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# Role of Integrin $\alpha 4\beta 7/\alpha 4\beta P$ in Lymphocyte Adherence to Fibronectin and VCAM-1 and in Homotypic Cell Clustering

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**Abstract.** Integrins are heterodimeric cell surface proteins that mediate both cell-cell and cell-extracellular matrix interactions. We and others recently identified cDNAs encoding a novel integrin  $\beta$  subunit,  $\beta 7$ , in lymphocytes. We have now detected  $\beta 7$  mRNA in mouse TK-1 T lymphoma cells, which are known to express the putative Peyer's patch homing receptor  $\alpha 4\beta P$ . We used an anti-peptide antiserum and a novel mAb against the  $\beta 7$  subunit to show that TK-1 cells express  $\beta 7$  as the only subunit associated with  $\alpha 4$ . We conclude that  $\beta 7$  and  $\beta P$  are identical. We also show that activated peripheral blood T cells express  $\alpha 4\beta 7$ .

We studied the function of  $\alpha 4\beta 7/\alpha 4\beta P$  in TK-1 cells, which do not express very late antigen (VLA)-4 ( $\alpha 4\beta 1$ ). Cells adhered to intact fibronectin and to a fibronectin fragment containing the CS-1 region, but not to a

fragment containing the RGD sequence. Adhesion to fibronectin was inhibited by antibodies to  $\alpha 4$ , suggesting that  $\alpha 4\beta 7$  is a fibronectin receptor. We confirmed that  $\alpha 4\beta 7$  binds to the CS-1 region of fibronectin using affinity chromatography. TK-1 cell adhesion to the vascular cell adhesion molecule VCAM-1 was also inhibited by antibodies to  $\alpha 4$ , implying that  $\alpha 4\beta 7$  also plays a role in the adherence of lymphocytes to endothelial cells. TK-1 cell binding to fibronectin and VCAM-1 is markedly increased by brief PMA stimulation. We also found that mAbs against  $\alpha 4$  and  $\beta 7$  induce homotypic clustering of TK-1 cells. Taken together these results suggest that  $\alpha 4\beta 7/\alpha 4\beta P$  recognizes some or all of the same widely distributed ligands recognized by VLA-4 ( $\alpha 4\beta 1$ ) and that the role of  $\alpha 4\beta 7/\alpha 4\beta P$  may not be restricted to lymphocyte homing.

**T**HE ability to interact with a variety of cells or with proteins of the extracellular matrix is crucial for a variety of leukocyte functions, including cell activation, phagocytosis, recruitment to sites of inflammation, recirculation, and homing (for reviews see Stoolman, 1989; Butcher, 1990; Springer, 1990). Many adhesion molecules expressed on T lymphocytes belong to the integrin family. Integrins are noncovalently linked heterodimers consisting of an  $\alpha$  and a  $\beta$  subunit that mediate cell-extracellular matrix as well as cell-cell interactions (Hynes, 1987; Ruoslahti, 1991). The  $\alpha$  and  $\beta$  subunits each have a large extracellular domain, a short transmembrane region, and a cytoplasmic tail. At least 13 different  $\alpha$  and eight different  $\beta$  subunits have been identified to date; these combine to produce at least 18 different integrin heterodimers. Both  $\alpha$  and  $\beta$  subunits participate in the determination of ligand specificity. The tripeptide RGD (Arg-Gly-Asp) is the recognition site for many of the integrins that bind to extracellular matrix proteins.

Members of the  $\beta 1$  integrin subfamily ( $\alpha 1\beta 1$  to  $\alpha 6\beta 1$ ), also referred to as the very late antigens (VLA-1 to VLA-6), bind and mediate adhesion to several extracellular matrix proteins such as laminin, collagens, and fibronectin (Hemler, 1990).

$\alpha 4\beta 1$  (VLA-4) has been shown to be involved in at least three different kinds of adhesive interactions. First,  $\alpha 4\beta 1$  mediates cell adhesion to fibronectin by binding to the alternatively spliced CS-1 region of fibronectin (Wayner et al., 1989; Mould et al., 1990; Guan and Hynes, 1990). Second,  $\alpha 4\beta 1$  mediates T cell adhesion to endothelium by binding to the vascular cell adhesion molecule VCAM-1 (Elices et al., 1990). T cell activation causes rapid enhancement of  $\alpha 4\beta 1$ -mediated binding to both fibronectin and VCAM-1 (Shimizu et al., 1990a,b). Third, certain antibodies directed against the  $\alpha 4$  subunit of  $\alpha 4\beta 1$  induce homotypic lymphocyte clustering (Campanero et al., 1990; Bednarczyk and McIntyre, 1990; Pulido et al., 1991). This clustering is dependent upon divalent cations. Clustering can be abolished by other antibodies directed against  $\alpha 4$  and by some anti- $\beta 1$  antibodies, but not by antibodies against other integrins or VCAM-1. It is possible that binding of anti- $\alpha 4$  antibodies induces a conformational change in  $\alpha 4\beta 1$  that allows  $\alpha 4\beta 1$  to bind to an unidentified counterreceptor on adjacent cells. Alternatively, binding of anti- $\alpha 4$  antibodies might cause clustering by triggering an  $\alpha 4\beta 1$ -mediated signal that activates other adhesion pathways.

In contrast to other integrin  $\alpha$  subunits, the 150-kD  $\alpha 4$  subunit can be cleaved at a site close to the middle of the molecule, resulting in variable amounts of 80- and 70-kD  $\alpha 4$  fragments (Sanchez-Madrid et al., 1986; Hemler et al., 1987; Takada et al., 1989). Both the intact and the cleaved forms of  $\alpha 4$  can associate with  $\beta 1$  on the cell surface to form functionally active integrins. The relative expression of the intact and cleaved forms greatly varies between different cell lines and is affected by T cell activation.

In some murine lymphocytes,  $\alpha 4$  can associate with a second  $\beta$  subunit,  $\beta P$  (Holzmann et al., 1989; Holzmann and Weissman, 1989). The molecular cloning and sequence of  $\beta P$  has not yet been reported. Antibodies directed against  $\alpha 4\beta P$  block adhesion of lymphocytes to Peyer's patch high endothelial venules (HEV)<sup>1</sup>, but not peripheral lymph node HEV, suggesting that  $\alpha 4\beta P$  may be a Peyer's patch lymphocyte homing receptor. The ligand(s) of  $\alpha 4\beta P$  have not been previously identified.

We and others have recently reported the cloning and sequencing of cDNAs coding for a novel human integrin  $\beta$  subunit,  $\beta 7$ , expressed in lymphocytes (Yuan et al., 1990; Erle et al., 1991a). It has recently been shown that the  $\beta 7$  subunit is part of the murine M290 antigen complex (Kilshaw and Murant, 1990; Yuan et al., 1991). M290 and its putative human homolog HML-1 are highly expressed on nearly all intraepithelial lymphocytes of the intestine (Cerf-Bensussan et al., 1987; Kilshaw and Murant, 1990). However, expression of HML-1 was reported on peripheral blood T cells after *in vitro* activation (Schieferdecker et al., 1990). The M290/HML-1  $\alpha$  subunits have not yet been identified, but these subunits do not appear to be related to any of the previously described  $\alpha$  subunits. The functions of M290/HML-1 remain to be determined.

We now report the detection of the  $\beta 7$  protein using a  $\beta 7$ -specific antiserum and a mAb. We present evidence that  $\alpha 4$  associates with  $\beta 7$  on mouse TK-1 lymphoma cells, that  $\alpha 4\beta 7$  and  $\alpha 4\beta P$  are identical, and that  $\alpha 4\beta 7$  is involved in adhesion to fibronectin and VCAM-1 and in homotypic T cell clustering. Our results also suggest that  $\beta 7$  can associate with at least two different  $\alpha$  subunits:  $\alpha 4$  and an unidentified  $\alpha$  subunit that is part of the M290/HML-1 complex.

## Materials and Methods

### Cell Culture

The murine T cell lymphoma line TK-1 was kindly provided by Dr. I. Weissman (Stanford University) (Holzmann et al., 1989). Cells were grown at 37°C, 5% CO<sub>2</sub>, and 100% humidity in RPMI 1640 medium (Cell Culture Facility, University of California, San Francisco, CA) supplemented with 10% FCS (Hyclone Laboratories Inc., Logan, UT). Human peripheral blood T cells were purified as previously described (Erle et al., 1991a). Purified T cells ( $1 \times 10^6$  cells/ml in RPMI 1640 medium and 10% FCS) were activated by incubation with the anti-CD3 mAb TR66 (1  $\mu$ g/ml, kindly provided by Dr. S. Abrignani, CIBA-GEIGY, Basel, Switzerland) for 20 min at 4°C, washing, and incubation with goat-anti-mouse IgG antiserum (1:500) (Boehringer Mannheim Corp., Indianapolis, IN) for 20 min at 4°C. After 48 h, human recombinant interleukin-2 (20 U/ml, Boehringer-Mannheim Corp.) was added to the cultures.

1. *Abbreviations used in this paper:* HEV, high endothelial venules; PCR, polymerase chain reaction.

