAM mIgG immobilized to the wells of a 96-well microtiter plate. Each point on the graph is the mean of duplicate samples and is representative of data obtained from several assays.

Fig. 3. Binding of ALCAM fusion proteins to CD6 expressing COS cells and to immobilized CD6. A, COS cells were transiently transfected with CD6 cDNA, stained by indirect immunofluorescence with the purified ALCAM fusion proteins and a fluorescein isothiocyanate labeled secondary antibody, and analyzed by flow cytometry. Mock transfected COS cells stained with the ALCAM fusion proteins are shaded, and CD6 transfected cells are represented by open profiles. B, binding of varying concentrations of ALCAM fusion proteins to CD6 mIgG immobilized to the wells of a 96-well microtiter plate. Each point on the graph is the mean of duplicate samples and is representative of data obtained from several assays.

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contains only the amino-terminal domain of ALCAM. All proteins were produced in COS cells (Fig. 1D).

When these fusion proteins were used to stain COS cell transfectants expressing CD6, proteins that contained the amino-terminal V-like domain were able to bind to the cells equally well (Fig. 3A). These results demonstrate that the amino-terminal domain of ALCAM is sufficient to confer specific binding to CD6.

In order to confirm the cell binding results and determine the relative binding strengths of the individual fusion proteins, an antigen capture ELISA was used to measure ALCAM-CD6 interactions. An additional fusion protein that lacked the most amino-terminal V domain (ALCAM V2CC, Fig. 1) but contained the remaining four domains was constructed and included in these studies to determine if other domains were involved in binding to CD6. In the ELISA, ALCAM Rg, ALCAM VVCC Rg, and ALCAM VVC Rg bound to immobilized CD6 equally well (Fig. 3B). However, proteins containing only the first or the first and second amino-terminal domains bound less well to CD6, with ALCAM VV Rg binding slightly better than ALCAM V Rg. Finally, the binding of ALCAM V2CC Rg to CD6 was detectable but very weak. The binding of this fusion protein to CD6 expressing cells, however, was not detectable (data not shown). These results support the cell binding data and show that the amino-terminal V domain of ALCAM mediates binding to CD6.

Production of Domain-specific Anti-ALCAM Monoclonal Antibodies—ALCAM fusion protein was used as an immunogen to produce mouse mAb specific for human ALCAM. We were able to map the domain specificity of the mAb by using the truncated fusion proteins in an ELISA (Table I). Although we were able to produce antibodies against each of the five Ig-like domains, the majority of antibodies, including the previously characterized J4–81 mAb (27, 28), appeared to recognize the second amino-terminal V-like domain (V2) of ALCAM. We have recently cloned the murine ALCAM cDNA,² and based on the comparison of the predicted amino acid sequence of human and murine ALCAM, a high degree of identity exists between the proteins. However, V2 of human ALCAM is the least conserved domain and would be expected to be more immunogenic in mice than the other domains. This might explain why the frequency of mouse mAb specific for V2 was higher than for the other domains when we produced hybridomas. Preliminary data indicate that HAL151, which recognizes the amino-terminal domain (V1) as well as some anti-V2 antibodies, are able to only partially block the binding of CD6 Rg to keratinocytes, which express high levels of cell surface ALCAM (data not shown).

These findings are consistent with our studies using Rg fusion proteins and further support the hypothesis that the amino-terminal domain of ALCAM contains the major binding site for CD6, although there may be an additional contribution from the second V-like domain (V2).

**DISCUSSION**

By constructing and expressing immunoglobulin fusion proteins containing single or multiple domains of ALCAM and CD6, we were able to localize the domains critical for the interaction between these proteins. As shown in this study, human ALCAM binds to the third, membrane-proximal SRCR domain of human CD6. Bott et al. (29) have characterized a group of anti-CD6 mAb and by cross-blocking and other criteria have determined that at least four epitopes are recognized by the reagents used in that study. Based on our results on mAb binding to the different truncated CD6 fusion proteins, we were able to determine that each of the four epitopes recognized by those antibodies as well as the additional mAb used in our study are located in the amino-terminal CD6 SRCR domain.

