The α4 Integrin Chain Is a Ligand for α4β7 and α4β1

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Summary

The heterodimeric α4 integrins α4β7 lymphocyte Peyer's patch adhesion molecule ([LPAM]-1) and α4β1 (very late antigen-4) are cell surface adhesion molecules involved in lymphocyte trafficking and lymphocyte–cell and matrix interactions. Known cellular ligands include vascular cell adhesion molecule (VCAM)-1, which binds to α4β1 and α4β7, and the mucosal addressin cell adhesion molecule (MAdCAM)-1, which binds to α4β7. Here we show that the α4 chain of these integrins can itself serve as a ligand. The α4 chain, immunoaffinity purified and immobilized on glass slides, binds thymocytes and T lymphocytes. Binding exhibits divalent cation requirements and temperature sensitivity which are characteristic of integrin-mediated interactions, and is specifically inhibited by anti-α4 integrin antibodies, which exert their effect at the cell surface. Cells expressing exclusively α4β7 (TK-1) or α4β1 (L1-2) both bound avidly, whereas α4-negative cells did not. A soluble 34-kD α4 chain fragment retained binding activity, and it inhibited lymphocyte adhesion to α4 ligands. It has been shown that α4 integrin binding to fibronectin involves an leucine–aspartic acid–valine (LDV) motif in the HepII/IIICS region of fibronectin (CS-1 peptide), and homologous sequences are important in binding to VCAM-1 and MAdCAM-1. Three conserved LDV motifs occur in the extracellular sequence of α4. A synthetic LDV-containing α4-derived oligopeptide supports α4-integrin–dependent lymphocyte adhesion and blocks binding to the 34-kD α4 chain fragment. Our results suggest that α4β7 and α4β1 integrins may be able to bind to the α4 subunit on adjacent cells, providing a novel mechanism for α4 integrin–mediated and activation-regulated lymphocyte interactions during immune responses.

The α4 integrins α4β7 (lymphocyte Peyer's patch adhesion molecule [LPAM][1]-1) and α4β1 (very late antigen [VLA]-4) are adhesion molecules important in lymphocyte migration and cell–cell binding. VLA-4 mediates leukocyte adhesion to fibronectin (1–3) and the cytokine-inducible endothelial cell ligand vascular cell adhesion molecule (VCAM)-1 (4–8). Differentially spliced forms of VCAM-1 with variable numbers of Ig domains have been identified in different species (9–12). LPAM-1 has been implicated as a lymphocyte homing receptor involved in binding to Peyer's patch (PP) high endothelial venules (HEV) in frozen sections (13, 14) and in lymphocyte homing to PP and the intestinal lamina propria (15). The ligand expressed on the HEV of PP is the addressin MAdCAM-1 (16, 17). Only α4β7-positive cells can bind to MAdCAM-1, whereas both α4β1 or α4β7 cells can bind to fibronectin and VCAM-1 (17, 18).

Despite the fact that lymphocytes do not express any of the known ligands for α4 integrins, certain antibodies to α4 were found to block cell–cell interactions between lymphoid subpopulations (19, 20) or to induce homotypic aggregation of lymphoid cells, which is blocked by other α4- or β7/β1-specific antibodies (21–26). It has been proposed that such aggregation may reflect that lymphocytes express specific coun-

[1] *Abbreviations used in this paper:* BOG, octylglucoside; Endo F, endoglycosidase F; FBS, fetal bovine serum; HEV, high endothelial vanules; HSA, heat-stable antigen; ICAM, intracellular adhesion molecule; LDV, leucine–aspartic acid–valine; LPAM, lymphocyte Peyer's patch adhesion molecule; PP, Peyer's patch; MAdCAM, mucosal addressin cell adhesion molecule; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

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terligand(s) for α4 integrins or, alternatively, that α4 integrins are capable of interacting in a homotypic fashion.

Here we report that the isolated α4 integrin chain itself, as well as leucine–aspartic acid–valine (LDV)-containing peptides of α4, can serve as ligands for recognition and binding by lymphocytes α4β1 and α4β7. Our results suggest a novel α4–α4 binding mechanism that could participate in diverse α4-dependent cell–cell interactions.

Materials and Methods

Cell Culture. The murine bend3 endothelioma cell line was kindly provided by Dr. W. Risau (Bad Nauheim, FRG) and was maintained in DMEM with high glucose (Life Technologies, Eggenstein, FRG) containing 10% low endotoxin fetal bovine serum (FBS) (Life Technologies). Cells were activated with LPS from *Salmonella enteritidis* (Sigma, Deisenhofen, FRG) at a dose of 1 μg/ml for 4 h at 37°C. The T cell lymphoma line TK-1 (13, 27), the lymphoma cell lines ESb 289 and Eb (28), and the B lymphoma line L1-2 were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Hepes, and 50 mM 2-ME (culture medium). The T cell hybridoma cell line BI 141 (29) was kindly provided by A. Reske-Kunz (University of Mainz, Mainz, FRG) and was maintained in Iscove's medium supplemented with 5% FBS, sodium pyruvate, and 50 mM 2-ME. All cells were kept at 37°C, 5% CO_2, and 100% humidity. Spleen and thymus cells were collected from 6–8-wk-old DBA/2 mice. Erythrocytes were lysed by brief incubation in 155 mM NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$ solution. T lymphocytes were purified from spleen by passage over nylon wool followed by treatment with B220 mAb RA32C2 (30) and rabbit complement (Camon, Wiesbaden, FRG). T lymphoblasts were induced from splenic lymphocytes by activation with 5 μg/ml Con A in RPMI 1640 supplemented with 10% FBS and 50 mM 2-ME for 48 h. Blast cells were purified by Percoll centrifugation (Pharmacia, Freiburg, FRG) and cell clumps were dissociated by treatment with PBS containing 5 mM EDTA.

Antibodies. The mAb 5/3 was obtained by immunizing rats with bend3 cells stimulated for 4 h with LPS. Animals were injected subcutaneously with live cells (1–2 × 10^7 per injection) in PBS and four booster injections were given with 2-wk intervals. Spleen cells from these rats were fused with Sp2/0 cells as described (31) and hybridomas that were able to block the binding of thymocytes to LPS-activated bend3 cells were selected. mAb 5/3 (IgG2b) was obtained from the parental blocking subclone 228/10 by limiting dilution cloning. mAb 7/1 (IgM) was obtained from the same subclone and has no blocking potential. It recognizes a cell surface molecule with unknown function and was used for control purposes. Other mAbs were: PS/2 (32) and R1-2 (13) directed against α4 integrins, FD441 (TIB 213) and FD 18.5 (33) recognizing the α chain of LFA-1, 30G12 against mouse CD45, MK2.7 against VCAM-1 (34), YN.1/1.7 against intracellular adhesion molecule-1 (ICAM-1) (35), 12-15 against mouse CD2 (36), DATK-32 against a combinational epitope of α4β7 (17), -Fib 30 against β7 (15), 324 against the L1 adhesion molecule (37), and 79 against HSA (38).