### RNA Extraction, cDNA Synthesis, Homology PCR, and Northern Blotting Analysis

Total RNA extraction, cDNA synthesis, and homology polymerase chain reaction (PCR) amplification of integrin  $\beta$  subunits was performed as previously described (Erle et al., 1991b). Briefly, total RNA was prepared from TK-1 cells using the LiCl-urea method and reverse transcribed to cDNA. Integrin  $\beta$  subunit partial cDNAs were amplified using the primer mixtures B1AF and B2AR that recognize two regions that are conserved in all known  $\beta$  subunit sequences (Erle et al., 1991b). Amplified cDNAs were cloned (using the pBluescript plasmid) and sequenced. RNA gel electrophoresis and Northern blotting analysis were performed as previously described with a probe made from a partial  $\beta 7$  cDNA clone containing nucleotides 857-2761 of the  $\beta 7$  cDNA using the random primer method (Erle et al., 1991a).

### Antisera and Antibodies

A peptide modeled after the carboxyl-terminal sequence of  $\beta 7$  (Erle et al., 1991a) plus an amino-terminal cysteine (CQDSNPLYKSAITTTINPRF-QAEDSPTL, kindly provided by Dr. L. Reichardt, University of California, San Francisco, CA) was coupled to keyhole limpet hemocyanin (Calbiochem Corp., San Diego, CA) using *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide (Pierce Chemical Co., Rockford, IL) and glutaraldehyde (Fisher Scientific Co., Pittsburgh, PA) (Harlow and Lane, 1988). A 1:1 mixture of the two conjugates was used to immunize a rabbit (CALTAG Laboratories, South San Francisco, CA). The antiserum was affinity purified using the same peptide coupled to Sepharose. Antisera against other integrin subunits were also obtained by immunizing rabbits with peptides modeled after the carboxyl termini and were generously provided to us by other investigators ( $\alpha 4$  antiserum, Drs. M. Johansson and E. Ruoslahti, La Jolla Cancer Research Foundation (La Jolla, CA);  $\beta 1$  antiserum, Dr. L. Reichardt;  $\beta 2$  antiserum, Dr. E. Small, University of California, San Francisco, CA).

The mAb LS722 was obtained by immunizing a rat with supernatants from chymotrypsin-treated mesenteric lymphocytes and boosting with TK-1 cells (Sikorski, E. E., and E. C. Butcher, manuscript in preparation). Hybridomas were selected by testing culture supernatant for their ability to specifically stain TK-1 cells but not thymocytes. Rat anti-mouse  $\alpha 4$  mAbs were generous gifts of Dr. I. Weissman (RI-2) (Holzmann et al., 1989) and Dr. P. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK) (PS/2) (Miyake et al., 1991b). The mAb M290 was a generous gift of Dr. S. Murant and Dr. P. Kilshaw (Institute for Animal Physiology and Genetic Research, Babraham, Cambridge) (Kilshaw and Murant, 1990). The rat anti-mouse hybridoma FD441, anti-LFA-1  $\alpha$  subunit, was obtained from the American Type Culture Collection (Rockville, MD) (ATCC TIB213) (Sarmiento et al., 1982). This mAb was purified and concentrated from culture supernatant by ammonium sulfate precipitation. The rat mAb KM201, anti-murine Pgp-1/CD44, was a generous gift of Dr. P. Kincade (Miyake et al., 1990). The anti-human  $\alpha 4$  mAb P4G9 was purchased from Telios Pharmaceuticals Inc., (San Diego, CA) (Wayner et al., 1989). mAb against human and murine VCAM-1 were kindly provided by Dr. J. Harlan (University of Washington, Seattle, WA) (4B9) (Carlos et al., 1990) and by Dr. P. Kincade (M/K-1) (Miyake et al., 1991b).

### Immunoprecipitation Experiments

Cells were surface labeled with <sup>125</sup>I (for TK-1 cells) or biotin (for human T cells). For <sup>125</sup>I labeling, 20–200  $\times 10^6$  TK-1 cells were washed and resuspended in 1 ml PBS, 0.1 ml 1% D-glucose, 0.1 ml enzymobeads suspension (Glucose oxidase/lactoperoxidase, BioRad Laboratories, Richmond, CA) and 1–2 mCi Na<sup>125</sup>I (DuPont/New England Nuclear, Boston, MA or Amersham Corp., Arlington Heights, IL). Cells were labeled for 20 min at room temperature under continuous gentle agitation and washed once in PBS. For biotin labeling, T cells were resuspended in 5 ml PBS containing 0.2 mg/ml *N*-hydroxysulfosuccinimide-biotin, (Pierce Chemical Co.) and incubated for 20 min on ice. Before lysis cells were washed twice in PBS in the presence of 25 mM L-lysine. After <sup>125</sup>I or biotin labeling, cells were lysed on ice in 1 ml immunoprecipitation buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1% Triton X-100, 0.1% SDS, 0.1% NP-40) supplemented with 1  $\mu$ M PMSF (GIBCO-BRL, Life Technologies, Gaithersburg, MD), 1  $\mu$ g/ml Pepstatin and 1  $\mu$ g/ml Leupeptin (Boehringer Mannheim Corp.). Cell lysates were precleared with protein A-Sepharose (Pharmacia-LKB Biotechnology, Piscataway, NJ) and incubated with antibodies at 4°C for 1 h. Rabbit anti-rat IgG antiserum (Zymed Laboratories Inc., San Francisco, CA) was added whenever a rat mAb was used. Immune com-

plexes were captured on protein A-Sepharose, washed, dissolved in Laemmli sample buffer, and analyzed by SDS-PAGE (7.5% acrylamide) (Laemmli, 1970). To detect biotinylated antigen, proteins were transferred from the gel onto a nylon membrane (Immobilon; Millipore Corp., Bedford, MA). The membrane was blocked with 3% BSA (Fisher Scientific Co.) and 2.5% nonfat dry milk (Carnation, Los Angeles, CA) in PBS for 4–16 h at room temperature, incubated in 0.2–1  $\mu\text{g}/\text{ml}$  streptavidin-HRP (Pierce Chemical Co.) in washing buffer (PBS/0.5% BSA/0.1% Tween 20) for 20 min, washed 5 times in washing buffer, incubated in ECL solution (Amersham Corp.) for 1 min, immediately wrapped in plastic, and exposed to film.

In immunodepletion experiments, the antigen of interest was removed from the lysate by two cycles of immunoprecipitation using a large excess of antiserum or mAb and Protein A-Sepharose. Residual proteins were precipitated from the depleted lysates as described above. For EDTA dissociation experiments, washed protein A-Sepharose antibody-antigen complexes were incubated in 20 mM EDTA, 50 mM TRIS, pH 7.4, and 0.1% SDS for 10 min. Supernatants containing released subunits were collected and analyzed by SDS-PAGE. Material that remained bound to protein A-Sepharose was solubilized in Laemmli's sample buffer and analyzed by SDS-PAGE.

### Adhesion Assays

For adhesion assays, 96-well Linbro plates (Flow Laboratories Inc., McLean, VA) were coated for 2–16 h at 4°C with 50  $\mu\text{l}$  of the following proteins dissolved in 100 mM  $\text{NaHCO}_3$ : rat collagen I (Sigma Chemical Co., St. Louis, MO); plasma fibronectin and vitronectin (purified from human plasma according to the methods of Engvall and Ruoslahti [1977] and Yátohgo et al. [1988] and generously provided by Dr. M. Busk, University of California, San Francisco, CA); a 120-kD fibronectin fragment containing the RGD sequence (Telios Pharmaceuticals Inc.), a 38-kD fibronectin fragment containing the CS-1 sequence (donated by Dr. A. Garcia-Pardo, Centro de Investigaciones Biológicas, Madrid, Spain) (Garcia-Pardo et al., 1990) and recombinant soluble human VCAM-1 (kindly provided by Dr. R. Lobb, Biogen Inc., Cambridge, MA) (Lobb et al., 1991). Plates were blocked with 1% BSA for 2 h at 37°C. In some experiments TK-1 cells were labeled with BCECF (2',7'-bis-[2-carboxyethyl]-5[6]-carboxy-fluorescein; Sigma Chemical Co.) and/or stimulated with PMA (50–100 ng/ml). TK-1 cells were added to the plates and allowed to adhere for 15 min to 2 h in a 37°C, 5%  $\text{CO}_2$  incubator. In inhibition experiments, TK-1 cells were preincubated for 30 min in the presence of mAbs or synthetic peptides before plating. The CS-1 peptide (KKTDELPLVTLPHPNLHGPEILDVPLSTVQK) (Mould et al., 1990) and a control peptide (CKSILQEENRRD-SWSYINSSNDD) were synthesized in our laboratory. Nonadherent cells were removed by washing the plates (Pulido et al., 1991) or by inverse centrifugation (Charo et al., 1985). In experiments where BCECF-labeled cells were used, adherent cells were lysed and quantified by fluorimetry. In experiments where unlabeled cells were used, adherent cells were counted under a microscope or by crystal violet staining and determination of absorbance using a plate reader. All assays were performed at least in duplicate.