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² M. A. Bowen, unpublished observations.
main. However, V2 may contribute to CD6 binding. In this was not detectable. These results show that the major binding
CD6 in an ELISA (Fig. 3). The terminal domain (ALCAM V2CCC Rg) bound very weakly to
with higher avidity to CD6. A fusion protein without the amino-
resulted in proteins that bound with higher avidity to CD6. A fusion protein without the amino-
terminal domain (ALCAM V2CCC Rg) bound very weakly to
CD6 or VV and CD6D3-S were amino-terminal sequenced.
Assessment of the binding interactions of other members of the IgSF that contain multiple domains and their ligands has shown that, like ALCAM, the amino-terminal Ig domain frequently contains the major binding site for counter-receptor. For example, the amino-terminal Ig domain of CD2 binds to its counter-receptor CD58 (30). Likewise, the amino-terminal domains of B7-1 and B7-2, with some contribution from the second domain, bind to CD28/CTLA4 (31–33).

Ben-Dor/DM-GRASP/SC-1, ALCAM’s chicken homologue, is capable of homophilic interactions (3–5). Size exclusion HPLC analysis experiments suggest that full-length ALCAM and CD6 form multimers in solution. 2 Thus, ALCAM and CD6 may be expected to form oligomers on the cell surface. This should provide for high avidity interactions on opposing cells and may explain why ALCAM-CD6 interactions can mediate cell adhesion (2). The proposed presence of pre-existing receptor and ligand oligomers on the surface of cells has implications for CD6 signaling. If CD6 multimerizes prior to interaction with ALCAM, receptor cross-linking such as required for cytokine receptors (34) cannot be sufficient for signal transduction. Rather, clustering of multiple receptors may be required, as proposed for CD28/CTLA4 (35). On T cells, clustering or cross-linking CD6 using mAb is not sufficient to cause Ca 2+ fluxing (29, 36), phosphorylation of CD6 on Tyr residues (19), or T cell proliferation. These effects require additional stimuli such as CD2 or CD3 ligation, phorbol 12-myristate 13-acetate, or the presence of accessory cells (10, 13, 29).

