Cloning, Mapping, and Characterization of Activated Leukocyte-Cell Adhesion Molecule (ALCAM), a CD6 Ligand

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Summary

Antibody-blocking studies have demonstrated the role of CD6 in thymocyte–thymic epithelial (TE) cell adhesion. Here we report that CD6 expressed by COS cells mediates adhesion to TE cells and that this interaction is specifically blocked with an anti-CD6 monoclonal antibody (mAb) or with a mAb (J4-81) that recognized a TE cell antigen. We isolated and expressed a cDNA clone encoding this antigen and show that COS cells transfected with this cDNA bind a CD6 immunoglobulin fusion protein (CD6-Rg). This antigen, which we named ALCAM (activated leukocyte–cell adhesion molecule) because of its expression on activated leukocytes, appears to be the human homologue of the chicken neural adhesion molecule BEN/SC-1/DM-GRASP. The gene was mapped to human chromosome 3q13.1-q13.2 by fluorescence in situ hybridization of cDNA probes to metaphase chromosomes. We prepared an ALCAM-Rg fusion protein and showed that it binds to COS cell transfectants expressing CD6, demonstrating that ALCAM is a CD6 ligand. The observations that ALCAM is also expressed by activated leukocytes and that both ALCAM and CD6 are expressed in the brain suggest that ALCAM–CD6 interactions may play a role in the binding of T and B cells to activated leukocytes, as well as in interactions between cells of the nervous system.

CD6 is a type I membrane protein (1) expressed by thymocytes, mature T cells, a subset of B cells known as B-1 cells, and by some cells in the brain (2–4). CD6 is a member of the scavenger receptor cysteine-rich (SRCR)[1] family of proteins (5), which includes the type I macrophage scavenger receptor (6) and the leukocyte antigens CD5 (7, 8), M130 (9), and WC1 (10), among others. The extracellular domain of CD6 is composed of three SRCR domains and has the same domain organization as CD5.

Antibody cross-linking studies have shown that CD6 can function as an accessory protein in T cell activation (3, 11–15). After T cell activation, CD6 becomes hyperphosphorylated on Ser and Thr residues (12, 16–18) and phosphorylated on Tyr residues (17). Anti-CD6 mAbs have been used in a clin-

ical setting to prevent renal and bone marrow transplant rejection (19, 20). Taken together, these data suggest that the interaction of CD6 with its physiological ligand plays a role in regulating the function of T cells in vivo.

We had previously reported the use of a CD6 immunoglobulin fusion protein (CD6-Rg) to identify a number of cell lines that express a CD6 ligand (21, 22). We found that both lymphoid and nonlymphoid cell lines, including human and murine thymic epithelial (TE) cells, expressed a putative CD6 ligand. This observation was consistent with the finding that an anti-CD6 mAb was able to partially block thymocyte–TE cell binding (23). In an effort to identify the CD6 ligand, antibodies from the 5th International Workshop on Human Leukocyte Differentiation Antigens (November 1993, Boston, MA) (24) known to bind to TE cells were screened for their ability to block the binding of CD6-Rg to TE cells (22). One antibody, J4-81 (25), was found to inhibit this interaction. Immunoprecipitation studies with CD6-Rg and J4-81 showed that these two proteins recognized a glycopro-

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[1] Abbreviations used in this paper: ALCAM, activated leukocyte–cell adhesion molecule; CD6-Rg, CD6 immunoglobulin fusion protein; FISH, fluorescence in situ hybridization; RAGE, receptor for advanced glycation end products; SRCR, scavenger receptor cysteine-rich; TE, thymic epithelial.

tein of ~100 kD. Partial tryptic digestion of this protein resulted in a similar set of peptide fragments, providing further evidence that J4-81 recognized a CD6 ligand (22).

In this report, we describe the use of CD6-expressing COS cells and antibody-blocking studies to show that CD6 can mediate adhesion to TE cells and that this interaction can be blocked with either the anti-CD6 mAb T12 or the J4-81 mAb. In addition, we report on the cloning and characterization of a cDNA that encodes the antigen recognized by J4-81 and the chromosomal localization of this gene. We found that this protein was also expressed by activated T cells, B cells, and monocytes, and so named it ALCAM, for activated leukocyte-cell adhesion molecule. Lastly, using soluble recombinant proteins and COS cell transfectants, we demonstrate that the antigen recognized by J4-81 is a CD6 ligand.