Peptides. Peptides were synthesized using Fmoc strategy and purified by preparative HPLC. They were characterized further by analytical HPLC and mass spectroscopy. Peptides were synthesized with an NH_2-terminal Cys residue. The following peptides were used: CS-1, CEILDVPST; LDV-α4(1), CNVSLDVHRKA; LDV-α4(2), CSFLLDVSSLS: LDV-α4(3), and CFNVLDVQTTT. For control, the peptide VAIYDDMESLPLTGT was used. Peptide–carrier protein conjugates for cell adhesion were produced by crosslinking *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)-activated rabbit IgG with peptides (39, 40). The following peptides were conjugated: CS-1, CDELPQLVTLPHPNLHGPEILDVPST; LDV-α4(1), CVLFYNVSLDVHRKAESPSRF; CMFSTKSAWLRNGGADQGPRG (α4 signal peptide) was used for control purpose. Peptide–carrier conjugates were coated to glass slides as described below.

Cytofluorography. The staining of cells with hybridoma supernatant– and PE-conjugated goat antibodies to rat immunoglobulins (SERVA, Heidelberg, FRG) has been described in detail elsewhere (41). Stained cells were analyzed with a FACScan® fluorescence-activated cell analyzer (Becton Dickinson, Heidelberg, FRG).

Affinity Purification of Cell Surface Antigens. mAbs were coupled to CNBr-activated Sepharose (Pharmacia) and used for the purification of antigen by affinity chromatography as previously described (42). Briefly, cells (ESb lymphoma, TK-1 cells, or pooled thymocytes and splenic lymphocytes) were lysed in 20 mM Tris/HCl, pH 8.0, containing 2% NP-40, 150 mM NaCl, 1 mM PMSF (lysis buffer). Lysates were cleared by centrifugation and passed over a normal rat IgG-Sepharose column followed by the specific antibody column. The column was washed with lysis buffer followed by buffer I (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.2% NP 40) and buffer II (20 mM Tris/HCl, pH 8.0, 0.05% NP-40). Subsequently, the column was washed with buffer III (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 50 mM β-octylglucoside [BOG]) and then eluted with 100 mM diethylamine/HCl, pH 11.5, 150 mM NaCl containing 50 mM BOG. Peak fractions of the eluate were neutralized and analyzed by SDS-PAGE after labeling with ^{125}I using Iodobeads (Pierce, Oud-Beijerlandt, The Netherlands) or by silver staining. VCAM-1 and ICAM-1 were isolated by affinity chromatography from lysates of spleen, liver, and kidney of animals treated with TNF-α for 16 h.

Cell-binding Assay. Isolated antigen in BOG was diluted 1:10 to 1:30 with 10 mM Tris/HCl, pH 8.0, 150 mM NaCl and coated to Labtek glass chamber slides (Nunc, Wiesbaden, FRG) for 16 h at 4°C. Fibronectin (Sigma) in PBS was coated at a concentration of 10 μg/ml for 16 h at 4°C. Wells were blocked with 2% BSA in PBS or 1% OVA in TBS for 2 h at room temperature, washed with HBSS containing 10 mM Hepes, 2 mM Ca^{2+}, and 2 mM Mg^{2+} (binding buffer), and used for the assay. For binding, cells (5–10 x 10^6/ml) were suspended in the same buffer and 0.2-ml aliquots were added to the coated slides. The binding assay was performed for 30 min at room temperature without shaking and the slides were fixed in 2% glutaraldehyde/PBS after briefly dipping into PBS. For antibody- or peptide-blocking studies, cells were preincubated with purified antibody (40 μg/ml final concentration or as otherwise indicated) or peptides (150 μg/ml or otherwise indicated) for 10 min at room temperature and then transferred to the chamber slides. In some experiments, cells and coated slides were incubated separately with antibody and then washed before the experiment. Cation dependence was determined by preincubation of cells with 5 mM EDTA or 5 mM EGTA in HBSS with 10 mM Hepes for 10 min and then used for the assay. For Mn^{2+} activation, the Ca^{2+} and Mg^{2+} ions in the buffer were substituted with 0.5 mM Mn^{2+}. For PMA activation, the cells were incubated in 50 ng/ml PMA for 10 min before the assay. Cell binding was measured by counting six independent fields at a magnification of 10 by video microscopy using IMAGE 1.47 software.

Biochemical Analysis. Lactoperoxidase-catalyzed iodination of surface glycoproteins on intact cells, cell lysis in the presence of NP-40, immunoprecipitation using protein G–Sepharose or protein A/MAR, treatment of precipitates with endoglycosidase F/N-glycosidase F (Boehringer Mannheim, Mannheim, FRG) has been

described elsewhere (41). SDS-PAGE was performed on 10% slab gels. Peptide mapping was carried out as described (43). Briefly, iodinated 80-, 70-, and 34-kD fragments, respectively, were electrophoretically separated, eluted from the gel, and digested with papain (2 μg/ml) in 125 mM Tris/HCl, pH 6.8, containing 0.5% SDS, 10% glycerol, and 0.0001% bromphenol blue for 2 h. Digestion was stopped by boiling, and 2-ME and SDS were added to a final concentration of 10% and 2%, respectively. The samples were separated on a 15% SDS-PAGE. Gels were dried and exposed to x-ray–sensitive films (Kodak X-Omat; Siemens, Mannheim, FRG).

Results

mAb 5/3 is Specific for the α4 Subunit. mAb 5/3 was produced by immunizing rats with activated bend3 endothelioma cells. The antibody was initially selected because of its ability to block the binding of thymocytes to the activated endothelioma cells. mAb 5/3 stained the endothelioma cells and also reacted with lymphocytes in FACS® analysis (Becton Dickinson). Functional studies showed that the antibody not only blocked the binding of lymphocytes to endothelioma cells but also to fibronectin. This observation suggested that it might react with α4 integrins. Biochemical analyses with the antibody were carried out using ^{125}I surface-labeled ESb lymphoma cells. These cells express α4 and an unknown β chain. Fig. 1 *A* (lane *1*) shows that mAb 5/3 precipitated a major band of ~150 kD and two additional bands of 80 and 70 kD. The 150-kD band represents the intact α4 chain and the smaller bands are proteolytic cleavage fragments of the α4 chain that exist in the membrane (44, 45). A β chain is not visible under the solubilisation conditions since in the mouse, the α4β heterodimers are not stable in the absence of Ca^{2+} and Mg^{2+} ions (13, 46). Treatment of the precipitate with endoglycosidase F (Endo F) to remove N-linked glycans decreased the apparent mass of the bands to 92, 60, and 50 kD, respectively (lane *2*). The 7/1 control antigen was reduced from ~50 to 34 kD after Endo F treatment (lanes *3* and *4*). Additional experiments indicated that mAb 5/3 and the established α4-specific mAb PS/2 precipitated identical protein bands from ESb cells (lanes *5* and *6*). Both mAbs were also compared for their ability to precipitate α4 integrins from TK-1 cells (α4β7) that were solubilized in the presence of Ca^{2+} and Mg^{2+} ions. Under these conditions, both mAbs showed the expected α4 chains plus the β7 chain (lanes *8* and *9*) that migrated at ~130 kD under nonreducing conditions.

