Journal of Cellular Biochemistry 83:304–319 (2001)

Critical Amino Acid Residues of the α4 Subunit for α4β7 Integrin Function

Yvonka Zeller, Sabine Mechtersheimer, and Peter Altevogt*

Tumor Immunology Programme, G0100, German Cancer Research Center, D-69120 Heidelberg, Germany

Abstract A characteristic feature of integrin–ligand interactions is the requirement for divalent cations. Putative cation binding sites have been identified in the α and β subunit of the α4 integrins, α4β1 and α4β7, and within their ligands which display the tripeptide LDV in fibronectin and homologous motifs in VCAM-1 and MAdCAM-1. The extracellular domain of the murine and human α4-subunit contains three conserved LDV motifs, designated LDV-1 to -3. Using site directed mutagenesis and transfection studies, we now examined the functional relevance of the LDV motifs for α4β7 integrins. We present evidence that LDV-1 mutants (D489N) behave like α4 wt cells, but LDV-3 mutants (D811N) are impaired in α4β7 integrin-triggered homotypic cell aggregation and in adhesion and spreading on α4 specific ligands. Further characterization of LDV-3 mutants revealed a defect in mAb-induced α4β7-cell surface cluster formation. Mutation of the LDV-2 motif (D698N) caused loss of α4β7 integrin cell surface expression. Our results indicate: (i) that LDV-3, located proximal to the cell membrane, is important for α4β7 integrin-triggered functions and for lateral clustering and (ii) that LDV-2 affects α4β7 heterodimer stability. J. Cell. Biochem. 83: 304–319, 2001.

Key words: cell aggregation; cell adhesion; alpha4 integrins; integrin clustering

Integrins belong to a superfamily of cell surface receptors, which mediate cell–cell or cell–extracellular matrix (ECM) interactions. They are heterodimeric transmembrane glycoproteins consisting of a noncovalently linked α and β subunit. Two α4 integrins are known, α4β1 (very late antigen [VLA] -4) and α4β7 (lymphocyte Peyer's patch adhesion molecule [LPAM]-1), respectively. The α4 integrins are widely expressed on different cell types, including hematopoietic progenitors, lymphocytes, natural killer cells, monocytes, eosinophils, and basophils [for review, see Wagner and Müller, 1998]. In addition, α4β1 is also expressed on various nonhematopoietic tumor cells such as melanomas and on fetal myoblasts [for review, see Holzmann et al., 1998]. The α4 integrins play an important role in inflammation and immune surveillance, and are involved in various developmental and physiological processes like lymphocyte migration, mouse skeletal muscle formation, placental or cardiac development, and hematopoiesis [Wagner and Müller, 1998; Arroyo et al., 1999]. Furthermore, α4 integrins are critically involved in the pathogenesis of diseases such as autoimmune encephalomyelitis [Yednock et al., 1992], arthritis [Morales-Ducret et al., 1992], and autoimmune gastritis [Barrett et al., 2000]. Investigation of the structural basis of α4 integrin–ligand interactions is of fundamental importance for understanding these physiological and pathophysiological processes and may lead to the development of new therapeutic agents aiming at modulating these binding interactions.

Ligands for α4β1 and α4β7 include fibronectin (FN) and vascular cell adhesion molecule-1 (VCAM-1), a cell surface molecule expressed

Abbreviations used: BSA, bovine serum albumin; ECM, extracellular matrix; FBS, fetal bovine serum; FN, fibronectin; HEV, high endothelial venules; LDV, leucin-aspartic acid-valine; LPAM, lymphocyte Peyer's patch adhesion molecule; MAdCAM, mucosal addressin cell adhesion molecule; mAb, monoclonal antibody; 2-ME, 2-mercaptoethanol; PBS, phosphate buffered saline; PE, phycoerythrin; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction VCAM, vascular cell adhesion molecule; VLA, very late antigen; wt, wildtype.

Grant sponsor: Deutsche Forschungsgemeinschaft to P.A.

*Correspondence to: Peter Altevogt, Tumor Immunology Programme, G0100, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.
E-mail: p.altevogt@dkfz-heidelberg.de

Received 4 December 2000; Accepted 27 March 2001

DOI 10.1002/jcb.1197

on activated endothelium. α4β7, but not α4β1 can bind to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on high endothelial venules (HEV) in mucosal associated lymphoid tissues [Holzmann et al., 1998; see Wagner and Müller, 1998]. Although lymphocytes do not express any of the known ligands for α4-integrins, certain antibodies to α4 were found to induce homotypic aggregation of lymphoid cells which is blocked by other α4 or β7/β1 specific antibodies [Campanero et al., 1990; Rüegg et al., 1992; Andrew et al., 1994]. The molecular mechanism of α4-triggered homotypic aggregation is still poorly understood.