Materials and Methods

Cell Lines, Tissue Culture, Antibodies. TE cells were cultured by an explant technique as described (26, 27). COS cells, breast carcinoma HBL-100, B cell lymphoma cell lines Ramos, Raji, and Daudi, T cell lymphoma cell lines CEM and MOLT4, erythroleukemia cell line K562, and monocyte-like cell lines HL60 and U937 were obtained from American Type Culture Collection (Rockville, MD) and maintained under standard tissue culture conditions in Iscove's modified Dulbecco's medium plus 10% fetal bovine serum. The NK-like cell line YT was obtained from E. Podack (University of Miami School of Medicine, Miami, FL). Antibodies used in this study were J4-81 (25) as ascites, Leu4 (anti-CD3)-PE (Becton Dickinson & Co., Mountain View, CA), G3-6 (anti-CD6; J. Ledbetter, Bristol-Myers Squibb, Seattle, WA), anti-L6 (Bristol-Myers Squibb), T12 (anti-CD6; American Type Culture Collection), and P3 (American Type Culture Collection).

Binding of TE Cells to COS Cell Transfectants Expressing CD6 (COS-CD6). COS-CD6 and COS-neo cells were prepared by cotransfecting a plasmid encoding CD6 (1) and the pSVneo plasmid (28). After G418 selection, CD6⁺ cells were cloned using a FACStar^Plus® (Becton Dickinson & Co.) fluorescence-activated cell sorter. COS-neo, COS-CD6, and TE cells were used in a suspension binding assay. To differentiate between COS cells and TE cells, TE cells were labeled with 1 μM calcein AM (Molecular Probes Inc., Eugene, OR) for 15 min at 37°C in PBS before harvest. Calcein AM–labeled cells are fluorescent and can be differentiated from unlabeled cells by fluorescence microscopy. Adhesion was quantitated by scoring the percentage of COS-CD6 cells that were conjugated with TE cells. For blocking adhesion, antibodies T12, J4-81, and P3 were used at or in excess of saturating binding titers.

Cloning and Characterization of the Gene for the Antigen Recognized by mAb J4-81. J4-81 immunoaffinity purification of ALCAM from HBL-100 cells (~3 × 10⁹) was performed as described (29). The purified protein and CNBr fragments were analyzed in a pulsed-liquid protein sequencer by previously described methods (30). NH₂-terminal sequences were screened against the SwissProt data base using Genetics Computer Group (Madison, WI) sequence analysis software. Chicken BEN probes were synthesized by PCR from a chicken embryo cDNA library (Clontech, Palo Alto, CA), random prime labeled with ³²P-dCTP, and used to screen a PHA-activated human T cell cDNA library. A partial cDNA clone was isolated; 200 bp of the 5' end of this cDNA was used to isolate a clone containing the entire coding region from a HL60 cDNA library. Data base searches with the cDNA-derived peptide sequence of ALCAM were performed using the BLAST program; alignments

and consensus sequence derivation of the four highest scoring hits were performed using the Pileup and Pretty programs from the Genetics Computer Group sequence analysis software.

Northern Blot Analysis. PBMC were isolated from whole blood with lymphocyte separation medium (Organon Teknika, Rockville, MD) according to the manufacturer's instructions. PBMC were stimulated with 1 μg/ml PHA (Boehringer Mannheim Corp., Indianapolis, IN). Total cellular RNA was prepared with TRIzol reagent (GIBCO BRL, Gaithersburg, MD). Monocytes were isolated from PBMC by adherence to plastic for 2.5 h. The RNAs were fractionated on a denaturing formaldehyde gel; 25 μg of RNA was loaded for all samples except for monocytes, where 15 μg was loaded. RNA was transferred to nitrocellulose TC (Schleicher & Schuell, Inc., Keene, NH) and probed with a random-primed, ³²P-dCTP-labeled ALCAM or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. Filters were exposed to film at −70°C and developed.