Fig. 1 *B* shows that mAb 5/3 stained TK-1 cells (α4β7) and L1-2 cells (α4β1) similar to mAb PS/2. In contrast to PS/2, which cross-reacts with human α4 integrins, the mAb 5/3 did not stain human PBLs, suggesting that the two antibodies reacted with different epitopes on the α4 chain.

To localize the mAb 5/3 epitope on the α4 subunit, the α4β7 heterodimer was isolated from iodinated TK-1 cells using mAb PS/2 bound to protein G. After washing, the bound antigen was eluted at pH 11.5, neutralized, and reprecipitated using mAb 5/3–Sepharose or control-Sepharose. Fig. 1 *D* shows that in the absence of divalent cations the heterodimer dissociated since the mAb 5/3 only reprecipitated the 150-kD α4 chain and the 80-kD subfragment (lane *3*). The control Sepharose did not precipitate any component of the α4β7 heterodimer (lane *2*). These results suggested that the mAb 5/3 epitope was located on the NH_2-terminal 80-kD portion of the α4 subunit.

The Purified α4 Subunit Binds Lymphocytes. We investigated whether the isolated α4 subunit could bind cells. The antigen was isolated on a mAb 5/3–Sepharose column initially from ESb lymphoma lysate and the washing steps were carried out in the absence of Ca^{2+} and Mg^{2+} ions to favor loss of the β chain. Bound material was eluted at pH 11.5 and the individual fractions were tested in ELISA. Antigen containing fractions were strongly reactive with mAb PS/2 to the α4 subunit (not shown). When the peak fraction was iodinated and reanalyzed by SDS-PAGE, the expected bands of 150, 80, and 70 kD were seen for the α4 subunit (see Fig. 1 *C*, lane *1*). In addition, minor bands at ~60, 46, and 34 kD, respectively, were observed. These bands were not detectable in the control antigen (7/1) obtained from the same lysate (Fig. 1, *C*, lane *2*), suggesting that they represented most likely degradation products generated during the elution procedure (see also discussion of the 34-kD band below).

The affinity-purified α4 subunit was coated to glass slides in increasing amounts and residual binding sites were blocked. As shown in Fig. 2 *A*, thymocytes adhered to the immobilized α4 subunit in a concentration-dependent fashion. No binding was seen to the control antigens 7/1 (Fig. 2 *A*) or HSA (see Fig. 2 *B*) isolated from the same lysate. As illustrated in Fig. 2 *B*, high levels of binding were observed with resting T lymphocytes or Con A–activated T cell blasts.

To rule out the possibility that small amounts of β chain were copurified under the isolation conditions, the α4 subunit was also isolated from the α4β7-positive TK-1 cells since antibodies to the β7 chain are available. The 5/3 column–eluted material could also promote cell binding and revealed in ELISA a strong reactivity with mAb PS/2 but not with the β7-specific mAb Fib 30 (15) or with mAb DATK32, respectively, recognizing a combinational epitope of the α4 and β7 subunit (17; data not shown).

Integrins Are Involved in the Binding to the α4 Subunit. Fig. 3 shows that the binding of thymocytes to the α4 subunit was cation dependent since pretreatment of the cells with EDTA or EGTA abolished binding. It was fully restored when Ca^{2+} and Mg^{2+} ions were added back to the assay (data not shown). Incubation at 4°C also inhibited adhesion. The binding of cells was enhanced by pretreatment with PMA and Mn^{2+}. The adhesion of thymocytes to fibronectin (Fig. 3) paralleled the binding to the α4 subunit and was equally affected.

The divalent cation requirement and temperature dependency suggested that the binding to the α4 subunit might involve integrins. To further support this conclusion, we tested α4-positive or α4-negative cells for their ability to bind. The T cell hybridoma BI 141 and the T lymphoma Eb do not express α4 integrins, as shown by FACS® staining with mAbs PS/2 or 5/3, respectively. We compared the binding of these