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REFERENCES

1. Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., de Bellard, M. E., Schmitt, M., Mahoney, J. A., Hartnell, A., Bradfield, P., and Crocker, P. R. (1994) Curr. Biol. 4, 965–972
2. Bowen, M. A., Patil, D. D., Li, X., Modrill, B., Malacko, A. R., Wang, W.-C., Margarid, H., Neubauer, M., Pesando, J. M., Frankan, U., Haynes, B. F., and Aruffo, A. (1995) J. Exp. Med. 181, 2213–2220
3. Pourquie, O., Corbel, C., Le Caer, J.-P., Rossier, J., and Le Douarin, N. M. (1992) Proc. Natl. Acad. Sci. U. S. A 89, 5251–5256
4. Tanaka, H., Matsu, T., Akihata, T., Tomura, M., Kubota, I., McFarland, K. C., Koir, B., Lee, A., Phillips, H. S., and Shelton, D. L. (1991) Neuron 7, 535–545
5. Burns, F. R., von Kannen, S., Guy, L., Raper, J. A., Kamholz, J., and Chang, S. (1991) Neuron 7, 209–220
6. Peduzzi, J. D., Irwin, M. H., and Geisert, E. E., J. (1994) Brain Res. 640, 296–307
7. Bajorath, J., Bowen, M. A., and Aruffo, A. (1995) Protein Sci. 4, 1644–1647
8. Aruffo, A., Melnick, M. B., Linsley, P. S., and Seed, B. (1991) J. Exp. Med. 174, 949–952
9. Reinerherz, E. L., Meuer, S., Fitzgerald, K. A., Hussey, R. E., Levine, H., and Schlossman, S. F. (1982) Cell 30, 735–743
10. Morimoto, C., Ruddle, C. E., Leukin, N. L., Hagan, M., and Schlossman, S. F. (1988) J. Immunol. 141, 2165–2170
11. Kamoun, M., Kadin, M. E., Martin, P. J., Nettleton, J., and Hansen, J. A. (1988) J. Immunol. 127, 987–991
12. Bastin, J. M., Granger, S., Tidman, N. J., Anosy, G., and McMichael, A. J. S. (1988) Clin. Exp. Immunol. 69, 597–606
13. Gangemi, R. M., Swack, J., A., Savioli, D. M., and Romain, P. L. (1989) J. Immunol. 143, 2439–2447
14. Mayer, B., Funke, J., Seed, B., Riethmuller, G., and Weiss, E. (1990) J. Immunol. 139, 193–202
15. Resnick, D., Pearson, A., and Krieger, M. (1994) Trends Biochem. Sci. 9, 5–8
16. Swack, J. A., Gangemi, R. M., Ruddle, C. E., Morimoto, C., Schlossman, S. F., and Romain, P. L. (1989) J. Immunol. 142, 1037–1049
17. Cardenas, L., Carrera, A. Yague, E., Pulido, R., Sanchez-Madrid, F., and de Landazuri, M. O. (1990) J. Immunol. 145, 1450–1455
18. Swack, J. A., Rudder, C. E., and Romain, P. L. (1990) J. Biol. Chem. 265, 7137–7143
19. Wee, S. F., Schieken, G. L., Kirihara, J. M., Tsu, T. T., Ledbetter, J. A., and Aruffo, A. (1993) J. Exp. Med. 177, 219–223
20. Whitmore, G. C., Starling, G. C., Bowen, M. A., Modrill, B., Siadak, A. W., and Aruffo, A. (1995) J. Exp. Med. 177, 18187–18190
21. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990) Cell 51, 1303–1313
22. Kiener, P. A., Moran-Davis, P., Rankin, B. M., Wahl, A. F., Aruffo, A., and Hildenbaugh, D. (1995) J. Immunol. 155, 4917–4925

Fig. 4. Analysis of the complexes formed between ALCAM V or ALCAM VV and CD6D3-S. ALCAM V, ALCAM VV, and CD6D3-S were produced by fusing the respective fusion proteins with thrombin to liberate the Ig portions of the soluble receptors. The oligomeric state of both the individual proteins and ALCAM-CD6 complexes were analyzed by HPLC. A, ALCAM V and CD6D3-S (3 μg each) were mixed, allowed to interact, and then analyzed by HPLC size exclusion chromatography (solid line) to determine the binding stoichiometry of the soluble receptors. The molar ratio of ALCAM to CD6 in the complex was determined by amino-terminal sequencing from a fraction of material collected at 19–20 min. Superimposed on the graph are profiles of ALCAM V (dotted line) and CD6D3-S (dashed line) that were analyzed separately. B, ALCAM VV and CD6D3-S (3 μg each) were mixed together and analyzed as above to determine the binding stoichiometry of the soluble receptors. The fraction of material analyzed was collected at 19–21 min. The profile of the intersecting receptors is depicted with a solid line; profiles of ALCAM VV (dotted line) and CD6D3-S (dashed line) alone are superimposed for comparison. The retention times (x axis, min) of the molecular weight markers were as follows: blue dextran, 12.7 min; ferritin, 16.6 min; catalase, 20.1 min; aldolase, 20.2 min; bovine serum albumin, 21.9 min; ovalbumin, 23.8 min; chromotrope 11.7 min; and RNase, 30.4 min.