As common for all integrins, α4 integrin–ligand interaction depends on divalent cations. Consistent with this, divalent cation binding sites were found in integrin α and β chains. Three putative divalent cation binding sites have been identified in the 5^{th}, 6^{th}, and 7^{th} homolougous sequence repeat of the amino-terminal domain of α4 that resemble the EF-hand motifs seen in other divalent cation binding proteins [Tuckwell et al., 1992]. Amino acid substitution of key residues within each of the three motifs resulted in impaired adhesion to FN and VCAM-1, confirming their importance for α4 integrin functions [Masumoto and Hemler, 1993]. However, these EF-hand like sequences lack one coordination site for complexing a divalent cation and it was assumed that an acidic amino acid residue present in the α4 ligand might complete the cation binding motif. This hypothesis was supported by identifying the binding sites in the α4 ligands, all of which contain an aspartic acid residue at a critical position. Although the concept of stabilized integrin–ligand interaction via a cation-bridge still exists, the current β-propeller model for integrin α-subunits shows the putative EF-hand sites on the lower face of the β-propeller domain opposite to the proposed ligand binding site [Springer, 1997]. According to this model, the EF-hand like motifs have been suggested to be crucial for the structural integrity of integrins rather than participating directly in ligand binding [Oxvig and Springer, 1998]. Further mutagenesis studies have identified additional amino acid residues in the extracellular portion of α4 important for α4β1 integrin functions [Irie et al., 1995; Munoz et al., 1996; Munoz et al., 1997]. Some of them are predicted to be involved in ligand binding [Irie et al., 1997], others have been suggested to stabilize α4-chain conformation [Guerrero-Esteo et al., 1998] or to affect the association of α4 integrins with other transmembrane proteins [Mannion et al., 1996]. We could show that a mutation of Asp-698 in the α4-chain interfered with the formation of a functional α4β1 heterodimer [Zeller et al., 1998]. Notably, Asp-698 is part of an leucin-aspartic acid-valine (LDV) motif. An LDV motif is the minimal essential binding sequence for α4 integrins in FN [Komoriya et al., 1991] and is homologous to the α4 binding motifs IDS and LDT defined in VCAM-1 and MAdCAM-1, respectively [Clements et al., 1994; Osborn et al., 1994; Vonderheide et al., 1994; Briskin et al., 1996]. A sequence analysis of the α4-chain revealed that three LDV motifs occur in the extracellular sequence of α4, which are conserved in mouse and human. Comparable to the ligands, we suggested a role for the α4 derived LDV motif in divalent cation binding. Support for this hypothesis came from results of other studies [Ma et al., 1995; Bazzoni et al., 1998]. In addition, it has been shown that the LDV sites within the α4-subunit could support cell binding of α4β1 or α4β7 positive cells, thus providing a possible mechanistic explanation for α4-triggered homotypic aggregation [Altevogt et al., 1995].

In the present report we decided to examine the functional relevance of the three LDV motifs for α4β7 integrin mediated functions. Using site-directed mutagenesis we have altered the three LDV motifs in the murine α4-subunit from LDV to LNV and investigated the effect on homotypic cell aggregation as well as adhesion and spreading of transfectants to FN, and MAdCAM-1. Our results indicate that two of the three mutations affected α4-integrin function, although in different ways.

METHODS

Cells

The murine T cell lymphoma line TK1, the TK1 derived subclone P1C2, and the human erythroleukemia cell line K562 (American Type Culture Collection, Rockville, MD; CCL 243) were maintained in RPMI 1640 medium supplemented with 5% (FBS), 2 mM L-glutamine, 10 mM Hepes, and 50 mM 2-ME (culture medium). Balb3T3 fibroblasts were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and 10 mM Hepes.

All cells were kept at 37°C, 5% CO_2, and 100% humidity.

P1C2 cell generation was based on the observation that in aggregation experiments (see below) more than 95% of the cells formed aggregates, whereas a small population remained as single cells in suspension. We enriched for these cells by separating them from cell aggregates using a FBS density gradient. This procedure was repeated until a homogeneous non-aggregating subpopulation of TK1 cells was obtained. The phenotype of P1C2 cells remained stable even after very long times (up to 4 month) of in vitro cultivation.