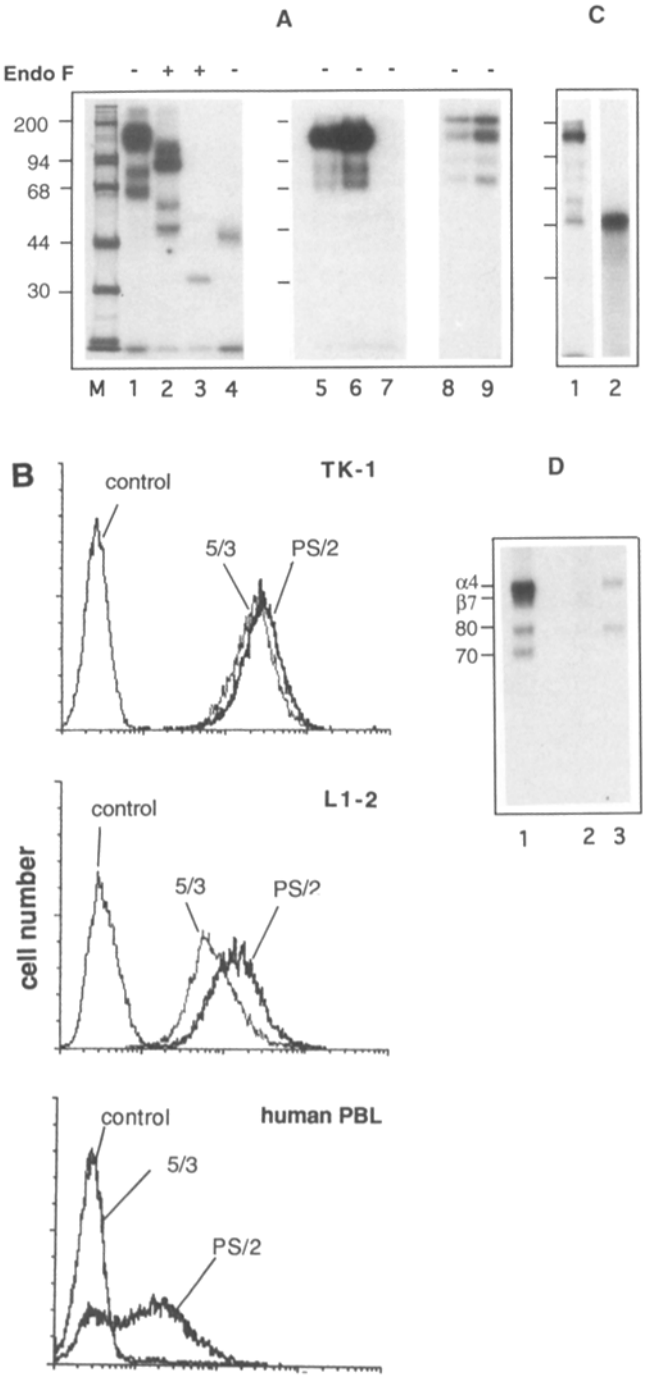

Figure 1. mAb 5/3 is a novel α4 subunit–specific antibody. (*A*) ESb lymphoma cells were labeled with ^{125}I and lysed in the absence of Ca^{2+} and Mg^{2+} ions. After incubation with primary mAbs, the immunocomplexes were precipitated using Sepharose-bound protein G or protein A/MAR (for mAb 7/1). Half of the precipitated material was treated with Endo F to remove N-linked glycans. TK-1 lymphoma cells were labeled with ^{125}I and lysed in the presence of Ca^{2+} and Mg^{2+} ions to preserve the α4β7 heterodimer. After incubation with primary mAbs, the immunocomplexes were harvested using Sepharose–protein G. Samples were analyzed by SDS-PAGE. Lanes *1*, ESb, mAb 5/3; lane *2*, ESb, mAb 5/3 Endo F treated; lane *3*, ESb, mAb 7/1 Endo F treated; lane *4*, ESb, mAb 7/1; lane *5*, ESb, mAb 5/3; lane *6*, ESb, mAb PS/2; lane 7, ESb, negative control protein G only; lane *8*, TK-1, mab 5/3; lane *9*, TK-1, PS/2. Note that samples in lanes *8* and *9* were run under nonreducing conditions. (*B*) Indirect immunofluorescence staining with mAb 5/3 and the α4 integrin mAb PS/2. Cells (TK-1, L1-2, human peripheral blood leukocytes) were stained by indirect immunofluorescence using mAb 5/3 or PS/2 followed by PE-conjugated goat anti–rat IgG. For negative control, the first antibody was omitted. (*C*) Analysis of affinity-purified α4 chain. The 4α subunit was isolated from ESb cells by affinity purification on 5/3-Sepharose in the absence of Ca^{2+} and Mg^{2+} ions and eluted with 50 mM diethylamine/HCl, pH 11.5, 150 mM NaCl containing 50 mM BOG. An aliquot of the sample was iodinated and reanalyzed (lane *1*). For control, the 7/1 antigen isolated from the same lysate is shown (lane *2*). Note that the 34-kD fragment (see text) is visible only after longer exposure of the gel and is not seen here. (*D*) Epitope localization for mAb 5/3. TK-1 lymphoma cells were labeled with ^{125}I and lysed in the presence of Ca^{2+} and Mg^{2+} ions to preserve the α4β7 heterodimer. After incubation with mAb PS/2 adsorbed to Sepharose–protein G the complex was eluted with 100 mM diethylamine/HCl, pH 11.5, 150 mM NaCl containing 50 mM BOG, neutralized and reprecipitated by the addition of mAb 5/3–Sepharose. Lane *1*, TK-1, mAb PS/2 (input); lane *2*, reprecipitated 7/1-Sepharose; lane *3*, reprecipitated 5/3-Sepharose. The positions of molecular mass marker proteins (^{14}C rainbow markers; Amersham) designated in kilodaltons are shown on the left margins.

cells with TK-1 ($\alpha4\beta7^+$) and L1-2 ($\alpha4\beta1^+$) cells. As shown in Fig. 4, both TK-1 and L1-2 cells adhered well to the α4 subunit, the binding being further increased after PMA pretreatment. In contrast, BI 141 and Eb cells showed low level binding that was slightly enhanced after pretreatment of the cells with PMA but remained low (Fig. 4).

Human PBL bound weakly to the α4 subunit. The binding was, however, strongly enhanced after PMA treatment of the cells (Fig. 4).

Inhibition of Binding in the Presence of α4-specific Antibodies. Fig. 5 *A* shows that antibodies to LFA-1 or to CD45 did not effect the cell binding to the α4 subunit. In contrast, antibodies PS/2 and R1-2 directed against α4 integrins were potent inhibitors. Similar blocking with α4-specific antibodies was observed when resting T lymphocytes or TK-1 cells instead of thymocytes were studied (data not shown).

mAb 5/3 also blocked the binding of thymocytes to the purified α4 subunit in a dose-dependent manner (Fig. 5 *B*). To determine whether the blocking effect occurred at the immobilized α4 subunit or at the cell surface, the coated glass slide and the cells were preincubated separately with mAb 5/3 and then washed to remove the antibody. As shown in Fig. 5 *B*, preincubation of cells with mAb 5/3 blocked cell binding to the α4 subunit. Also the binding to fibronectin was fully blocked (not shown). Preincubation of the α subunit coated to the glass slide did not affect the binding of cells (Fig. 5 *B*). This suggested that in order to block, the antibody had to first bind to the lymphocyte cell surface. The data also indicated that two distinct epitopes on the α4 chain existed: a binding epitope that can be blocked by mAb 5/3 and an attachment epitope(s) that is not blocked by this antibody.

The binding of human PBLs to the α4 subunit was blocked in the presence of mAb PS/2 (which cross-reacts with human cells) but not mAb 5/3 (data not shown). This is in agreement with the observation that mAb 5/3 does not cross-react with human cells (Fig. 1 *B*).

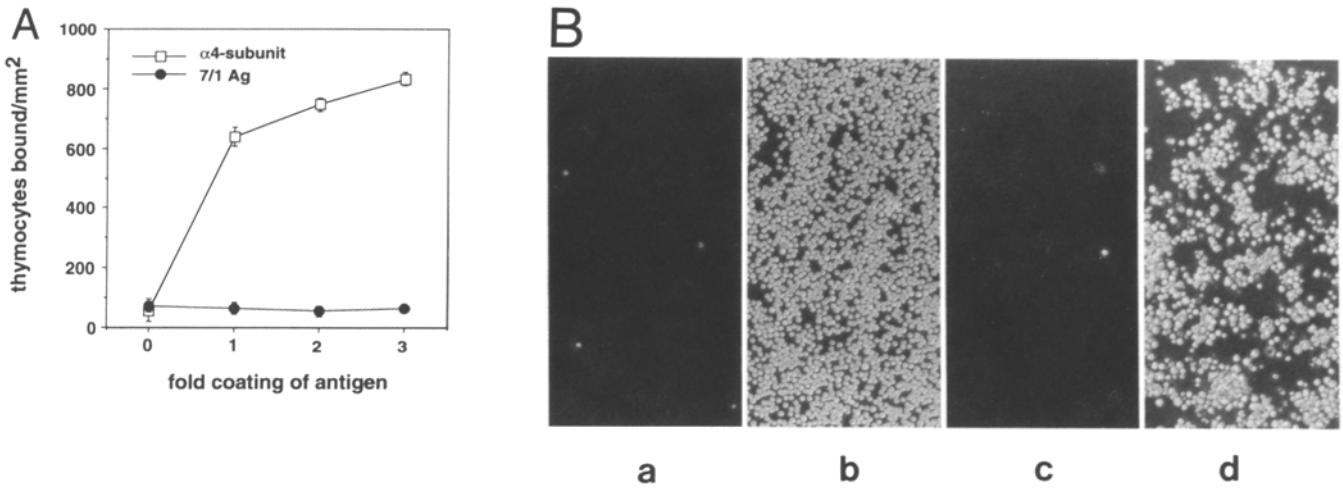

Figure 2. Binding of lymphocytes to the α4 subunit. The integrin α4 chain or control antigen in BOG were coated to glass slides and were blocked with 1% OVA. Cells in HBSS/10 mM Hepes containing Ca^{2+} and Mg^{2+} were tested for binding. (*A*) Dose–response curve for the binding of thymocytes to the α4 subunit or to 7/1 Ag (control antigen). (*B*) Binding of splenic T lymphocytes binding to HSA (*a*) or to the α4 subunit (*b*). Binding of Con A–activated splenic T cells to HSA (*c*) or to the α4 subunit (*d*).