PHA Blast Preparation, Staining. PBMC were isolated and activated with PHA as described above and stained on selected days with mAb J4-81 (1:200) or CD6-Rg (50 μg/ml) followed by a FITC-labeled second antibody and anti-CD3-PE; cytofluorometric analysis of J4-81 staining T cells was performed on a FACScan® (Becton Dickinson & Co.) by gating on CD3⁺ cells.

Chromosomal Mapping of the Gene Encoding ALCAM. Two overlapping cDNA clones of the human ALCAM gene containing 2.2- and 1.9-kb inserts in CDM8 were used as probes for fluorescence in situ hybridization (FISH) as described (31). The probe was labeled with biotin-dUTP by nick translation and was hybridized at a concentration of 500 ng/50 μl per slide to pretreated and denatured metaphase chromosomes from a human PHA-stimulated lymphocyte culture. Salmon sperm DNA served as carrier, but no human genomic DNA was used as competitor. After incubation, washing, signal detection with avidin-FITC (Vector Laboratories, Inc., Burlingame, CA), and one round of amplification with biotinylated goat anti-avidin D antibody (Vector Laboratories, Inc.), chromosomes were counterstained with propidium iodide and analyzed under an epifluorescence microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) as described (31). Hybridization signals were considered specific only when the signal was observed on both chromatids of a chromosome. Results were recorded by digital imaging using a cooled charge-coupled device camera and displayed in pseudocolors.

Binding of CD6-Rg to ALCAM-expressing COS Cells and Binding of ALCAM-Rg to CD6-expressing COS Cells. COS cells were either mock transfected with parent vector CDM8 or with cDNA clones encoding CD6 or ALCAM by the DEAE-dextran method (32). 3 d after transfection, the cells were detached with PBS/0.5 mM EDTA, washed with PBS, and resuspended in PBS/1% BSA/0.1% NaN₃ (PBA) containing the following concentrations of mAbs or fusion proteins: J4-81 (anti-ALCAM) 1:200 dilution of ascites, G3-6 (anti-CD6) 5 μg/ml, CD6-Rg 50 μg/ml, and ALCAM-Rg 25 μg/ml. After 30 min on ice, cells were washed twice with PBA and resuspended in PBA containing either goat anti-mouse or human IgG-FITC for 30 min on ice. The cells were washed twice in PBA, once with PBS, fixed in PBS/1% formaldehyde, and analyzed by flow cytometry.

Results and Discussion

CD6 on Transfected COS Cells Promotes TE Cell Adhesion. To directly examine the role of CD6 in thymocyte-TE cell adhesion, we prepared a stable COS cell transfectant expressing CD6. COS-CD6 supported TE cell adhesion, while

COS cells transfected with the parent vector alone (COS-neo) did not (Fig. 1). The adhesion of TE cells to COS-CD6 cells was inhibited by pretreating the COS-CD6 cells with anti-CD6 mAb T12 or by pretreating TE cells with J4-81 mAb (Fig. 1 B), suggesting that CD6-mediated adhesion to TE cells involves the molecule recognized by mAb J4-81. These findings provide further evidence that CD6 plays a role in the binding of thymocytes to TE cells and are consistent with our previous data showing that pretreating TE cells with J4-81 blocks their ability to bind CD6-Rg (22).

Cloning and Characterization of the Antigen Recognized by mAb J4-81 (ALCAM). A J4-81 affinity column was used to purify the protein from HBL-100, a breast carcinoma cell line that expressed high levels of a CD6 ligand (21) and the antigen recognized by J4-81 (22). Amino acid sequences obtained from intact protein and internal peptide fragments were found to have substantial homology with the chicken neural adhesion molecule BEN/SC-1/DM-GRASP (33–35), which was also reported to be expressed by activated chicken leukocytes (36). DNA fragments corresponding to chicken BEN were obtained by PCR and used to screen a PHA-activated human T cell cDNA library. A single clone that did not contain the complete coding region was isolated. A 200-bp PCR

fragment derived from the 5′ region of this clone was used to isolate an ~1.9-kb cDNA clone that contained the complete coding sequence from an HL60 cDNA library (the nucleotide sequence has been deposited in GenBank under accession No. L38608). The two cDNA clones exhibit polymorphisms at three nucleotide positions in the region of overlap, two of which result in differences at the protein level (G to A from HL60 to the T cell clone at position 836 results in the substitution of N231 by S; C to T from HL60 to the T cell clone at position 965 results in the substitution of M274 by T).