Antibodies and Reagents

The antibodies used in this study included 5/3 and PS/2 directed against the α4 integrin subunit, Fib 504, Fib 30, and LS722 against β7, DATK32 recognizing a combinatorial epitope of α4β7, HMβ-1 against the mouse β1-integrin chain, 30G12 against mouse CD45, M290 against mouse αE integrins and 12–15 against CD2 were described before [Andrew et al., 1994; Altevogt et al., 1995; Zeller et al., 1998]. Monoclonal antibodies (mAbs) were used in a purified form or as hybridoma supernatants. A MAdCAM-1-Ig fusion protein, consisting of the extracellular domains of human MAdCAM-1, fused to human IgG Fc was a gift from Dr. Mike Briskin (LeukoSite, Inc., Cambridge). FN and its fragment FN40 were obtained from Life Technologies, Inc.

Mutagenesis and DNA Transfection

The cDNAs representing the wild-type murine α4 integrin chain (α4 wt) and the mutated α4 chain carrying a D→N substitution at position 489 (mut 1) has been described before [Zeller et al., 1998]. The α4 mutants mut 2 and mut 3 contain a D→N substitution at position 698 and 811, respectively. These mutants were prepared using the mouse α4-cDNA clone in Bluescript SK+ (a gift from Dr. B. Holzmann, Technical University of Munich, Germany) as template for site-directed mutagenesis (Chameleon kit, Stratagene, Heidelberg, FRG). Sequencing of the products confirmed that only the intended mutation was introduced. The α4-cDNAs were cloned into the pcDNA3 expression vector (Invitrogen) and used for transfection studies. The mouse β7-cDNA clone in pCDM8 was obtained from Dr. G. Krissansen (University of Auckland, New Zealand). P1C2 or K562 cells were transfected via electroporation at 960 μF and 260 mV using a gene pulser (Bio-Rad). After selection with 0.6 mg/ml G418 (Life Technologies, Grand Island, NY), resistant cells were subjected to several rounds of fluorescence-activated cell sorting (FACS, see below) to enrich α4β7 expressing stable transfectants. CDNAs were also stably transfected in Balb3T3 fibroblasts as previously described [Zeller et al., 1998].

Flow Cytometry

The cell-surface staining of cells with saturating amounts of mAbs, either hybridoma supernatants or purified antibodies, and phycoerythrin (PE)-conjugated goat antibodies to rat Ig (SERVA, Heidelberg, FRG) has been described elsewhere [Zeller et al., 1998]. For the detection of cytoplasmic staining, cells were first incubated on ice with 0.5% formaldehyde in phosphate buffered saline (PBS). After washing in PBS cells were permeabilized with 0.05% saponin (Sigma) for 10 min at room temperature. The cells were washed again and stained as above. Stained cells were analyzed with a FACScan fluorescence-activated cell analyzer (Becton Dickinson, Heidelberg, FRG). For enrichment of α4β7-transfectants, the cells were stained under sterile conditions with mAb DATK32 and sorted on a FACS Vantage using counter staining with propidium iodide (PI) to exclude dead cells.

Biochemical Analysis

Lactoperoxidase-catalyzed iodination of intact cells and immunoprecipitation for the analysis of integrin heterodimer stability was carried out as as described before [Zeller et al., 1998]. Briefly, iodinated cells (2×10^7) were washed and lysed in TBS containing either 0.3% CHAPS (Sigma), 1% IPEGAL (CA-630, Sigma) or 1% IPEGAL in the presence of 2 mM Ca^{2+} and Mg^{2+} ions. Lysates were prepared by centrifugation in an Eppendorf centrifuge and precleared with rat Ig coupled to sepharose. Immunoprecipitations were carried out using mAb 5/3 coupled to sepharose or mAb Fib 504 preadsorbed to Protein G-Sepharose for 1 h at 4°C. The precipitates were washed in the respective lysis buffers and eluted from the sepharose by boiling for 2 min in non-reducing SDS-sample buffer. SDS–PAGE was performed on 7.5% slab gels. Gels were dried and exposed to X-ray sensitive films (Kodak

Biomax-MS) using the Biomax MS intensifying screen.

Isolation of RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

The isolation of total RNA from cells and the subsequent RT-PCR were performed as described [Ebeling et al., 1996]. Primers used in the RT-PCR reaction were as follows: GACATTAGCTTTCTCCTGGAG (α4 forward), TCACACTAACATTTGGAGCCA (α4 reverse), ATA GGTT TTGGCTCCTTCGTG (β7 forward), and AGTGGAGAGTGCTCA-AGAGTCACAGT (β7 reverse). PCR products were separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Cell Aggregation Assays