### Affinity Chromatography

Affinity chromatography was performed as previously described (Pytela et al., 1987). CS-1 peptide (see above) and a control peptide ( $\beta 7$  carboxyl terminus peptide, see above) were coupled to CNBr-activated Sepharose-4B (Sigma Chemical Co.) following the manufacturer's instructions (7 mg peptide/g Sepharose).  $1-2 \times 10^8$  TK-1 cells were stimulated for 300 min with PMA (100 ng/ml), labeled with  $^{125}\text{I}$  as above and lysed in affinity chromatography buffer (Tris 50 mM, pH 7.4, NaCl 150 mM,  $\text{CaCl}_2$  2 mM,  $\text{MnCl}_2$  1 mM) supplemented with 100 mM *n*-octylglucoside (Boehringer-Mannheim Corp.) and 10 mg/ml BSA (Fisher Scientific). Lysates were precleared with underivatized Sepharose 4B and applied to the columns at 4°C. Columns were washed with 5 vol affinity chromatography buffer containing 25 mM *n*-octyl-glucoside. Specifically bound proteins were eluted with EDTA (Tris 50 mM, pH 7.4, NaCl 150 mM, EDTA 10 mM). Aliquots of the fractions were analyzed by SDS-PAGE. Fractions of interest were dialyzed against immunoprecipitation buffer and analyzed in immunoprecipitation experiments.

### Cell Clustering Assay

TK-1 cells were washed twice in PBS and resuspended in RPMI 1640 medium supplemented with 10% FCS.  $5 \times 10^4$  cells in 100  $\mu\text{l}$  were plated in 96-well tissue culture plates in the presence of different mAbs and in-

cubated at 37°C. Cultures were observed under the microscope and clustering was scored using a modification of the system of Rothlein and Springer (1986): 0 indicated that no cells were aggregated; 1+ indicated up to 20% of the cells were in aggregates; 2+, 20–40%; 3+, 40–60%; 4+, 60–80%; and 5+, 80–100%. All assays were performed in duplicate.

### Flow Cytometry

Unstimulated TK-1 cells or TK-1 cells incubated for 30 min in the presence of 100 ng/ml PMA were collected, washed twice, and resuspended in PBS at  $2 \times 10^6$  cells/ml. Saturating amounts of mAbs were added to the cells. After a 20-min incubation at 4°C, cells were washed and resuspended in PBS. Phycoerythrin-labeled rabbit anti-rat IgG (H+L) F(ab)' (Zymed Laboratories Inc.) was added and the cells were incubated for 20 min at 4°C. After washing, cells were fixed (Coulter Immunology, Hialeah, FL) and analyzed with a flow cytometer (FACScan; Laboratory for Cell Analysis, University of California, San Francisco, CA).

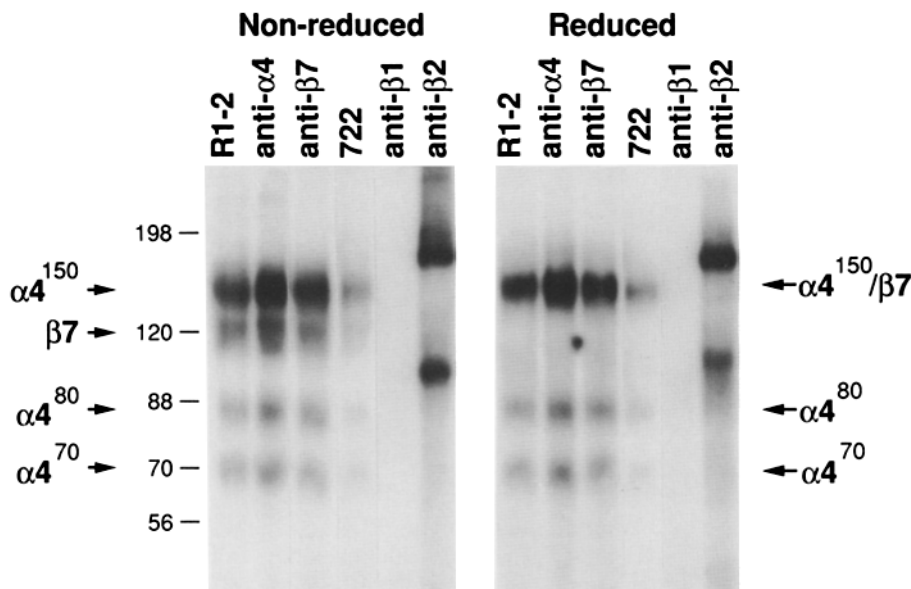
## Results

### TK-1 Cells Express $\beta 7$ mRNA

We used the homology PCR technique (Erle et al., 1991b) to identify integrin  $\beta$  subunit cDNAs from TK-1 cells, which are known to express the  $\beta\text{P}$  subunit (Holzmann and Weissman, 1989). This technique relies upon the use of oligonucleotide primer mixtures designed to recognize all known integrin  $\beta$  subunit cDNAs. We obtained 20 partial integrin  $\beta$  subunit cDNA clones from TK-1 cells. The sequence obtained from 15 of these clones is very similar to the human  $\beta 7$  sequence (96% identity of deduced amino acid sequences), and presumably represents mouse  $\beta 7$  (see Discussion). This sequence is identical to the sequences of  $\beta 7$  clones previously obtained from two other mouse cell lines (Erle et al., 1991b). The sequence obtained from the five other TK-1 cell cDNA clones is identical to the previously reported mouse  $\beta 2$  sequence (Kofler, 1991). In TK-1 cells, unlike other cells we have previously studied using homology PCR (Erle et al., 1991b), no  $\beta 1$  cDNA clones were obtained. This is consistent with a previous report that  $\beta 1$  mRNA is not detectable in TK-1 cells using Northern blotting (Holzmann and Weissman, 1989). We were able to detect  $\beta 7$  mRNA of  $\sim 3.2$  kb in size in TK-1 cells using Northern blotting (data not shown). Since only  $\beta 7$  and  $\beta 2$  cDNA clones were obtained from TK-1 cells, we hypothesized that  $\beta 7$  and  $\beta\text{P}$  might be identical.

### $\beta 7$ Associates with $\alpha 4$ on TK-1 Cells

We raised an antiserum against the integrin  $\beta 7$  subunit by immunizing a rabbit with a peptide containing the 27 carboxyl-terminal residues of the deduced human  $\beta 7$  amino acid sequence. SDS-PAGE analysis of material immunoprecipitated from surface-labeled TK-1 cells with the anti- $\beta 7$  antiserum revealed four protein bands at  $\sim 150$ , 120, 80, and 70 kD under nonreducing conditions (Fig. 1). A similar pattern of bands was obtained using two antibodies against the  $\alpha 4$  subunit: the mAb R1-2 and a polyclonal antiserum directed against the carboxyl terminus of  $\alpha 4$ . The novel mAb LS722, raised against mesenteric lymphocytes, also produced four similar bands. Based on previous data concerning  $\alpha 4$ -containing integrins (Hemler et al., 1987), we provisionally identified the 150-kD band as the uncleaved  $\alpha 4$  subunit, the 120-kD band as the  $\beta 7$  subunit, and the 80- and 70-kD components as the fragments resulting from the cleavage of  $\alpha 4$ . No material was precipitated using anti- $\beta 1$  antibody, con-

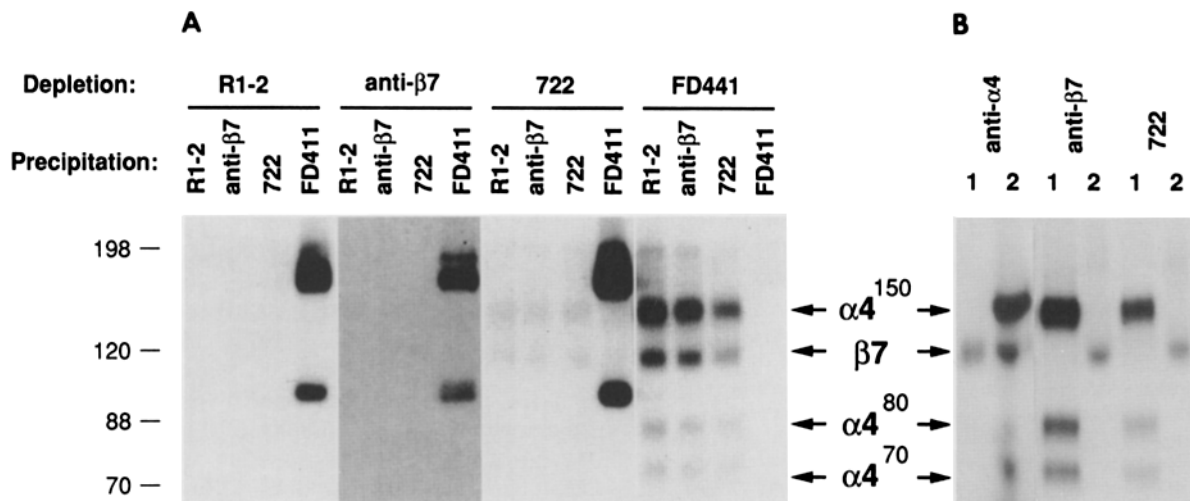


**Figure 1.** Immunoprecipitation of integrin complexes from TK-1 cells. mAbs R1-2 (directed against the  $\alpha 4$  subunit) and LS722 (722) and polyclonal antisera raised against peptides modeled after the carboxyl termini of the  $\alpha 4$ ,  $\beta 7$ ,  $\beta 1$ , and  $\beta 2$  subunits were used to immunoprecipitate proteins from  $^{125}\text{I}$  surface-labeled TK-1 mouse T lymphoma cells. TK-1 cells were incubated with PMA 100 ng/ml for 30 min at 37°C before labeling. Immunoprecipitates were released from protein A-Sepharose with Laemmli's sample buffer and analyzed using nonreducing and reducing SDS-PAGE, as indicated. Relative molecular masses (in kD), position of molecular weight markers, positions of the intact  $\alpha 4$  subunit ( $\alpha 4^{150}$ ), the  $\beta 7$  subunit, and the cleaved  $\alpha 4$  subunit fragments ( $\alpha 4^{80}$  and  $\alpha 4^{70}$ ) are indicated. Similar results were obtained when unstimulated TK-1 cells were used for immunoprecipitation (data not shown).