The predicted amino acid sequence of the human homologue of chicken BEN (ALCAM), a type I membrane protein, consists of a 27–amino acid NH_2-terminal hydrophobic signal peptide, which is cleaved from the mature protein, followed by a 500–amino acid extracellular domain, a 24–amino acid hydrophobic transmembrane domain, and a 32–amino acid cytoplasmic domain. Glycosylation of ALCAM probably accounts for much of the difference between the predicted molecular mass of ~65 kD and the 100–105-kD molecular mass observed by immunoprecipitation (22, 25), as there are nine potential sites for N-linked glycosylation (not shown). Based on homology, the extracellular domain of ALCAM
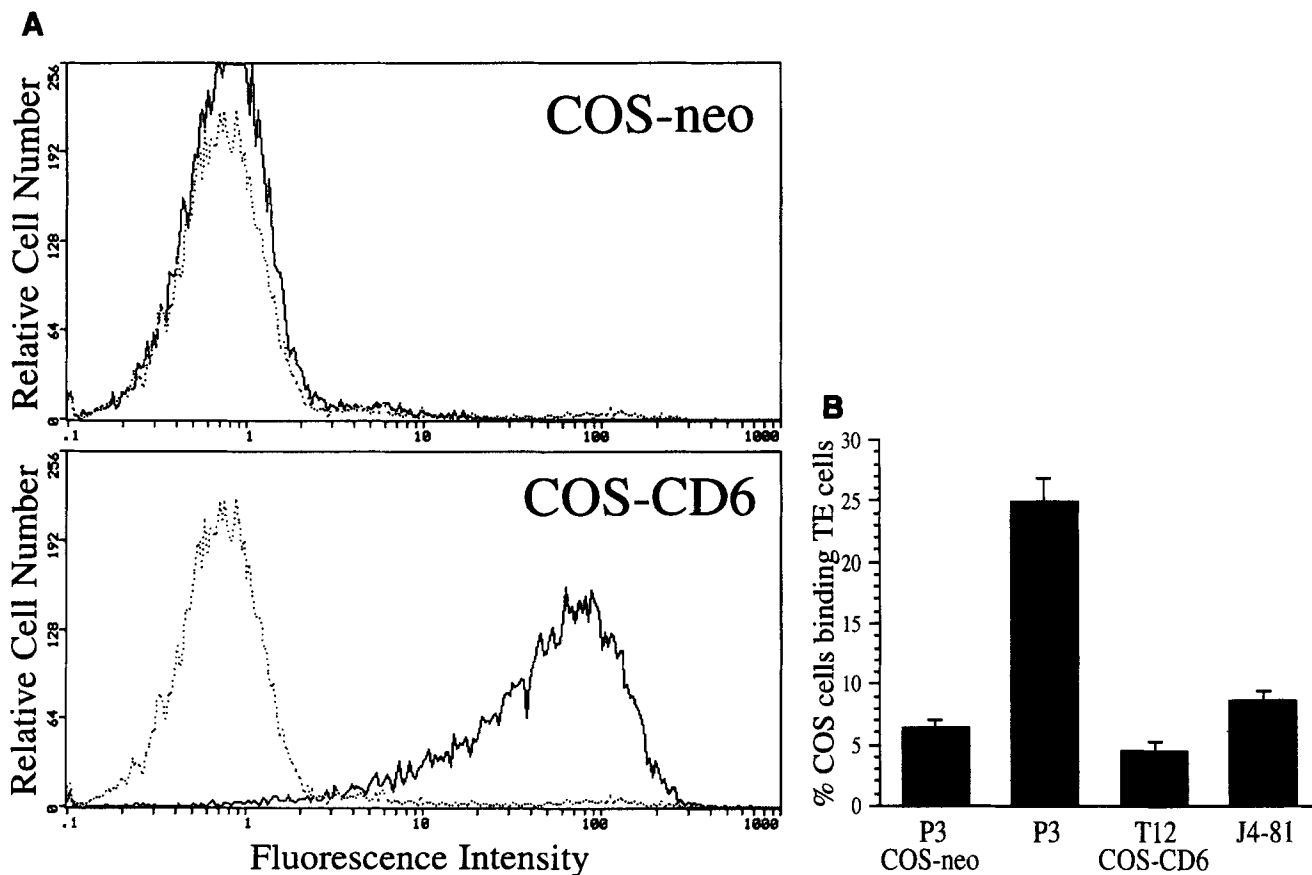