For aggregation experiments 3×10^5 TK1 or P1C2 cells or 9×10^4 K562 cells (more than 95% viability) were added in 100 μl culture medium to each well of a 96-well flat bottomed microtiter plate. MAb DATK32 or 30G12 for control was added to a final concentration of 0.2 μg/ml. Cells were incubated at 37°C and 5% CO_2 atmosphere. Cell aggregation was followed by observation under an inverted microscope and pictures were taken with a video camera at the indicated time points. For co-aggregation analysis, TK1 cells were fluorescently labeled with FITC as described [Butcher and Weissman, 1980], washed three times in PBS and mixed with equal number of unlabeled P1C2 cells, P1C2 transfectants, or TK1 cells for control. An aliquot of the mixture was taken for immediate FACS analysis to verify the ratio of labeled to unlabeled cells. The remaing cells were seeded into six-well plates and allowed to aggregate in the presence of stimulating mAb DATK32. After overnight culture the cells were harvested carefully in order to preserve the formed aggregates using Eppendorf tips from which the tip had been cut off. The cells were transfered to a 5 ml cushion of 100% FBS in a 15 ml tube and the cell aggregates were allowed to sediment for 10 min at room temperature. Cell aggregates that had sedimented to the bottom of the tube were isolated by sucking of the FBS, 500 μl PBS was added and the cells were dispersed by gentle pipetting. The composition of labeled and unlabeled cells in the aggregates was quantitated by FACS analysis. Each assay was carried out in triplicates.

Cell Adhesion Assays and Cell Spreading

FN or MAdCAM-1-Fc were coated to LAB-TEK glass chamber slides (Nunc, Wiesbaden, Germany) at a concentration of 10 μg/ml for 16 h at 4°C. Wells were blocked either with 1% BSA in PBS or with 10 μg/ml human serum IgG in PBS for 1 h at room temperature, washed with HBSS containing 10 mM HEPES, 2 mM Ca^{2+}, and 2 mM Mg^{2+} and used for the assay. For adhesion, cells (2×10^6/ml) were suspended in the same buffer and 0.2 ml aliquots were added to the coated slides and incubated for 10 min at 37°C. After incubation, the slides were washed once and fixed in 2% glutaraldehyde/PBS. For antibody blocking studies, cells were preincubated with purified antibody at a final concentration of 10 μg/ml for 10 min at room temperature and then transfered to the glass chamber slides. For Mn^{2+} activation, the Ca^{2+} and Mg^{2+} ions in the buffer were substituted with 0.5 mM Mn^{2+}. Cell binding was measured by counting six independent 10× fields by video microscopy using IMAGE 1.55 software. To analyze cell spreading, the plated cells were incubated at 37°C and pictures were taken after 4 h to evaluate spreading.

Immunofluorescence Analysis

The mAb DATK32 was Cy3-labeled using a labeling kit (Amersham Pharmacia Biotech, Freiburg, Germany). K562 transfectants, pretreated with 10 μg/ml human IgG to block Fc receptors, or TK1 and P1C2 transfectants were incubated in culture medium containing Cy3-conjugated DATK32 at 37°C. To analyze the distribution of α4β7 on the cell surface, cells were washed after 1 h, plated on poly-L-lysine coated glass slides (Bio-Rad) and fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. After a final wash, the coverslips were rinsed in deionized water and mounted in Elvanol. The cells were observed using a fluorescence microscope (Zeiss, Göttingen, Germany). Cells (50–100) were examined and images of representative cells were digitally recorded.

RESULTS

Characterization of an α4β7 $^-$ Variant of TK1 Cells

TK1 cells express the integrin α4β7at the cell surface and have been used before to study α4-triggered homotypic aggregation [Rüegg et al.,

1992; Andrew et al., 1994]. We used the mAb DATK32, which recognizes a combinational epitope on α4β7, to induce TK1 cell aggregation. As early as 30 min after addition of 0.2 μg/ml of mAb DATK32 the formation of cell aggregates could be observed and large aggregates were formed within 7 h, which were still visible after 24 h (Fig. 1A).

We generated a non-aggregating variant of TK1 cells, called P1C2 cells, as described in Materials and Methods. Although P1C2 cells were unable to aggregate after addition of mAb DATK32 (Fig. 1A), the anti-CD45 induced aggregation via mAb 30G12 which is α4-integrin independent [Andrew et al., 1994] remained intact, but the size of aggregates was reduced. To characterize P1C2 cells further, they were examined for the expression of different cell surface molecules by flow cytometry. In contrast to TK1 cells, P1C2 cells showed no cell surface expression of α4 and β7 as revealed by staining with an α4- or β7-specific mAb (Fig. 1B). The expression levels of other cell surface receptors, CD2 (see Fig. 1B), LFA-1, αE integrins, and ICAM-1 (not shown), were not changed significantly compared to parental TK1 cells. Immunoprecipitation analysis using ^{125}[I]-labeled cell lysates and α4- and β7-specific mAbs confirmed the complete lack of α4β7 expression on P1C2 cells (not shown). TK1 and P1C2 cells were also tested for expression of α4 and β7 message using RT-PCR analysis. As shown in Figure 1C, the β7 mRNA was present in both cell types, but α4 message was absent in P1C2 cells. Indeed, the cytoplasmic staining of permeabilized P1C2 cells with a β7-specific mAb revealed the intracellular presence of β7 protein, but in reduced amounts compared to TK1 cells. The α4 subunit was detected exclusively in TK1 cells after intracellular staining with an α4-specific mAb (data not shown).