Characterization of an α4 Chain Fragment. Upon prolonged storage, we noticed that the isolated α4 subunit was degraded, yet could still mediate cell binding when coated to glass slides. When bound and reeluted from the mAb 5/3 column, the material retained the ability to bind cells. We therefore purified the active fragment by Mono Q ion exchange chromatography. Individual fractions were analyzed for cell binding ability. As shown in Fig. 6, the binding activity eluted with the major protein peak from the column. When iodinated and analyzed by SDS-PAGE, a prominent band migrating at ~34 kD under reducing conditions was detected (Fig. 7 *A*, lane *1*). Treatment with Endo F showed that the 34-kD protein fragment was resistant (lanes *4* and *5*), whereas the 7/1 control antigen (lanes *2* and *3*) was cleaved under these

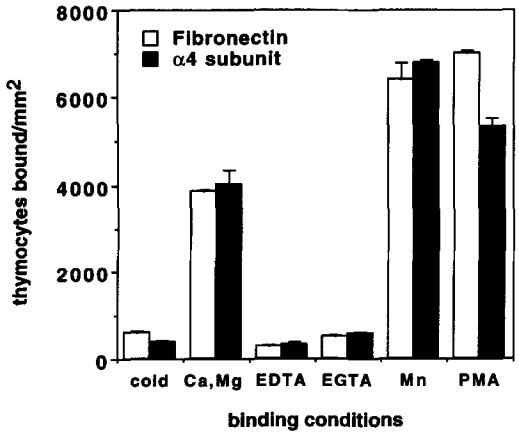

Figure 3. Requirements for the binding of thymocytes to the α4 subunit. Temperature and ion requirements for the binding of thymocytes to the α4 subunit or fibronectin.

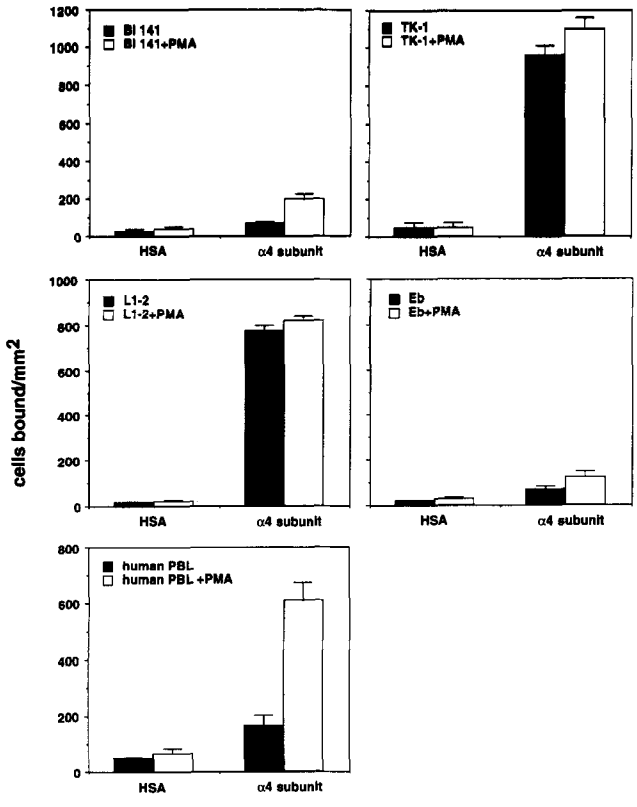

Figure 4. Differential binding to the α4 subunit by α4-positive and -negative cells. Binding of BI 141 (α4 negative), Eb cells (α4 negative), TK-1 cells (α4β7), or L1-2 cells (α4β1) and human PBL to the α4 subunit or to HSA. Cells were also pretreated with PMA for 15 min before the assay.

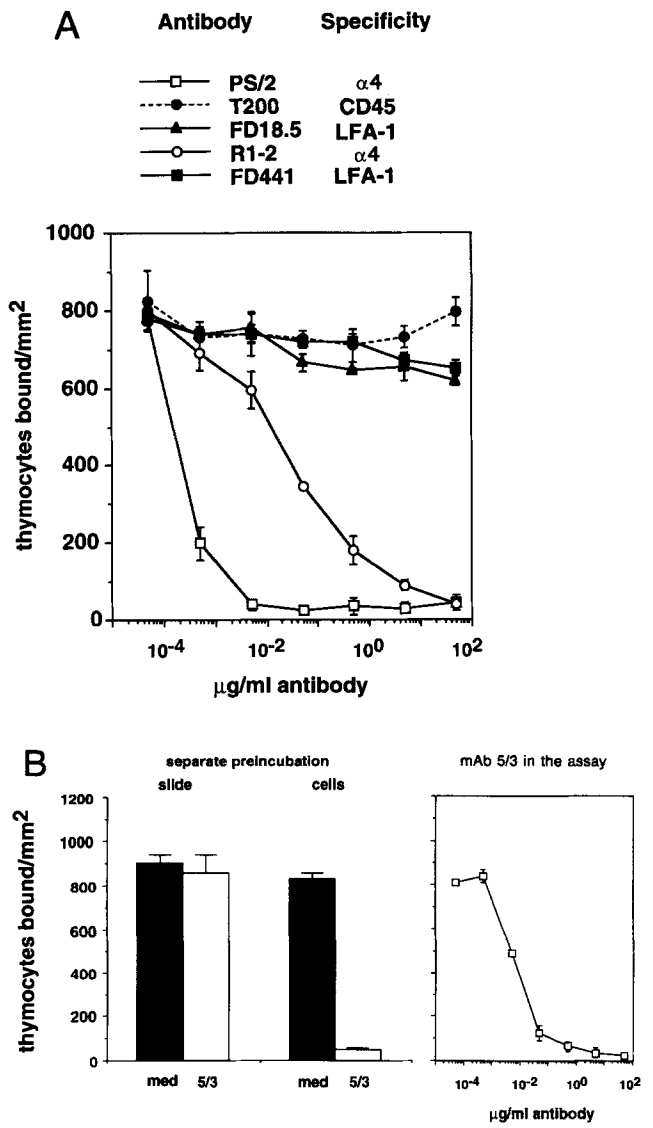

Figure 5. Antibody inhibition of thymocyte binding to the α4 subunit. (*A*) Inhibition of thymocyte binding to the α4 chain by antibodies to α4 integrins (R1-2 and PS/2) but not by LFA-1 antibodies (FD441 and FD18.5) or a CD45 control antibody. (*B*) Blocking of mAb 5/3 requires binding to cells. Either thymocytes or the α4 subunit–coated glass slide were preincubated with mAb 5/3 for 15 min, washed twice, and used in the binding assay.