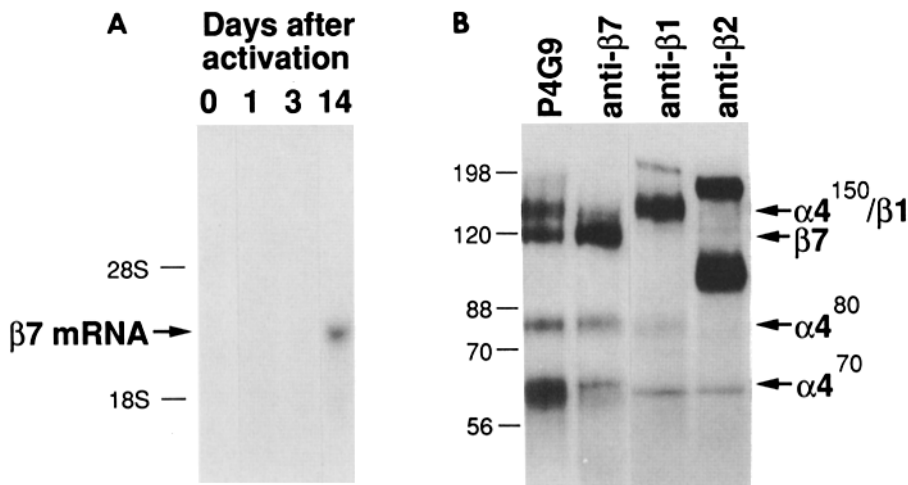
firming that TK-1 cells do not express  $\beta 1$  integrins. Analysis of the material immunoprecipitated with an anti- $\beta 2$  antiserum revealed two radiolabeled bands at  $\sim 170$  and 95 kD, consistent with the known molecular masses of the  $\alpha L$  and  $\beta 2$  subunits of LFA-1. The anti- $\beta 7$  antiserum does not cross react with  $\beta 2$  despite the similarity between the carboxyl termini of these two molecules. Under reducing conditions the putative  $\beta 7$  subunit comigrated with the intact  $\alpha 4$  subunit. Integrin  $\beta$  subunits, which have many intrachain disulfide bonds, typically migrate more slowly after reduction.

We used immunodepletion to demonstrate that antibodies

to  $\alpha 4$  and  $\beta 7$  and the mAb LS722 all recognize the same antigen complex on TK-1 cells (Fig. 2 A). TK-1 lysate was immunodepleted using antibodies against  $\alpha 4$  (R1-2) and  $\beta 7$  (anti-peptide antiserum) and the mAb LS722. The mAb FD441, which recognizes the unrelated integrin LFA-1 ( $\alpha L\beta 2$ ), was used as a control. Remaining antigen was then immunoprecipitated from the depleted lysates using each of the four antibodies. We found that the anti- $\alpha 4$  antibody R1-2 depleted all  $\beta 7$  antigen from the lysate, and the  $\beta 7$  antiserum depleted all  $\alpha 4$  antigen. This demonstrates that  $\alpha 4$  and  $\beta 7$  are associated. We also found that mAb LS722 depleted both  $\alpha 4$



**Figure 2.** Analysis of  $\alpha 4\beta 7$  by immunodepletion and EDTA-induced dissociation. (A) Immunodepletion analysis of integrins expressed on TK-1 cells. Equal aliquots of a lysate made from surface-labeled TK-1 cells were immunodepleted using mAb R1-2 (anti- $\alpha 4$ ), the anti- $\beta 7$  antiserum, mAb LS722, and mAb FD441 (anti-LFA-1  $\alpha$  subunit). Remaining antigen was then immunoprecipitated with the same four antibodies and analyzed by nonreducing SDS-PAGE. (B) EDTA-induced dissociation of immunoprecipitated complexes. An anti- $\alpha 4$  antiserum, the  $\beta 7$  anti-peptide antiserum, and mAb LS722 were used to immunoprecipitate protein complexes from TK-1 cells. Immunoprecipitated complexes (still bound to protein A-Sepharose) were dissociated with 20 mM EDTA and soluble proteins were collected (lane 1). Proteins that had not been released were then solubilized in Laemmli's sample buffer (lane 2). Proteins were analyzed by nonreducing SDS-PAGE.



**Figure 3.** Analysis of  $\beta 7$  mRNA and protein expression in peripheral blood T cells. (A)  $\beta 7$  mRNA level increases after T cell activation. Total RNA (15  $\mu$ g/lane) was obtained from human peripheral blood T cells before (day 0) and 1, 3, and 14 d after activation by T cell receptor complex crosslinking (see Materials and Methods).  $\beta 7$  mRNA was detected by Northern blotting hybridization with a  $^{32}$ P-labeled probe made using a 1,905-nucleotide fragment of  $\beta 7$  cDNA. Positions of the 28S and 18S ribosomal RNA bands are indicated at the left. Ethidium bromide staining was used to verify that similar amounts of total RNA were loaded in each lane. (B) Immunoprecipitation of  $\beta 7$ -containing integrins from activated T cells. 14 d after

activation, T cells were surface labeled using sulfo-NHS-biotin. Antibodies against  $\alpha 4$  (P4G9) and  $\beta 7$ ,  $\beta 1$ , and  $\beta 2$  (anti-peptide antisera) were used for immunoprecipitation. Immunoprecipitated proteins were analyzed by reducing SDS-PAGE. The biotinylation procedure results in an artifactual band migrating at  $\sim 65$ –70 kD, near the 70-kD  $\alpha 4$  fragment.

and  $\beta 7$ , while antibodies to  $\alpha 4$  and  $\beta 7$  depleted the LS722 antigen. This demonstrates that mAb LS722 recognizes the  $\alpha 4\beta 7$  complex.

We used a modification of the immunoprecipitation protocol to show that mAb LS722 recognizes the  $\beta 7$  subunit of the  $\alpha 4\beta 7$  complex. Antisera against  $\alpha 4$  and  $\beta 7$  and mAb LS722 were used for immunoprecipitation. After washing, the protein A-Sepharose antibody-antigen complexes were incubated with EDTA, which has previously been shown to dissociate  $\alpha 4$  from  $\beta P$  (Holzmann and Weissman, 1989). When the anti- $\alpha 4$  antiserum was used, the 120-kD  $\beta 7$  subunit was released from the complex by EDTA (Fig. 2 B, anti- $\alpha 4$ , lane 1). When the anti- $\beta 7$  antiserum and the mAb LS722 were used, EDTA treatment released the 150-, 80-, and 70-kD  $\alpha 4$  subunits (anti- $\beta 7$  and 722, lanes 1). This confirms that the anti- $\beta 7$  antiserum and the mAb LS722 both recognize the 120-kD  $\beta 7$  protein of the complex.

### $\beta 7$ Expression on In Vitro Activated Human Peripheral T Cells

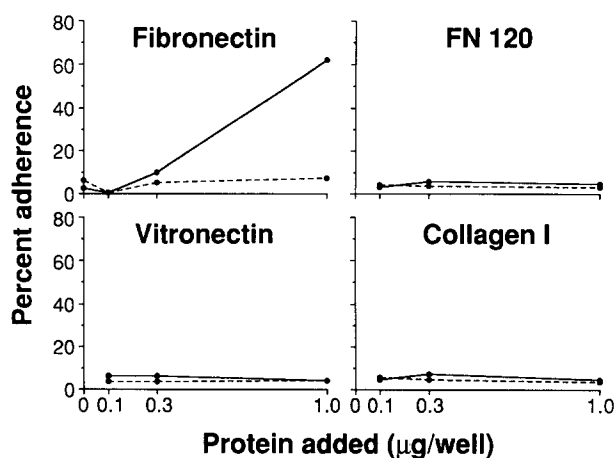
We previously described the activation-dependent induction of  $\beta 7$  mRNA in human peripheral T lymphocytes after stimulation with ionomycin and PMA (Erle et al., 1991a). Here we activated T cells via the T cell receptor-CD3 complex using cross-linked anti-CD3 mAb and studied  $\beta 7$  mRNA and protein expression. Northern blot analysis was performed on total RNA extracted from purified human peripheral blood T cells before activation (d 0) and 1, 3, or 14 d after activation (Fig. 3 A).  $\beta 7$  mRNA was undetectable at 0, 1, and 3 d but was present 14 d after activation.