Expression of Wild-Type and Mutant α4 Subunits in P1C2 Cells

To study the role of LDV motifs for α4 integrin functions, α4 wt and α4 chains either mutated in LDV-1, -2, or -3 were transfected into P1C2 cells. Mutated α4 chains were generated by D→N substitution at position Asp489 (mut 1), Asp698 (mut 2), or Asp811 (mut 3), thereby changing the LDV motifs to LNV. Figure 2 shows a schematic representation of the α4 chains used for transfection. The level of α4 expression was comparable for α4 wt, mut 1, mut 3, and TK1 cells as detected by fluorescent staining (Fig. 3A). The transfected α4 chain was paired with the endogenous β7 subunit at the cell surface as revealed by staining with the respective mAbs. We failed to establish mut 2 transfectants in P1C2 cells.

LDV-2 Mutation Prevents α4β7 Surface Expression

We reasoned that the failure to establish mut 2 transfectants in P1C2 cells might be related to the inability of this mutant to form a functional heterodimer. Indeed, we showed previously that Asp698 (mut 2) is critical for heterodimer formation of α4 integrins [Zeller et al., 1998]. In order to analyse this in more detail, all three mutants were stably transfected into Balb3T3 fibroblasts. We had observed before that transfection of the α4 subunit into α4-negative Balb3T3 fibroblasts can induce expression of α4β7 at the cell surface [Zeller et al., 1998]. As shown in Figure 3B, α4-wt, mut 1, and mut 3 transfected Balb3T3 cells revealed cell surface staining with α4-, β7-, and α4β7 specific mAbs. In contrast, mut 2 transfectants showed α4 but not β7 and α4β7 specific staining. We concluded that the mutation in Asp698 (mut 2) of the α4-subunit had affected the formation of the α4β7 heterodimer or its transport to the cell surface. Most likely the mut 2 α4 chain was expressed as an α4β1 heterodimer in these transfectants.

Lack of Co-Aggregation Between TK1 and P1C2 Cells

Although P1C2 cells were unable to aggregate due to the lack of α4β7 we investigated the possibility that the cells carried an unidentified ligand structure X that could engage in cell aggregation. To this end, TK1 cells were fluorescently labeled and allowed to co-aggregate with unlabeled TK1 or P1C2 cells following triggering with mAb DATK32. Cell aggregates were isolated by 1*g* sedimentation on a FBS cushion. Sedimented cell aggregates were dispersed and the composition of cells was determined by FACS analysis. As shown in Figure 4A labeled and unlabeled TK1 cells were equally represented in cell aggregates. In sharp contrast, P1C2 cells did not enter the clusters at all (Fig. 4B). Similar experiments using P1C2 transfectants revealed that P1C2 α4 mut 1 cells readily formed co-aggregates (Fig. 4C) whereas P1C2 α4 mut 3 cells showed a significant