Figure 1. Binding of COS-CD6 to TE cells is CD6 specific. (*A*) Histograms of CD6 expression on COS-neo (control) and COS-CD6 cells were determined by indirect immunofluorescence with the anti-CD6 mAb T12 (11) (*solid line*) or a control antibody P3 (*dotted line*). (*B*) The bar graph depicts COS-neo and COS-CD6 binding to TE cells in the presence of either the T12, J4-81, or P3 mAbs. The mean and SEM of three separate experiments are shown.

can be divided into five Ig-like domains. The two NH₂-terminal domains are of the V set, and the three Ig domains that follow are from the C2 set (33–35). Comparison of the amino acid sequence of ALCAM with others in the data base showed that it was most homologous to neurolin (37), a protein expressed by neural axons of the goldfish visual system (38% identity/55% similarity), RAGE (38), a receptor for advanced glycation end products (28/43%), and MUC18 (39),

a cell surface protein whose expression correlates with the metastatic potential of melanoma cells (23/49%) (Fig. 2 *A*).

ALCAM Is Expressed by Activated Leukocytes. By Northern blot analysis, an ALCAM cDNA probe hybridized with a ~5.2-kb mRNA expressed by mitogen-activated PBMC and a number of T cell, B cell, monocytic, and tumor-derived cell lines; activated monocytes showed two additional minor species of ~10 and 8.5 kb (Fig. 2 *B*). ALCAM mRNA was
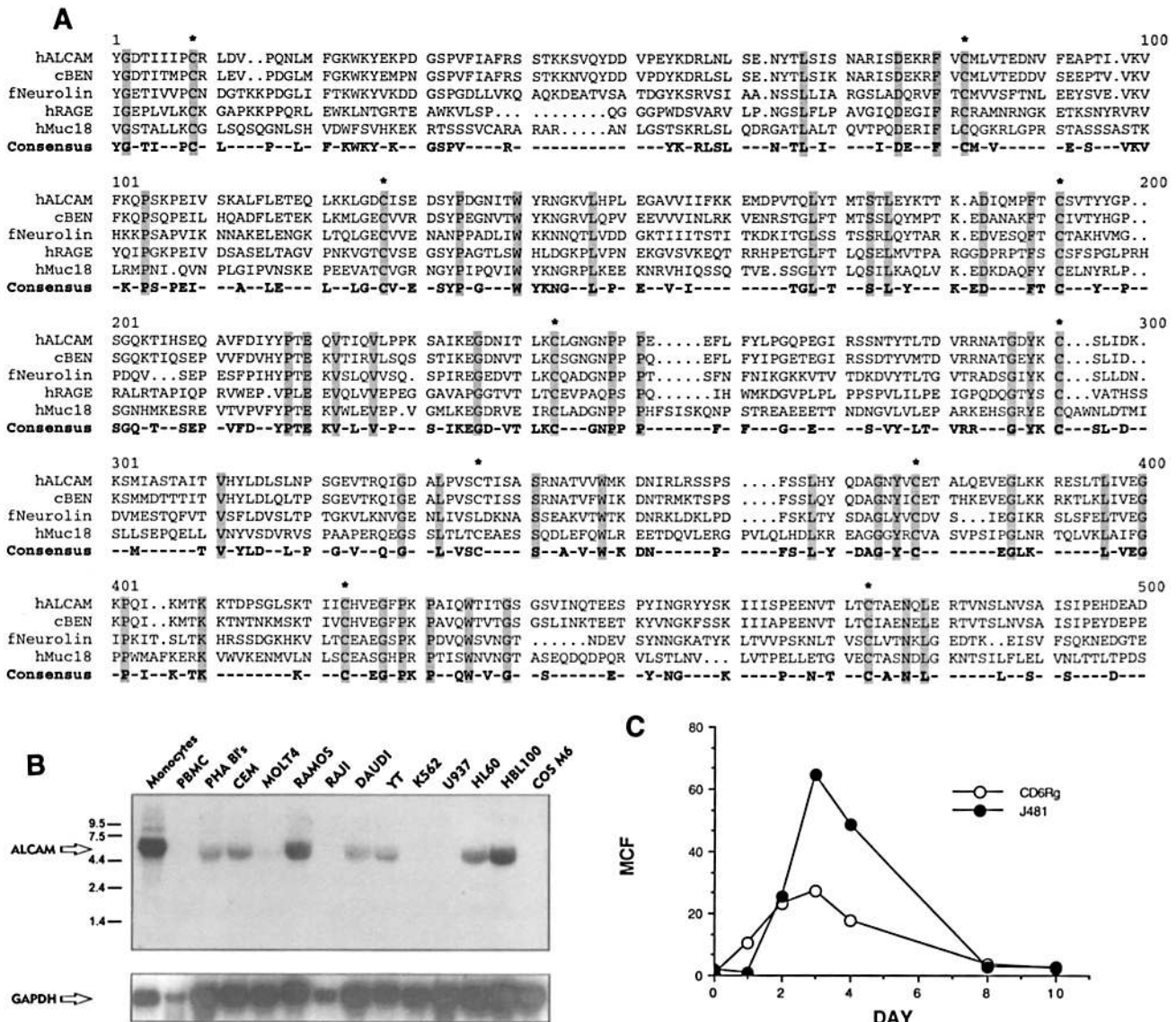