We used the anti- $\beta 7$  antiserum to analyze the expression of  $\beta 7$ -containing integrins on T cells 14 d after activation (Fig. 3 B). Antibodies to  $\alpha 4$  (P4G9), and  $\beta 7$ ,  $\beta 1$ , and  $\beta 2$  (anti-peptide antisera) were used for immunoprecipitation. The anti- $\beta 7$  antiserum immunoprecipitates proteins of  $\sim 150$ , 140, 80, and 70 kD (reducing conditions). The 80- and 70-kD bands are very likely to represent the cleaved form of  $\alpha 4$  since no other known integrin  $\alpha$  subunit is cleaved to produce fragments of these sizes. The results suggest that intact or cleaved  $\alpha 4$  can associate with either  $\beta 1$  or  $\beta 7$  in acti-

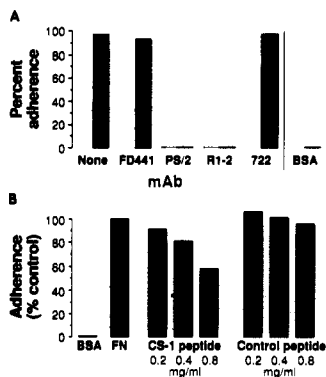
vated T cells. It appears that  $\alpha 4\beta 7$  is the major  $\beta 7$ -containing integrin in these cells. However, these results do not exclude the presence of other  $\beta 7$ -containing integrins on activated T cells (see Discussion).

### $\alpha 4\beta 7$ Mediates TK-1 Cell Adhesion to Fibronectin

We used TK-1 cells to examine possible interactions between  $\alpha 4\beta 7$  and extracellular matrix proteins. Neither unstimulated nor PMA-stimulated TK-1 cells adhered to vitronectin, collagen I, collagen IV, laminin, fibrinogen, or the RGD-containing 120-kD fibronectin fragment (Fig. 4 and data not shown). In contrast, PMA-stimulated TK-1 cells did adhere to intact fibronectin. PMA stimulation had a marked effect



**Figure 4.** Adherence of unstimulated and PMA-stimulated TK-1 cells to intact fibronectin, the 120-kD chymotryptic fragment of fibronectin (FN 120), vitronectin, and collagen I. 96-well plates were coated with matrix proteins (0, 0.1, 0.3, or 1.0  $\mu$ g/well) and then blocked with BSA. BCECF-labeled TK-1 cells were allowed to adhere for 2 h in the absence (---) or presence (—) of PMA (100 ng/ml) and nonadherent cells were removed by centrifugation of the inverted plate. Adherence was measured by fluorimetry.



**Figure 5.** Inhibition of TK-1 cell adherence to fibronectin by antibodies to  $\alpha 4$  and by a CS-1 peptide. (A) Antibodies to  $\alpha 4$  specifically inhibit adherence of TK-1 cells to the 38-kD tryptic fragment of fibronectin. Unlabeled TK-1 cells were preincubated with mAb against the LFA-1  $\alpha$  subunit (FD441, 5  $\mu$ g/ml), the  $\alpha 4$  subunit (PS/2, 1:10 dilution of supernatant or RI-2, 5  $\mu$ g/ml), or the  $\beta 7$  subunit (722, 5  $\mu$ g/ml). Cells were allowed to adhere to the 38-kD fibronectin fragment (0.5  $\mu$ g/well) for 30 min at 37°C in the absence (□) or presence (■) of PMA (50 ng/ml). Non-adherent cells were removed by gentle washing. Adherent cells were counted under a microscope. Adherence to wells treated with BSA alone is shown for comparison. (B) CS-1 peptide blocks adherence of PMA-stimulated TK-1 cells to intact fibronectin. TK-1 cells were allowed to adhere to fibronectin (1.0  $\mu$ g/well) in the presence of PMA (100 ng/ml) and up to 0.8 mg/ml of CS-1 peptide or control peptide (see Materials and Methods). Nonadherent cells were removed by washing and adherent cells were fixed and stained with crystal violet. Adherence was measured by absorbance (adherence of cells in the absence of peptide is defined as 100%).

on adhesion to fibronectin; this effect was apparent within 15 min and maximal at ~30 min (data not shown). To investigate whether the alternative cell attachment domain CS-1 was involved in the observed binding to fibronectin, we studied the adhesion of TK-1 cells to the 38-kD tryptic fragment of fibronectin, which contains the CS-1 domain (Fig. 5 A). This attachment was mediated by an  $\alpha 4$ -containing integrin since it was blocked by the anti- $\alpha 4$  mAbs RI-2 and PS/2. These antibodies also blocked attachment to intact fibronectin (data not shown). The anti- $\beta 7$  mAb LS722 had

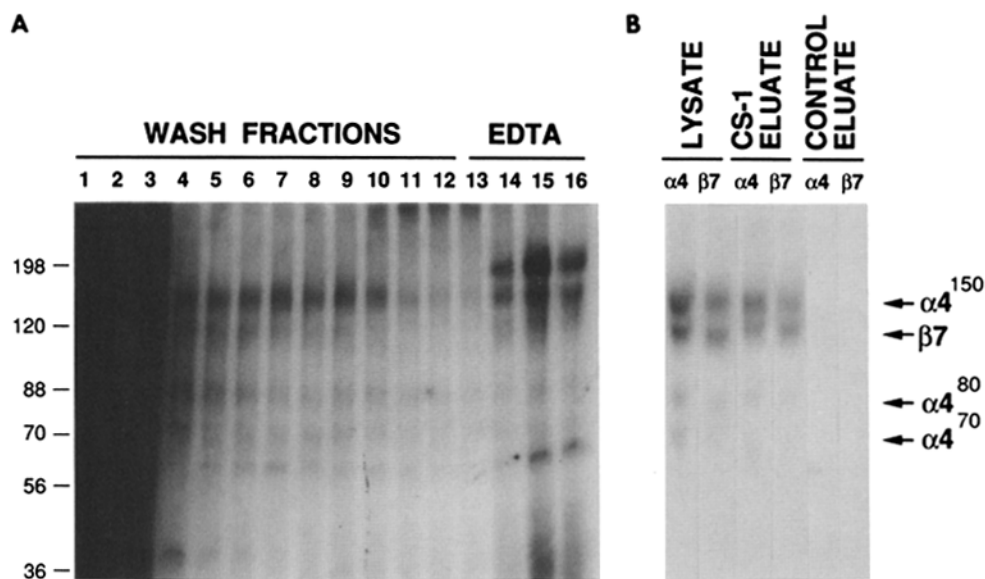
no effect on binding to fibronectin. This suggests that LS722 recognizes a  $\beta 7$  epitope not involved in fibronectin binding. Control antibodies had no effect on adhesion of TK-1 cells to fibronectin (Fig. 5 A and data not shown). We also investigated the adhesion of PMA-stimulated TK-1 cells to intact fibronectin in the presence of a blocking peptide modeled after the CS-1 region of fibronectin (Fig. 5 B). This peptide had a dose-dependent inhibitory effect on the adhesion of PMA-stimulated TK-1 cells to fibronectin (40% inhibition at 0.8 mg/ml).

### $\alpha 4\beta 7$ Binds to the Immobilized CS-1 Peptide in Affinity Chromatography

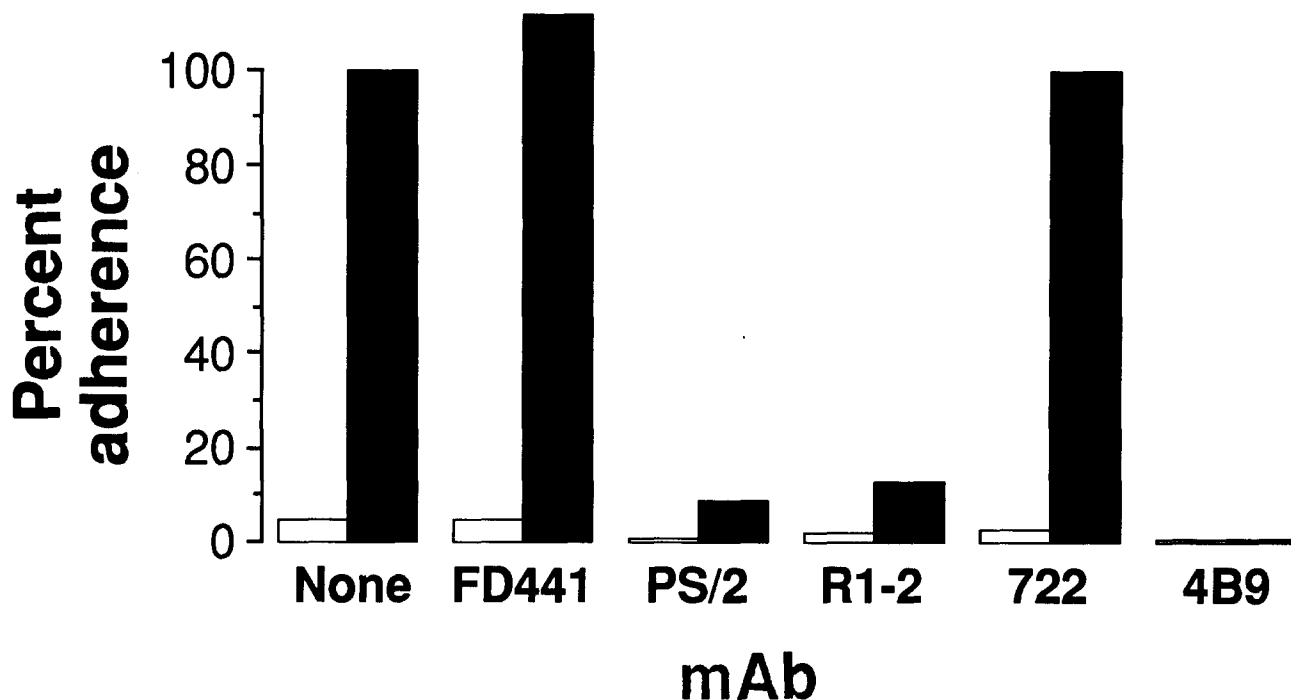
We used affinity chromatography to determine whether  $\alpha 4\beta 7$  binds to the CS-1 region of fibronectin, as suggested by the TK-1 adhesion experiments. Lysates from PMA-stimulated and surface-labeled TK-1 cells were applied to CS-1 peptide and control peptide columns. After washing, specifically bound material was eluted with EDTA. Wash fractions and EDTA-eluted fractions were analyzed by SDS-PAGE (Fig. 6 A). Proteins of ~150, 120, 80, 70, and 55 kD were present in decreasing amounts in successive wash fractions and dramatically increased with EDTA elution. An additional unidentified protein of ~190 kD was eluted by EDTA. EDTA-eluted material from the CS-1 peptide and control peptide columns was analyzed by immunoprecipitation (Fig. 6 B).  $\alpha 4\beta 7$ , but not the 190-kD protein, was immunoprecipitated from the CS-1 eluate using antisera against  $\alpha 4$  and  $\beta 7$ . In contrast, these antibodies did not immunoprecipitate any detectable material from eluates of a column made with a control peptide. These data show that  $\alpha 4\beta 7$  specifically binds to the CS-1 peptide.