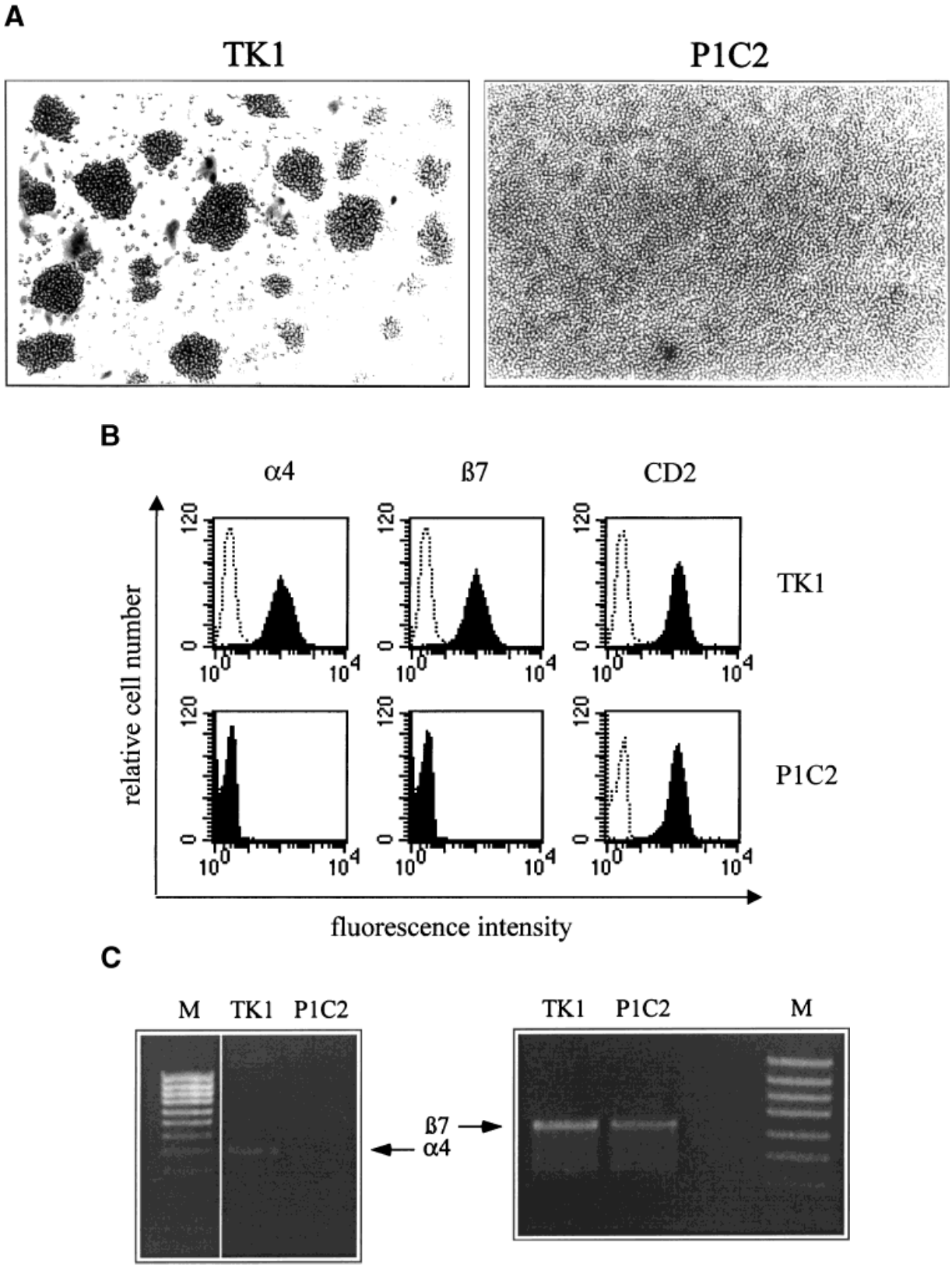


Fig. 1. Characterization of P1C2 cells. **A:** TK1 or P1C2 cells were incubated with 0.2 μg/ml of mAb DATK32 at 37°C. Seven hours later pictures were taken. Four experiments were performed with identical results. **B:** Cell surface staining of cells with the α4-specific mAb 5/3, the β7-specific mAb Fib504 or the CD2-specific mAb 12-15, respectively. **C:** Analysis of α4 and β7 mRNA by RT-PCR.