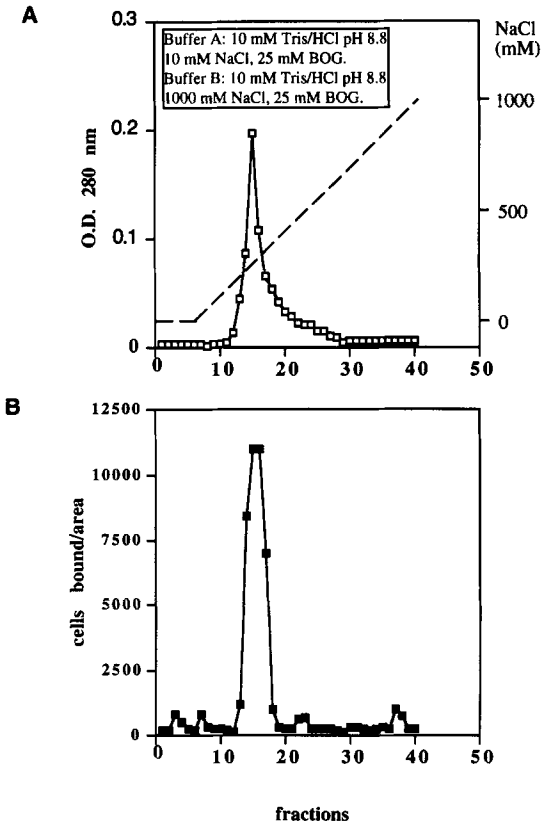

Figure 6. Isolation of a 34-kD fragment derived from the α4 subunit. Ion exchange chromatography on a Mono Q column. Degraded α4 subunit was dialyzed against buffer A, bound to the column, and eluted with a linear gradient of buffer B. An aliquot of each fraction was diluted 1:20 in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl and coated to Labtek glass chamber slides for 16 h at 4°C. The binding of thymocytes was tested as outlined in Materials and Methods.

conditions. The 34-kD protein migrated at ~25 kD under nonreducing conditions that implicated the presence of intrachain disulfide bridges (not shown). It could be reprecipitated as expected with mAb 5/3–Sepharose (Fig. 7 *B*, lane *3*) but not by control antibodies coupled to Sepharose (lanes *2*, *4*, and *5*), suggesting that it was a fragment of the α4 subunit.

The NH_2 terminus of the 34-kD fragment was blocked. To determine the location within the α4 subunit, peptide mapping analyses were carried out (Fig. 7 *C*). The iodinated 80- and 70-kD fragments of the α4 subunit and the 34-kD fragment were excised from the gel and digested with papain. Gel analysis of the digested material showed shared bands in the 80- and 34-kD fragments, respectively, which were absent in the 70-kD fragment (Fig. 7 *C*). These results suggested that the 34-kD fragment was derived from the 80-kD portion of the α4 molecule. This assignment is consistent with the localization of the mAb 5/3 epitope on the 80-kD subfragment (see Fig. 1 *D*).

When the cell-binding ability of the intact α4 subunit and the 34-kD fragment were compared, no differences were observed. Also, the binding to the fragment was blocked by α4-specific antibodies and it required the presence of α4 integrins on the cell surface. Thus, the 34-kD fragment had retained the mAb 5/3 epitope and the attachment epitope involved in cell binding.

The Fragment Blocks α4 Integrin–dependent Cell Binding. The 34-kD fragment was soluble in PBS and was studied for its potential to block α4-dependent cell binding. In the presence of 2 μg/ml of the fragment the binding of thymocytes to fibronectin was inhibited by 60 ± 3%, the binding to purified VCAM-1 by 43 ± 5% (mean of three experi-