### $\alpha 4\beta 7$ Mediates Adhesion of TK-1 Cells to Purified VCAM-1

The endothelial cell surface protein VCAM-1 is a ligand



**Figure 6.** CS-1 peptide affinity chromatography. (A) SDS-PAGE analysis of fractions from a CS-1 peptide column. PMA stimulated TK-1 cells were surface labeled with  $^{125}$ I and lysed in n-octyl-glucoside-containing buffer. The lysate was applied to a CS-1 peptide column. The column was washed with wash buffer (fractions 1-12) followed by EDTA elution (fractions 13-16). (B) Immunoprecipitation of proteins eluted from CS-1 and control peptide columns.  $\alpha 4$  and  $\beta 7$  anti-peptide antisera were used to immunoprecipitate  $\alpha 4\beta 7$  from the TK-1 total lysate and from EDTA-eluted fractions from CS-1 and control peptide columns. Column fractions and immunoprecipitates were analyzed by nonreducing SDS-PAGE.



**Figure 7.** Antibodies to  $\alpha 4$  inhibit adherence of PMA-stimulated TK-1 cells to VCAM-1. 96-well plates were coated with recombinant soluble human VCAM-1 (0.5  $\mu\text{g}/\text{well}$ ) and blocked with BSA. Unlabeled TK-1 cells were preincubated with mAb against the LFA-1  $\alpha$  subunit (FD441, 5  $\mu\text{g}/\text{ml}$ ), the  $\alpha 4$  subunit (PS/2, 1:10 dilution of supernatant or R1-2, 5  $\mu\text{g}/\text{ml}$ ), or the  $\beta 7$  subunit (722, 5  $\mu\text{g}/\text{ml}$ ). To determine the effect of the antibody to VCAM-1, wells were preincubated with 10  $\mu\text{g}/\text{ml}$  of the mAb 4B9. Cells were allowed to adhere for 30 min at 37°C in the absence ( $\square$ ) or presence ( $\blacksquare$ ) of PMA (50 ng/ml). Adherent cells were counted under a microscope.

for the  $\alpha 4$ -containing integrin  $\alpha 4\beta 1$ . To determine whether  $\alpha 4\beta 7$  might also bind VCAM-1 we studied TK-1 cell adhesion to recombinant soluble human VCAM-1, which is similar to mouse VCAM-1 (Miyake et al., 1991a). There was minimal or no binding of unstimulated TK-1 cells to VCAM-1. After PMA stimulation, there was essentially complete cell binding to VCAM-1 (Fig. 7). The binding was  $\alpha 4$  dependent since the adhesion was completely inhibited by either of the two anti- $\alpha 4$  mAbs (R1-2 or PS/2). The anti-VCAM-1 mAb 4B9 also completely inhibited TK-1 adhesion to VCAM-1. The anti- $\beta 7$  mAb LS722 had no effect, suggesting that the epitope recognized by LS722 is not involved in binding to VCAM-1. Antibodies to other adhesion molecules (LFA-1, Pgp-1/CD44, leukocyte common antigen) had no effect on TK-1 cell adhesion to VCAM-1 (Fig. 7 and data not shown). These results strongly suggest that VCAM-1 is a ligand for  $\alpha 4\beta 7$ .

#### mAbs Against $\alpha 4$ and $\beta 7$ Induce Clustering of TK-1 Cells

$\alpha 4\beta 1$  is involved in homotypic cell clustering.  $\alpha 4\beta 1$ -mediated T cell clustering can be triggered by mAbs against particular epitopes on the  $\alpha 4$  subunit (Bednarczyk and McIntyre, 1990; Campanero et al., 1990; Pulido et al., 1991). To study whether  $\alpha 4\beta 7$  may also be involved in homotypic T cell clustering we incubated TK-1 cells in the presence of mAbs against  $\alpha 4$  (PS/2 and R1-2) and  $\beta 7$  (722) (Table I and Fig. 8). Clustering was detectable by 2 h and became more pronounced at later time points. Control antibodies recognizing other cell-surface molecules did not induce clustering. Addition of EDTA completely abolished antibody-in-

duced TK-1 cell clustering (data not shown). The TK-1 cell clustering induced by R1-2, PS/2 or LS722 was not inhibited by preincubation with mAbs against LFA-1 (FD441) or VCAM-1 (M/K-1), demonstrating that the observed effects were LFA-1 and VCAM-1 independent (data not shown).

#### TK-1 Cells Express High Levels of $\alpha 4$ and $\beta 7$ but Little or No M290 Antigen by Flow Cytometry

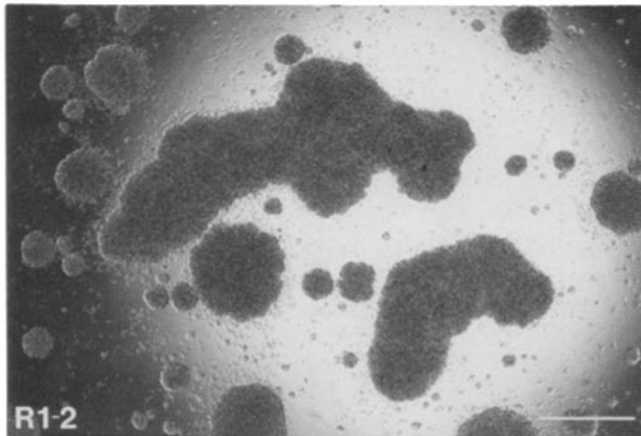
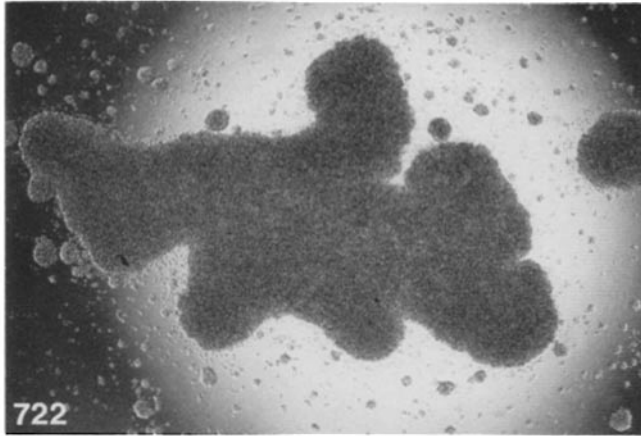
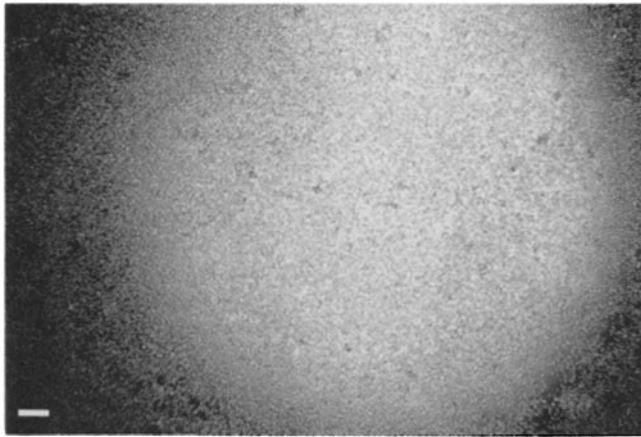
We used flow cytometry to detect adhesion molecules on TK-1 cells and to determine whether PMA stimulation had an effect on  $\alpha 4\beta 7$  expression (Fig. 9). Both  $\alpha 4$  (R1-2 and PS/2 mAbs) and  $\beta 7$  (722) are detectable by flow cytometry. While brief (30 min) PMA stimulation has a dramatic effect on  $\alpha 4\beta 7$  function (Figs. 4, 5, and 7), it has no detectable

**Table I.** Aggregation of TK-1 Cells by mAbs against  $\alpha 4$  and  $\beta 7$ \*

mAb	Specificity	Incubation time			
		30 min	3 h	8 h	11 h
None	—	0	0	0	0
R1-2	$\alpha 4$ subunit	0	++	++++	++++
PS/2	$\alpha 4$ subunit	0	++	++	++++
722	$\beta 7$ subunit	0	+++	++++	++++
FD441	$\alpha L$ subunit	0	0	0	0
M/K-1	VCAM-1	0	0	0	0
KM201	Pgp-1/CD44	0	0	0	0

\* Aggregation of TK-1 cells at 37°C in the presence of mAb (1  $\mu\text{g}/\text{ml}$  of purified antibody or 1:10 dilution of PS/2 hybridoma supernatant). The extent of aggregation was estimated as described in Materials and Methods.