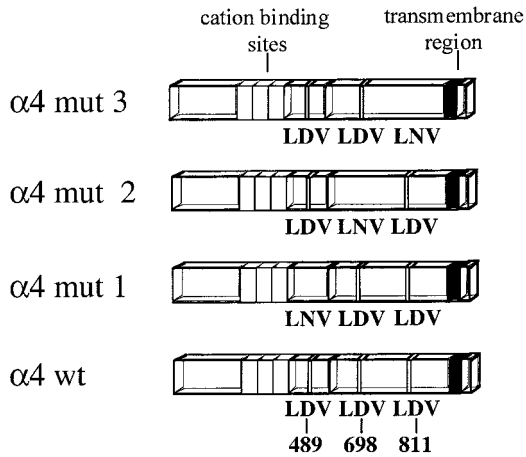


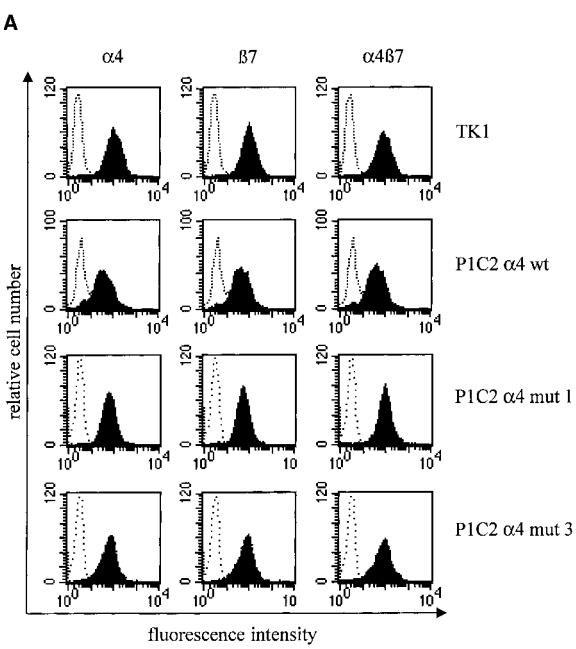
Fig. 2. Structures of mutant α4-subunits used in this study. Mutated α4 chains were generated by D→N substitution at position Asp489 (mut 1), Asp698 (mut 2), or Asp811 (mut 3). The mutations rendered the LDV motifs to LNV. Known divalent cation binding sites are also indicated.

reduction in co-aggregation (Fig. 4). Thus in P1C2 cells, the loss of α4β7 caused a loss of homotypic aggregation but also a loss of ability to co-aggregate with α β4 positive cells. Expression of the α4 mut 1 but not the α4 mut 3 chain could completely restored the ability. This suggested that the α4 mut 3 chain carried some type of defect.

Mut 3 Transfectants are Altered in Aggregation

To further analyze this phenomenon, aggregation experiments were performed with P1C2 transfectants and the results are summarized in Figure 5. After addition of mAb DATK32 the α4 wt and mut 1 transfectants formed large aggregates within 7 h comparable to TK1 cells. Mut 3 cells showed no DATK32-dependent aggregation and thus behaved like α4β7 negative P1C2 cells. In contrast, all transfectants, P1C2 and control TK1 cells could aggregate after stimulation with the CD45-specific mAb 30G12. Interestingly, aggregation of mut 3 and P1C2 cells was delayed.

We also tested the effect of the LDV muta7-tions in a heterologous expression system. Human α4β7 negative K562 cells were transfected with the α4 wt or mutated α4 subunits in combination with the β7 subunit. Stable transfectants expressing α4 wt, α4 mut 1, or mut 3 together with β7 were established. Cell surface expression of α4β7 integrins was analyzed by indirect immunofluorescence staining using mAb DATK32. As depicted in Figure 6, all transfectants expressed α4β7 at comparable



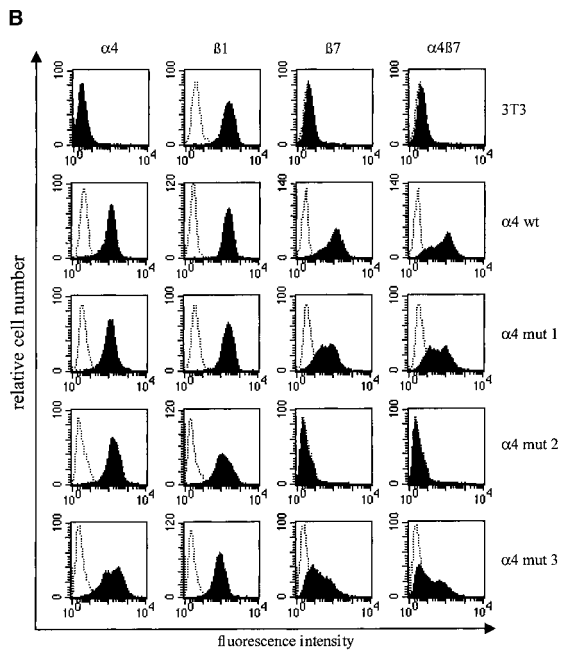


Fig. 3. Expression of α4 and β7 chains on transfected P1C2 or Balb/3T3 cells. **A:** Analysis of transfected P1C2 cells. Indirect immunofluorescence staining of the cells using mAb 5/3 (anti α4), Fib504 (anti β7) or DATK32 (anti α4β7) followed by PE-conjugated goat anti-rat IgG. For negative control the first antibody was omitted (dotted line). Note that comparable staining results were obtained with other α4 mAbs (PS/2) or β7 specific mAbs (Fib30, LS722). **B:** Analysis of transfected Balb/3T3 cells by FACS analysis with the indicated antibodies was carried out as described in (A).