(D1). We determined that the anti-D1 mAb characterized here do not block the binding of ALCAM Rg to CHO-CD6 cells (data not shown), supporting our conclusion that D1 does not mediate binding to ALCAM. Additional studies in the mouse have shown that anti-D3 and not anti-D1 mAbs were effective blockers of murine CD6-human ALCAM interactions (20). We have previously reported that T12, an IgM mAb specific for CD6, can block the binding of CD6 expressing cells to thymic epithelial cells (2). Because T12 (IgM) recognizes CD6 D1, we conclude that either it is able to block cell-cell adhesion by steric hindrance due to its relatively large size or that thymic epithelial cells express additional ligands for CD6 that bind to D1.

CD6, in contrast, binds to the amino-terminal immunoglobulin V-like domain of ALCAM (Fig. 3, A and B). Fusion proteins containing this first domain of ALCAM were able to bind to CD6. The inclusion of the other ALCAM Ig-like domains to the first amino-terminal V domain resulted in proteins that bound with higher avidity to CD6. A fusion protein without the amino-terminal domain (ALCAM V2CCC Rg) bound very weakly to CD6 in an ELISA (Fig. 3B), but binding to CD6 expressing cells was not detectable. These results show that the major binding activity of ALCAM for CD6 resides in the amino-terminal domain. However, V2 may contribute to CD6 binding. In this study we were able to determine that the stoichiometry of ALCAM-CD6 binding was 1:1 when complexes of ALCAM V or VV and CD6D3-S were amino-terminal sequenced.
Domain Interactions of ALCAM-CD6

23. Wee, S. F., Wang, W.-C., Farr, A. G., Nelson, A. J., Patel, D. D., Haynes, B. F., Linsley, P. S., and Aruffo, A. (1994) Cell Immunol. 158, 353–364
24. Kearney, J. F., Radbruch, A., Liesegang, B., and Rajewsky, K. (1979) J. Immunol. 123, 1549–1550
25. Lane, R. D. (1985) J. Immunol. 81, 223–228
26. Maresh, G. A., Wang, W. C., Beam, K. S., Malacko, A. R., Hellström, I., Hellström, K. E., and Marquardt, H. (1994) Arch. Biochem. Biophys. 311, 95–102
27. Patel, D. D., Wee, S.-F., Whichard, L. P., Bowen, M. A., Pesando, J. M., Aruffo, A., and Haynes, B. F. (1995) J. Exp. Med. 181, 1563–1568
28. Pesando, J. M., Hoffman, P., and Abed, M. (1996) J. Immunol. 157, 3689–3695
29. Bott, C. M., Doshi, J. B., Morimoto, C., Romain, P. L., and Fox, D. A. (1993) Int. Immunol. 7, 783–792
30. Arulanandam, A. R. N., Wijkstra, J., Wyss, D., Wagner, G., Kister, A., Pallai, P., Recny, M., and Reinherz, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11613–11617
31. Peach, R. J., Bajorath, J., Naemura, J., Leytze, G., Green, J., Aruffo, A., and Linsley, P. S. (1995) J. Biol. Chem. 270, 21181–21187
32. Guo, Y., Wu, Y., Zhao, M., Kong, X. P., and Liu, Y. (1995) J. Exp. Med. 181, 1345–1355
33. Fargeas, C. A., Truneh, A., Reddy, M., Hurle, M., Sweet, R., and Sekaly, R. P. (1995) J. Exp. Med. 182, 667–675
34. Cunningham, B., Ultsch, M., De Vos, A., Mulkerrin, M., Clauser, K., and Wels, J. (1991) Science 254, 821–825
35. Linsley, P. S., Ledbetter, J. A., Peach, R. J., and Bajorath, J. (1995) Res. Immunol. 146, 130–140
36. Ledbetter, J. A., Norris, N. A., Grossmann, A., Grosmaire, L. S., J une, C. H., Udkun, F. M., Cosand, W. L., and Rabinovitch, P. S. (1989) Mol. Immunol. 26, 137–145