**Figure 8.** Antibody-induced TK-1 cell clustering. TK-1 cells ( $5 \times 10^4$  cells in  $100 \mu\text{l}$ ) were incubated in the absence of antibody (–) or in the presence of mAb ( $1 \mu\text{g/ml}$ ) against  $\beta 7$  (722) or  $\alpha 4$  (R1-2). Cells were photographed after 8 h of incubation. Bar,  $500 \mu\text{m}$ .

effect on the amount of  $\alpha 4\beta 7$  expressed on the cell surface. Although the M290 antigen complex is known to include the  $\beta 7$  subunit (Kilshaw and Murant, 1990; Yuan et al., 1991), little or no M290 antigen is detectable on these cells. This result implies that M290 does not recognize  $\alpha 4\beta 7$ . Considered in the light of previous reports about the structure of the M290 antigen complex, our findings suggest that  $\beta 7$  can associate with multiple  $\alpha$  subunits. In TK-1 cells,  $\beta 7$  associates only with  $\alpha 4$ . In M290<sup>+</sup> intraepithelial lymphocytes,  $\beta 7$  ap-

parently associates with one or more as yet unidentified  $\alpha$  subunits to form the M290 antigen complex.

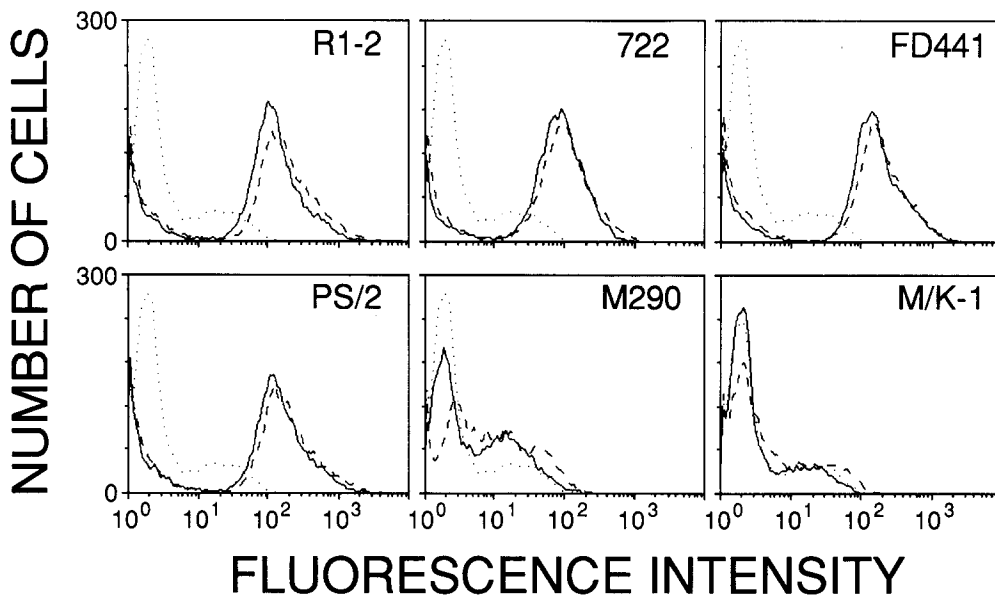
## Discussion

We and others previously identified the integrin  $\beta 7$  subunit cDNA from human lymphocytes using homology PCR (Yuan et al., 1990; Erle et al., 1991a). The work presented here began as an effort to identify the  $\beta 7$  protein. We were particularly interested in determining the relationship between  $\beta 7$  and the lymphocyte integrin subunit  $\beta P$ , identified by immunological techniques (Holzmann and Weissman, 1989). We chose to study mouse TK-1 T lymphoma cells because these cells are known to express  $\beta P$ . Using homology PCR, we identified one cDNA encoding mouse  $\beta 2$ , and a second cDNA encoding a portion of a mouse  $\beta$  subunit that is 96% identical to human  $\beta 7$ . Homologous  $\beta$  subunits from different mammalian species are generally 92–98% identical in this region (Erle et al., 1991b). Distinct  $\beta$  subunits are much less closely related in all known cases: for example, human  $\beta 7$  is only 61% identical to its closest known relative,  $\beta 2$ , in this region. Based upon these observations, we have designated the partial cDNA amplified from TK-1 cells as mouse  $\beta 7$ . The  $\beta 7$  sequence obtained from TK-1 cells is completely identical to sequences we previously obtained from mouse WR 2.3 T lymphoma cells and P388D1 macrophage-like cells (Erle et al., 1991b). Human  $\beta 7$  mRNA is also present in both T cells and macrophage-like cells (Erle et al., 1991a).

To identify the  $\beta 7$  subunit protein, we raised an antiserum against a synthetic peptide modeled after the deduced amino acid sequence of the carboxyl terminus of human  $\beta 7$ . This antiserum clearly cross reacts with mouse  $\beta 7$ , since it immunoprecipitates similar complexes from mouse and human lymphocytes (Figs. 1 and 3). The complex from TK-1 cells includes proteins of  $\sim 150$ , 120, 80, and 70 kD. We used EDTA to dissociate the complex and demonstrated that the  $\beta 7$  anti-peptide antiserum recognizes the 120-kD protein. This protein migrates more slowly under reducing conditions, which is characteristic of integrin  $\beta$  subunits and presumably relates to disruption of extensive intrachain disulfide bonding. The anti-peptide antiserum is apparently specific for  $\beta 7$ : the most closely related known  $\beta$  subunit,  $\beta 2$ , is 67% identical to an 18-amino acid portion of the peptide, but the antiserum clearly does not immunoprecipitate  $\beta 2$  (Figs. 1 and 3). By immunodepleting the TK-1 lysate with antibody to the  $\alpha 4$  subunit (Fig. 2 A), we showed that the other components of the  $\beta 7$  complex are intact  $\alpha 4$  (150 kD) and cleaved  $\alpha 4$  (80 and 70 kD). No other  $\alpha$  subunits associate with  $\beta 7$  in TK-1 cells.

We also used immunoprecipitation and immunodepletion to show that the novel mAb LS722, raised against mesenteric lymphocytes (Sikorski, E. E., and E. C. Butcher, manuscript in preparation), recognizes  $\alpha 4\beta 7$  (Figs. 1 and 2). mAb LS722 recognizes the  $\beta 7$  subunit, and not  $\alpha 4$ , since EDTA releases the  $\alpha 4$  subunit from the antibody-antigen complex (Fig. 2 B). As expected, mAb LS722 reacts with TK-1 cells, which express  $\alpha 4\beta 7$  (Fig. 9), but does not react with other mouse cells that express  $\alpha 4\beta 1$  but not  $\alpha 4\beta 7$  (unpublished observations).

Our results indicate that  $\alpha 4\beta 7$  is identical to  $\alpha 4\beta P$ . The  $\alpha 4\beta P$  complex was first identified when TK-1 cells were



**Figure 9.** Analysis of adhesion molecule expression on TK-1 cells by flow cytometry. TK-1 cells were incubated for 30 min in the absence (— —) or presence (—) of PMA (100 ng/ml) and then analyzed using flow cytometry. mAbs against  $\alpha 4$  (PS/2 and R1-2),  $\beta 7$  (722), LFA-1 (FD441), VCAM-1 (M/K-1), and the mAb M290 were used. Background fluorescence (phycoerythrin-labeled goat anti-rat antibody only) is represented by the dotted lines.

noted to express  $\alpha 4$  in the absence of its only previously described partner,  $\beta 1$  (Holzmann and Weissman, 1989). The alternative partner for  $\alpha 4$  in TK-1 cells was designated as  $\beta P$ . Our results indicate that  $\beta 7$  is the partner for  $\alpha 4$  in these cells, and we therefore infer that  $\beta 7$  and  $\beta P$  are identical.

We also studied  $\beta 7$  expression on human peripheral blood T lymphocytes by Northern blotting and immunoprecipitation (Fig. 3).  $\beta 7$  is expressed late after *in vitro* T cell activation. Immunoprecipitation analysis suggests that  $\alpha 4\beta 7$  may be the major  $\beta 7$ -containing integrin expressed on activated T cells, although we cannot exclude the possibility that other  $\beta 7$ -containing integrins may also be expressed on these cells. In fact, activated cells are weakly positive for HML-1, an antibody believed to recognize a  $\beta 7$ -containing integrin (Schieferdecker et al., 1990, and data not shown). HML-1 antigen and its putative mouse homolog M290 antigen are apparently different from  $\alpha 4\beta 7$  because TK-1 cells express high levels of  $\alpha 4\beta 7$  but express little or no M290 antigen (Fig. 9). This implies that the M290 antibody is not directed against the  $\beta 7$  subunit itself. Instead, M290 may recognize an  $\alpha$  subunit other than  $\alpha 4$  that associates with  $\beta 7$  in some cells.