Figure 2. Sequence analysis of the predicted amino acid sequence of ALCAM, Northern blot analysis of ALCAM mRNA expression, and ALCAM cell surface expression by activated T cells. (*A*) Alignment of the immunoglobulin-like extracellular domains of ALCAM (residues 35–512), BEN, neurolin, RAGE, and MUC18. The lowercase letter in front of the protein name designates the species (*h*, human; *c*, chicken; *f*, fish). Consensus residues are those shared by three or more proteins. Invariant residues are shaded, and Cys residues are highlighted with an asterisk. The numbering of the peptide sequences shown, obtained from published manuscripts, are as follows: BEN, 8–484 (33); neurolin, 1–466 (37); RAGE, which contains three Ig domains, 30–307 (38); MUC18, 40–525 (39). (*B*) 15 μg of total RNA from adherent peripheral blood monocytes and 25 μg of total RNA from resting and PHA-activated (72 h) PBMC, the T cell lymphomas CEM and MOLT4, the erythroleukemia cell line K562, the B cell lymphomas Ramos, Raji, and Daudi, the myelomonocytic cell lines HL60 and U937, the large granular lymphoma YT, the human breast carcinoma HBL-100, and COS cells were used to prepare an RNA blot. Random primed ³²P-labeled ALCAM or GAPDH cDNAs were used as probes. (*C*) PBMC were activated in vitro with PHA, and the ability of T cells to bind to either CD6-Rg or J4-81 was monitored for 10 d by two-color immunofluorescence and flow cytometry. The mean channel fluorescence versus day was plotted.

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not detected in unactivated PBMC. To examine if the presence of ALCAM transcripts in activated T cells corresponded to the expression of a J4-81- and CD6-Rg–binding protein, PBMC were activated with PHA, and aliquots of the bulk culture were examined by flow cytometry for 10 d. Expression of a J4-81- and CD6–binding protein on T cells was observed 2 d after activation, was maximal at 3 d, and declined to undetectable levels 8 d after activation (Fig. 2 C). The binding of J4-81 and CD6-Rg to activated T cells exhibited the same expression kinetics, further supporting the hypothesis that these two molecules recognize the same molecular target.

Assignment of Human ALCAM Gene to Chromosome Bands 3q13.1-q13.2. FISH experiments using ALCAM cDNA clones as probes localized the gene to a single site on the proximal long arm of human chromosome 3. Of the 25 metaphase spreads that were analyzed, 20 exhibited a specific fluorescent signal on both chromatids at this site, and 13 out of 20 had signals on both chromosome 3 homologues. The chromosomes were identified by an R-banding pattern produced by the incorporation of BrdU after synchronization of the cell culture. The ALCAM signal was assigned to bands 3q13.1-q13.2 (Fig. 3).