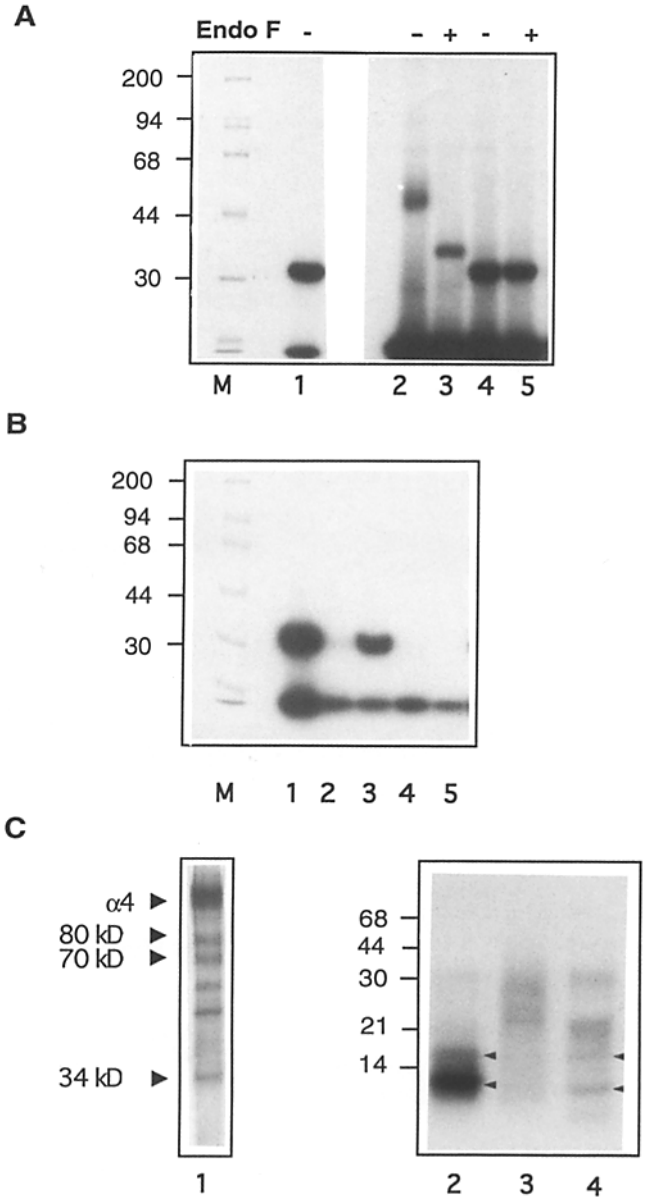

Figure 7. Biochemical characterization of the α4 Subunit Fragment. (*A*) Lane *1*, an aliquot of the peak fraction eluted from the Mono Q was iodinated and analyzed. The iodinated 34-kD fragment or the control antigen 7/1 was treated for 1 h with Endo-F and analyzed by SDS-PAGE. Lane *2*, 7/1 antigen; lane *3*, 7/1 antigen plus Endo F; lane *4*, 34-kD α4 fragment; lane *5*, 34-kD α4 fragment plus Endo F. (*B*) Reprecipitation of the iodinated fragment: lane *1*, 34-kD fragment input; lane *2*, mAb 7/1 Sepharose; lane *3*, mAb 5/3 Sepharose; lane *4*, mAb 12-15 (CD2)-Sepharose; lane *5*, mAb 324 (L1)-Sepharose. (*C*) Affinity-isolated α4 subunit was iodinated and separated by SDS-PAGE (lane *1*). The α4 subunit (150-kD), 80-kD, 70-kD, and 34-kD fragments, respectively, are indicated by arrowheads. The bands were excised from the gel and subjected to peptide mapping. Lane *2*, 34-kD fragment, papain digested; lane *3*, 70-kD fragment, papain digested; lane *4*, 80-kD fragment, papain digested. Small arrowheads at the right margin of lanes *2* and *4* denote identically migrating proteolytic fragments.

ments). Binding of thymocytes to the α4 fragment itself was blocked by >90% under these conditions, whereas the binding to purified ICAM-1, which is LFA-1 mediated, was not affected.

The α4-subunit Contains LDV Peptide Motifs. The ability of the fragment to partially block the binding to VCAM-1 and fibronectin prompted us to search for structural similarities between the α4 subunit and its ligands. The LDV peptide sequence within the CS-1 peptide of fibronectin was shown to be the minimal sequence to promote α4β1-mediated cell binding (39, 47). Related sequences like IDS or LDT appear to be important in the binding to VCAM-1 or MAdCAM-1, respectively (48–51; Briskin, M. J., and E. C. Butcher, manuscript submitted for publication). We used these amino acid motifs to screen the mouse α4 sequence. As shown in Table 1, the α4 subunit contains three LDV motifs that are conserved in the human α4 sequence. The LDV-α(1) is likely to be shared between the intact subunit and the 34-kD fragment.

Peptide-mediated Inhibition of Cell Binding. To test whether the LDV sites were important in the cell binding to the α4 subunit, peptides covering all three LDV sites were synthesized and used for inhibition studies. Fig. 8 *A* shows that at 150 μg/ml, the LDV-α4(1) peptide was the only peptide that could efficiently inhibit the binding of thymocytes to the 34-kD fragment. The CS-1 peptide from fibronectin could also block the cell adhesion; however, it required approximately fivefold higher concentration. Similar results were observed when the intact α4 subunit was used as substrate (data not shown). A mixture of the LDV-α4 peptides could partially block the adhesion of thymocytes to fibronectin (Fig. 8 *B*). The binding to fibronectin was also inhibited by CS-1 peptide, as well as by mAb 5/3. The degree of inhibition seen with both reagents suggested that VLA-5, beside α4

Table 1. *Amino Acid Motifs of α4 Integrin Ligands Involved in Cell Binding*

Species	Protein	Sequence
Hu/Ra/Bo	FN CS1	P E I L D V P S T V
Ch	FN CS1	P D M L D V P S V D
Xe	FN CS1	P E I L D V P T D E
Hu/Ra/Bo/Ch	FN H1	T T A V D S P S N L
Hu/Ra	VCAM-1 D1	R T Q I D S P L N G
Mu	VCAM-1 D1	R T Q I D S P L N A
Hu	VCAM-1 D4	R T Q I D S P L S G
Mu/Ra	VCAM-1 D4	R T Q I D S P L N G
Mu	MAdCAM-1 D1	W R G L D T S L G S
Mu	α4 (1)	N V S L D V H R K A
	α4 (2)	S F L L D V S S L S
	α4 (3)	F N V L D V Q T T T
Hu	α4 (1)	N M S L D V N R K A
	α4 (2)	S F L L D V S S L S
	α4 (3)	F N I L D V Q T T T

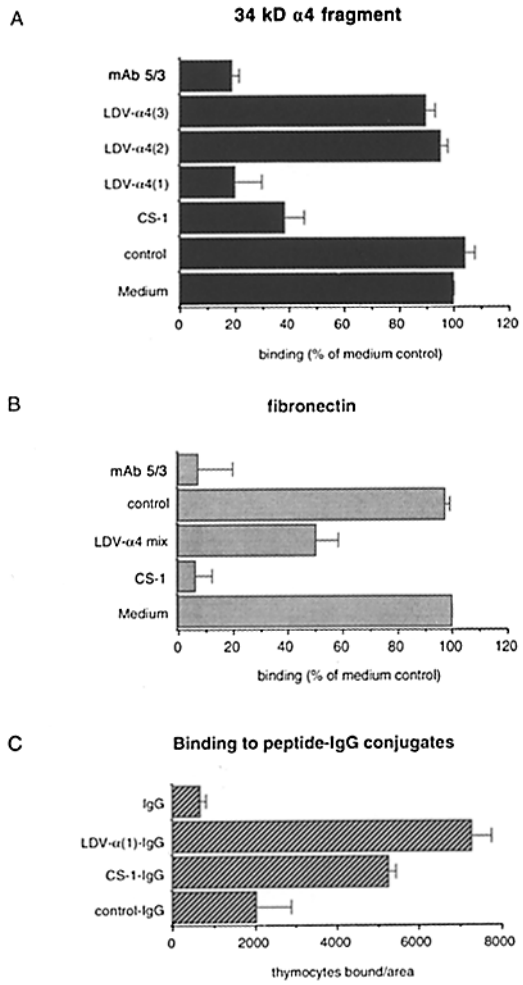

Figure 8. The role of LDV peptide motifs in the binding to the α4 subunit. (*A*) Inhibition of thymocyte binding to the 34-kD fragment using peptides. Cells were preincubated with peptides at 150 μg/ml (CS-1 peptide at 700 μg/ml) for 10 min and then tested for binding to the 34-kD α4 fragment. (*B*) Inhibition of thymocyte-binding to fibronectin using peptides at the same concentration as in *A*. (*C*) Binding of cells to peptide–IgG conjugates. CS-1 peptide, the LDV-α4(1) peptide, and a negative control peptide were coupled to rabbit IgG, coated to glass slides, and tested for the binding of thymocytes as described in Materials and Methods.

integrins an additional fibronectin receptors on thymocytes (52) is either not active or present in too low amounts to contribute to the binding.

The LDV-α4 Peptide Promotes Cell Binding. To demonstrate binding of cells to the LDV-α4(1) site, the peptide CVLFYNVSLDVHRKAESPSRF was conjugated to rabbit IgG as carrier and was analyzed for its cell-binding capacity. As shown in Fig. 8 *C*, the LDV-α4 peptide was as potent as the CS-1 peptide in promoting cell adhesion. A control peptide–IgG or rabbit IgG alone had only little or no effect.