We used TK-1 cells to study the function of  $\alpha 4\beta 7/\alpha 4\beta P$ . These cells are especially well suited for this purpose because they do not express detectable amounts of  $\beta 1$ -containing integrins, including  $\alpha 4\beta 1$ . Holzmann and Weissman (1989) used Northern blotting and two different antisera against  $\beta 1$  to demonstrate that TK-1 cells do not express  $\beta 1$ . In experiments reported here, we were unable to detect  $\beta 1$  mRNA with the homology PCR technique and used a third antiserum to confirm that  $\beta 1$  proteins are not present on TK-1 cells. We also chose to study TK-1 cells because these cells have been used previously to study the function of  $\alpha 4\beta P$  (Holzmann and Weissman, 1989; Holzmann et al., 1989). These studies demonstrated that the adhesion of TK-1 cells to Peyer's patch high endothelial venules is dependent on  $\alpha 4\beta P$ . The nature of the  $\alpha 4\beta P$  ligand(s) expressed on these endothelial cells is unclear. We were interested in attempting to identify well-defined ligands of  $\alpha 4\beta 7/\alpha 4\beta P$ .

Since most known integrins mediate adhesion to one or more extracellular matrix proteins, we first tested the adhe-

sion of TK-1 cells to a variety of these proteins. PMA-stimulated TK-1 cells adhered to intact fibronectin and to the 38-kD tryptic fragment of fibronectin containing the CS-1 region, but not to vitronectin, collagen I or IV, laminin, fibrinogen, or the 120-kD fragment of fibronectin that contains the RGD region (Figs. 4 and 5 and data not shown). Binding to fibronectin was specifically inhibited by anti- $\alpha 4$  mAbs and by a soluble CS-1 peptide. Affinity chromatography experiments using a CS-1 peptide and a control peptide column confirmed that  $\alpha 4\beta 7$  binds to the CS-1 region (Fig. 6).  $\alpha 4\beta 7$  is slowly released from the column even during washing with a buffer containing a physiological concentration of salt (lanes 1-12); this indicates that the  $\alpha 4\beta 7$ -CS-1 interaction, like many other integrin-ligand interactions, is of low affinity (Pytela et al., 1987). Taken together, these results demonstrate that  $\alpha 4\beta 7/\alpha 4\beta P$  mediates cell adhesion to fibronectin by binding to the CS-1 region.  $\alpha 4\beta 1$  is the only other integrin that has been shown to bind to this region (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990).

Because of this striking functional similarity between  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$ , we wondered whether  $\alpha 4\beta 7$  might also share other ligands with  $\alpha 4\beta 1$ .  $\alpha 4\beta 1$  has been convincingly demonstrated to mediate lymphocyte adherence to endothelium by binding to the vascular cell adhesion molecule VCAM-1 (Elices et al., 1990; Carlos et al., 1990). To determine whether  $\alpha 4\beta 7$  might have a similar function, we studied TK-1 cell adhesion to human recombinant soluble VCAM-1 (Fig. 7). PMA-stimulated TK-1 cells adhered very efficiently to human VCAM-1 and adhesion was specifically and completely inhibited by antibodies directed against  $\alpha 4$  and VCAM-1. This result strongly suggests that  $\alpha 4\beta 7$  can mediate adhesion to endothelium by binding to VCAM-1.

TK-1 cell adhesion to fibronectin and to VCAM-1 was greatly enhanced by brief PMA stimulation. It has previously been shown that brief stimulation of T cells with PMA or other agents results in increased binding activity of several integrins, including LFA-1,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ , and  $\alpha 6\beta 1$ , without affecting levels of expression of these molecules (Dustin and Springer, 1989; Shimizu et al., 1990a). The effect of PMA

stimulation on  $\alpha 4\beta 7$  is similar: binding is increased without any change in level of surface expression of either  $\alpha 4$  or  $\beta 7$  (Fig. 9). The effect of PMA stimulation was apparent within 15 min and maximal at  $\sim 30$  min (data not shown); these kinetics are not consistent with new synthesis of  $\alpha 4\beta 7$  or other proteins. In particular, we demonstrated that brief PMA stimulation does not induce expression of  $\alpha 4\beta 1$  on TK-1 cells (Fig. 1). Instead, our data strongly suggest that PMA affects the function of  $\alpha 4\beta 7$  molecules already present on the cell surface.

We also investigated the phenomenon of antibody-induced cell clustering in TK-1 cells. It has previously been shown that some mAbs directed against the  $\alpha 4$  subunit of  $\alpha 4\beta 1$  induce clustering of lymphocytes (see introduction). We showed that mAbs directed against either subunit of  $\alpha 4\beta 7$  can induce dramatic TK-1 cell clustering (Table I and Fig. 8).  $\alpha 4\beta 7$ -induced clustering, like  $\alpha 4\beta 1$ -induced clustering, is dependent upon the presence of divalent cations and is VCAM-1 and LFA-1 independent. The anti- $\beta 7$  mAb LS722, like some previously described anti- $\alpha 4$  antibodies (Pulido et al., 1991), induces cell clustering but has no effect in fibronectin and VCAM-1 adhesion assays (Figs. 5 and 7). It is not yet known how antibodies to  $\alpha 4\beta 7$  and  $\alpha 4\beta 1$  induce cell clustering. It is intriguing to speculate that the in vitro clustering phenomenon might reflect a role for  $\alpha 4\beta 7$  in lymphocyte-lymphocyte interactions in vivo.

While our data do suggest that  $\alpha 4\beta 7/\alpha 4\beta P$  and  $\alpha 4\beta 1$  share similar ligands, there are likely to be important differences between these integrins. The distribution of  $\alpha 4\beta 7/\alpha 4\beta P$  and  $\alpha 4\beta 1$  is clearly different. Individual lymphoma cell lines may express either one or both of these integrins (Holzmann and Weissman, 1989). Flow cytometry indicates that  $\alpha 4\beta 1$  is poorly represented on mucosa-associated lymphocytes whereas  $\alpha 4\beta 7$  is highly expressed in mucosal sites (Picker et al., 1990; and Sikorski, E. E., and E. C. Butcher, manuscript in preparation). Expression of these integrins is also regulated differently. While  $\alpha 4\beta 1$  is easily detectable on resting T cells, we were able to detect  $\alpha 4\beta 7$  only after T cell activation. Integrins are known to be involved in signaling, and it is possible that  $\alpha 4\beta 7/\alpha 4\beta P$  and  $\alpha 4\beta 1$  might transduce different signals after binding similar ligands. Integrin signaling is believed to involve interaction of the integrin cytoplasmic domain with other cytoplasmic elements (see Hynes, 1987; Ruoslahti, 1991). The cytoplasmic domains of human  $\beta 7$  and  $\beta 1$  are only 47% identical and differ most strikingly because of the presence of five additional amino acids at the carboxyl terminus of  $\beta 7$  (Erle et al., 1991a). It remains to be determined if this difference has functional implications.

While previous work (Holzmann and Weissman, 1989; Holzmann et al., 1989) suggested that  $\alpha 4\beta P$  might function as a Peyer's patch lymphocyte homing receptor, our findings demonstrate that  $\alpha 4\beta 7/\alpha 4\beta P$  recognizes widely distributed ligands. There are at least two fundamentally different ways in which these two sets of observations can be reconciled. First, it is possible that additional, as yet unidentified  $\alpha 4\beta 7$  ligands are expressed only on Peyer's patch HEV. These ligands might play a more important role than fibronectin or VCAM-1 in Peyer's patch homing. Second, it may be that an adhesion molecule other than  $\alpha 4\beta 7/\alpha 4\beta P$  is responsible for initial specific binding to Peyer's patch HEV, while  $\alpha 4\beta 7/\alpha 4\beta P$  is responsible for stabilizing this interaction. The initial binding might involve an as yet unidentified lym-

phocyte adhesion molecule which recognizes the mucosal vascular addressin (Berg, 1991). This model is reminiscent of the interaction of neutrophils with venular endothelium: initial adhesion is mediated by members of the selectin family of adhesion molecules, followed by a more durable interaction between neutrophil  $\beta 2$  integrins and endothelial cell ICAMs (Kishimoto et al., 1989; Lawrence and Springer, 1991; Hallmann et al., 1991; von Andrian et al., 1991). The stabilizing interaction of T cells with Peyer's patch HEV can apparently be mediated by either  $\alpha 4\beta 7/\alpha 4\beta P$  or by  $\alpha 4\beta 1$  since adherence of TK50 cells, which express only  $\alpha 4\beta 1$ , to Peyer's patch HEV can be inhibited by antibody against  $\alpha 4$  (Holzmann and Weissman, 1989). This suggests that  $\alpha 4\beta 7/\alpha 4\beta P$  may be only one of multiple adhesion receptors involved in Peyer's patch homing.

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