This gene localization is distinct from that for CD6 on chromosome 11 (40). Chromosome 11 also carries the neural cell adhesion molecule locus at 11q23.1 encoding the neural cell adhesion molecule (41). Of the genes closely related to ALCAM, the gene for the melanoma-associated glycoprotein MUC18 (42) has not been mapped, but another member of the Ig superfamily of adhesion molecules, B-CAM, which

encodes the human F8/G253 antigen, is located on chromosome 19q13.2q-13.3 (43). While these mapping results indicate that members of this gene family are not all clustered in the human genome, one gene for another membrane glycoprotein that is also a member of the Ig superfamily (MOX2) is located in the vicinity of ALCAM on human chromosome 3 band q12-q13 (44, 45). The human ALCAM gene is a potential candidate for mutations in Mendelian disorders that affect the nervous system and the immune system. No disease suggestive for further testing has yet been mapped to this region of chromosome 3. The polymorphisms identified in the ALCAM cDNA will be useful for future candidate gene exclusion studies.

ALCAM is a CD6 Ligand. To directly examine if the ALCAM cDNA clone isolated from HL60 cells directs the expression of a protein that binds J4-81 and CD6-Rg, the cDNA was transfected into COS cells. 3 d after transfection, the COS cells were examined for their ability to bind to J4-81 (aALCAM) or CD6-Rg by flow cytometry. As shown in Fig. 4 A, COS cells expressing ALCAM were able to bind to both J4-81 and CD6-Rg. To further confirm that ALCAM is a CD6 ligand, a chimeric gene encoding ALCAM-Rg was constructed. ALCAM-Rg was expressed as a covalent homodimer with a molecular mass of ~200 kD and was recognized by mAb J4-81 (data not shown). To demonstrate the binding of ALCAM-Rg to CD6, COS cells were transfected with a cDNA clone encoding CD6 (1) and examined by flow cytometry 3 d later. COS cells transfected with CD6 bound both G3-6 (aCD6) and ALCAM-Rg (Fig. 4 B). These results demonstrate that CD6 and ALCAM are a receptor/ligand pair.
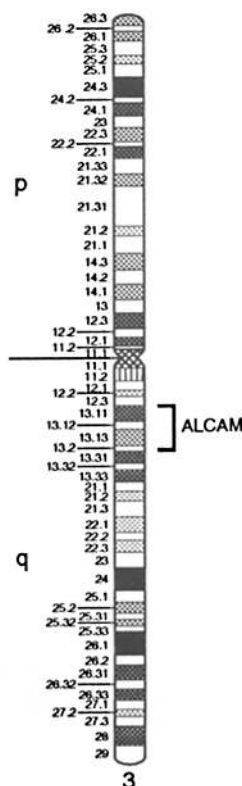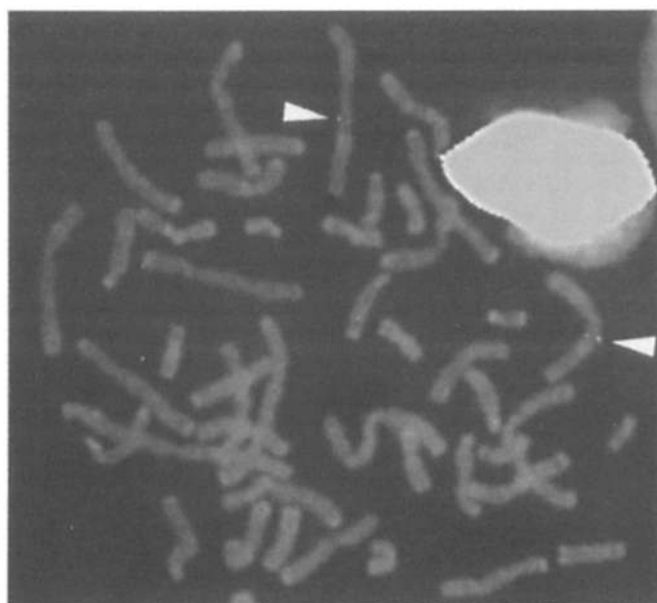