Discussion

The α4 integrin ligands comprise a diverse group of molecules with different structure and location. VCAM-1 and MAdCAM-1 belong to the Ig superfamily, whereas fibronectin is a plasma and extracellular matrix component. Invasin, another α4 integrin–binding protein, is an outer membrane protein of *Yersinia pseudotuberculosis* that uses β1 integrins, including VLA-4 on human T cells as a receptor to enter the cell (53, 54). In this report, we demonstrate that the α4 subunit itself can be a ligand for α4 integrin–dependent lymphocyte binding.

Our studies were initiated by a novel mAb against α4 integrins, which was obtained by immunizing with the brain-derived endothelioma bend3. The identification of such an antibody was not surprising since bend3 cells could be stained with mAb PS/2 and α4 integrins can be detected on brain endothelial cells in vivo (Engelhardt, B., and E. C. Butcher, unpublished data). Studies using the novel mAb 5/3 in comparison to the established mAb PS/2 indicated that both antibodies recognized the α4 chain and that the epitope for mAb 5/3 was located on the NH_2-terminal 80-kD fragment of the α4 subunit.

To investigate the functional ability of the α4 subunit, we used the mAb 5/3 for affinity purification of the antigen from cell lysates. Surprisingly, when immobilized to glass slides, the purified α4 subunit could avidly bind thymocytes or various lymphoid cell populations, whereas control antigens isolated from the same lysate could not. The cell adhesion was temperature dependent, required divalent cations, and was enhanced by PMA or Mn^{2+} ions, suggesting that integrins were involved. Antibody-blocking studies demonstrated that mAbs to α4 integrins but no other antibodies were capable of inhibiting the binding to the α4 subunit. Both α4β7- and α4β1-positive mouse cells but also human peripheral blood lymphocytes could bind to the α4 chain, whereas α4-negative cells could not. Binding was also seen to a 34-kD soluble fragment derived from the α4 subunit. Biochemical data indicated that it was derived from the 80-kD portion of the α4 subunit. The requirements for the binding to the fragment were similar to the intact α4 chain. This observation ruled out the possibility that the cell binding was mediated by residual intact αβ heterodimers in the α4 preparations. Based on the amount of protein, the fragment was as active as the intact α4 subunit, indicating that it had retained a cell attachment site(s) that was present in the α4 subunit. The fragment could block α4 integrin–dependent binding of thymocytes to VCAM-1 and fibronectin but did not impair the binding of thymocytes to ICAM-1, which is LFA-1 dependent. The cross-blocking results suggested the involvement of a structurally related epitope in the binding of α4 integrins to its ligands.

Binding of α4β1 integrins to fibronectin involves the fibronectin CS-1 peptide, located in the alternatively spliced type III connecting segment (1–3, 39, 47). The LDV amino acid motif is the minimal sequence that promotes α4-dependent cell binding (39, 55). As shown in Table 1, homologous sequences to LDV are present in mouse and human VCAM-1. Mutation analysis of VCAM-1 in this position has shown that a D-A exchange introduced into the IDS sequence abolished the binding of α4 integrins (48). Other studies have

shown that residues within a conserved amino acid motif in domains 1 and 4 (included the IDS) are required for binding to VLA-4 (49, 50). CS-1 peptide also blocks the $\alpha4\beta1$-mediated binding of cells to VCAM-1 (51). The first domain of MAdCAM-1 contains the related LDT motif. Recently, a point mutation within MAdCAM-1 has been identified, which results in an L-R amino acid exchange in position 61 of the protein (Briskin, M. J., and E. C. Butcher, manuscript submitted for publication). This alters the motif from LDT to RDT in the mutant. When expressed in Chinese hamster ovary cells, the mutant MAdCAM-1 protein could no longer bind cells in a $\alpha4$-dependent manner unless the cells were activated with PMA or Mn^{2+} ions. Interestingly, grafting of the CS-1 peptide sequence onto a non-$\alpha4$ integrin-binding protein can restore $\alpha4$-mediated binding (56), suggesting that the LDV epitope can act independently of its environment. We analyzed the $\alpha4$ subunit for similar binding motifs. The sequence analysis of the mouse $\alpha4$ subunit revealed three LDV sites that are also present in the human $\alpha4$ subunit but are not found, for example, in the $\alpha6$ sequence. To analyze whether these sites were relevant for the binding, we initiated peptide inhibition studies. Based on these results, the LDV-$\alpha(1)$ site appeared to be functionally most active. At higher concentrations (>500 μg/ml), the CS-1 peptide was also able to block the binding to the $\alpha4$ subunit in a specific fashion, supporting the notion that the LDV motif could indeed be important. Moreover, the LDV-$\alpha(1)$ site, when coupled to IgG carrier protein, was as potent as the fibronectin CS-1 peptide in promoting cell binding. Thus, the $\alpha4$ integrin chain contains a potent cell-binding motif with the potential to support adhesion of lymphocytes through cell surface $\alpha4$ integrins. It is presently unknown whether this ability is also preserved in the intact $\alpha4$ integrin heterodimer. Although our studies do not address the potential importance of conformational alterations that might be induced during isolation and coating, it should be pointed out that little is known about the range of states assumed by $\alpha4$ integrins expressed on the cell surface; the existence of free α chains has been suggested based on studies of cell lines expressing $\alpha4$ in the absence of known or detectable novel pairing β chain (18, 57).

Recently, Weissman and colleagues have reported that transfection of $\alpha4$ into a $\beta1$-expressing melanoma cell results in enhanced cell–cell interactions in an assay involving adhesion of suspended B16 melanoma cells to an adherent melanoma monolayer (58). Enhanced adhesion was dependent on $\alpha4$ expression on both the suspended and monolayer populations, leading to the hypothesis that $\alpha4$ integrins might in this setting participate in homotypic molecular interactions. Our study demonstrating that the $\alpha4$ integrin chain itself contains motifs capable of serving as potent $\alpha4$ integrin binding ligands is consistent with this model, and it raises the possibility that regulated $\alpha4\beta1$ and $\alpha4\beta7$ interaction with cell surface $\alpha4$ represents an important new pathway for the control of cell–cell interactions. Formal proof of the involvement of $\alpha4$ as a ligand in particular cell–cell adhesion events must await mutagenic analysis, permitting expression of $\alpha4$ integrins displaying recognition but not ligand activity, or vice versa.

In summary, this paper characterizes a novel ligand for $\alpha4$ integrins, namely the $\alpha4$ subunit itself. It will be important to determine under what physiological conditions $\alpha4$ is available as a ligand for cell–cell interactions, and whether this activity can be regulated coordinately or independently of conventional $\alpha4$ integrin functions. In conjunction with the recent evidence that $\alpha3$ integrins can interact in a homotypic fashion (59), our results suggest a novel mechanism by which integrins may participate in the complex regulation of cell–cell interactions.

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