Figure 3. Human chromosomal mapping. FISH of cDNA probes to R-banded chromosomes generated signals on both chromatids (*arrows*) on chromosome 3 at bands q13.1-q13.2. The region is shown by brackets on a G-banding ideogram.
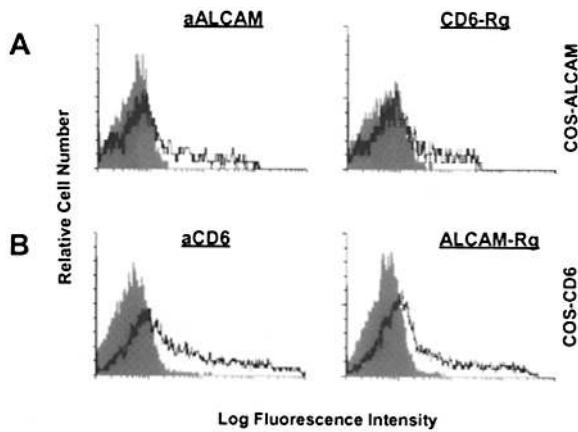
Figure 4. Binding of ALCAM to CD6. (A) Flow cytometry histograms that show the binding of the anti-ALCAM mAb J481 and the CD6-Rg fusion protein to COS cell transfectants expressing ALCAM. (B) Flow cytometry histograms that show the binding of anti-CD6 mAb G3-6 and ALCAM-Rg to COS cell transfectants expressing CD6. The shaded histograms represent level of background binding of mAbs or fusion proteins to mock-transfected COS cells.

Chicken BEN has been reported to mediate homotypic adhesion (33–35). We found that ALCAM-Rg was indeed able to bind COS cells that expressed ALCAM (not shown). This result supports previous observations of ALCAM–ALCAM interactions and indicates that ALCAM mediates both homophilic and heterophilic adhesion.

The data presented in this report showing that ALCAM–CD6 interactions mediate the adhesion of CD6-expressing cells to TE cells suggest that ALCAM–CD6 binding might play a role in regulating T cell maturation. Other molecules including CD2/LFA-3, intracellular adhesion molecule-1/LFA-1, very late antigen-3, -4, -6, and fibronectin can serve as adhesion molecules in thymocyte–stromal cell binding (46–49). The identification of a CD6 ligand will allow further studies to determine the relative contribution of CD6 in thymocyte–TE cell binding and T cell maturation. ALCAM

may also be involved in B cell development, since studies in the chicken showed that ALCAM (BEN) is expressed on endothelial cells in the bursa of Fabricius (50), which is an organ in birds where B cells develop. At this time, there is no information on the ability of ALCAM to transduce intracellular signals. The finding that the anti-CD6 mAb T12 can activate T cells in the presence of accessory cells and in the apparent absence of cross-linking FcRs suggests that activation of T cells by signaling through CD6 could be important in autoimmune reactivity (11). In support of this, anti-CD6 mAbs can enhance or inhibit the autologous MLR (11, 14) (Singer, N. G., and D. A. Fox, manuscript submitted for publication). Since ALCAM is expressed by activated T cells and monocytes, as well as by a number of T and B cell lines, we propose that ALCAM–CD6 interactions play a role in functional interactions between CD6^{+} T and B cells and activated leukocytes, and that ALCAM–ALCAM binding mediates adhesive interactions among activated leukocytes.

ALCAM (BEN) in the chicken is expressed predominantly during early embryonic development and in the brain (50). ALCAM functions as a homophilic adhesion molecule and has also been shown to support neurite outgrowth (34, 35). We have previously reported on the expression of human ALCAM by neurons in the brain (22). Interactions between the immune and nervous systems may be important in the pathology of certain chronic neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis (51–53). The finding that ALCAM and CD6 are both expressed by cells in these systems may indicate a function for this receptor/ligand pair in cellular interactions between the immune and nervous systems.

This report describes the novel interaction between a member of the SRCR family of proteins and a member of the Ig supergene family. This interaction contrasts with the finding that CD72 is a ligand for CD5 (54), a protein that has weak homology to C-type lectins and suggests that SRCR domains are able to interact with members of several different protein families.

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