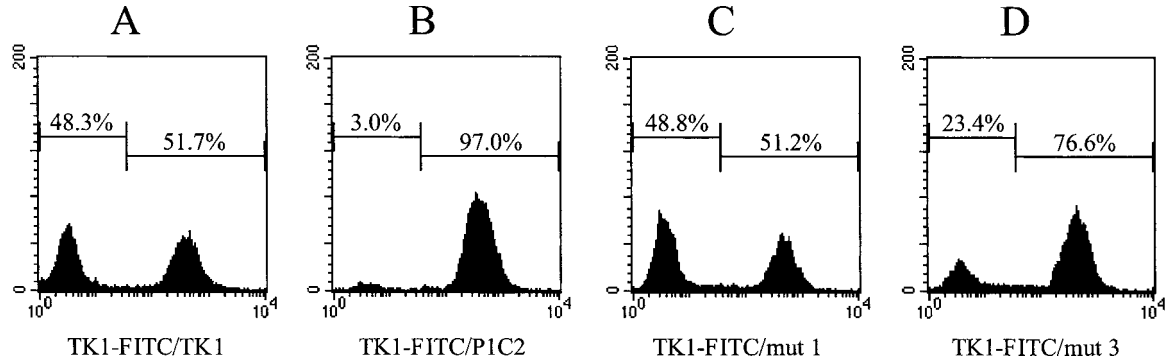


Fig. 4. Co-aggregation of TK1 and P1C2 cells. TK1 cells were FITC-labeled and mixed either with unlabeled TK1 (**A**) unlabeled P1C2 (**B**), unlabeled P1C2 α4 mut 1 (**C**) or unlabeled P1C2 α4 mut 3 (**D**) cells. Equal numbers of FITC-labeled and unlabeled cells in the mixture were confirmed by FACS analysis before the assay. Aggregation was induced by the addition of mAb DATK32 for 16 h. Cell aggregates were separated from non-aggregated cells by 1*g* sedimentation on a FCS cushion and the percentage of FITC-labeled vs. unlabeled cells in the aggregates was determined. Data presented are representative from four individual experiments with identical results.

levels, whereas α4β7 surface expression on parental K562 cells was not detectable. Analogous to the P1C2 cell system, we failed to generate α4 mut 2/β7 integrin expressing K562 cells as revealed by staining with mAb DATK32. However, by staining with mAb 5/3 the mouse α4 integrin chain could be detected at the cell surface, presumably paired with the endogenous human β1 chain (data not shown).

In aggregation experiments K562 α4 wt transfectants could aggregate in an α4β7-dependent manner similar to TK1 cells. Also mut 1 cells showed DATK32-triggered cell aggregation, whereas mut 3 cells and α4β7 negative K562 cells could not aggregate (Fig. 6). Taken together, the results indicated that a mutation in LDV-3, but not in LDV-1, affected DATK32-triggered homotypic cell aggregation and co-aggregation.

Biochemical Analysis of α4β4 Heterodimer Stability

We next investigated whether the point mutations in LDV-3 had altered the α4β7 conformation and heterodimer stability. In previous work, we used differential sensitivity in detergent to analyze the stability of α4β1[Zeller et al., 1998]. We compared TK1 cells with P1C2 mut 3 transfectants. After cell surface iodination, aliquots of each cell type were solubilized in 0.3% CHAPS, 1% IPEGAL CA-630, or 1% IPEGAL CA-630 in the presence of 2 mM Ca and Mg ions, respectively. Immunoprecipitation of each lysate with the α4 specific mAb 5/3 showed the typical pattern of α4 integrins on SDS–PAGE consisting of the 150 kDa band representing the intact α4 chain and bands at 80 and 70 kDa, respectively, which are proteolytic cleavage fragments of the α4 subunit (see Fig. 7). The coprecipitated β7 chain migrated at ~110 kDa. As seen in Figure 7, the amount of β7 in the α4 specific precipitates was highest when cells were solubilized in the presence of divalent cations. The association with the β7 subunit was weaker in the absence of divalent cations but was detectable and comparable in wt-α4 or mut 3 transfected cells. Similar results were obtained when the precipitation was carried out using an antibody against the β7 chain or with K562 transfectants (data not shown). These results together with the staining data obtained using different α4 and β7 specific antibodies suggested that the point mutations in the α4 subunit had not grossly disturbed the α4β7 heterodimer conformation.

Mut 3 Transfectants are Altered in Adhesion

To study other α4 integrin-triggered functions, the K562 as well as the P1C2 transfectants were used in static cell adhesion assays. The binding of the different cells to the α4-substrates FN and MAdCAM-1 was investigated. As shown in Figure 8A, non-activated TK1 cells could only weakly bind to MAdCAM-1-Ig immobilized to glass slides. After activation of the α4β7 integrins with 0.5 mM Mn^{2+} ions, binding of TK1 cells was increased approxi-

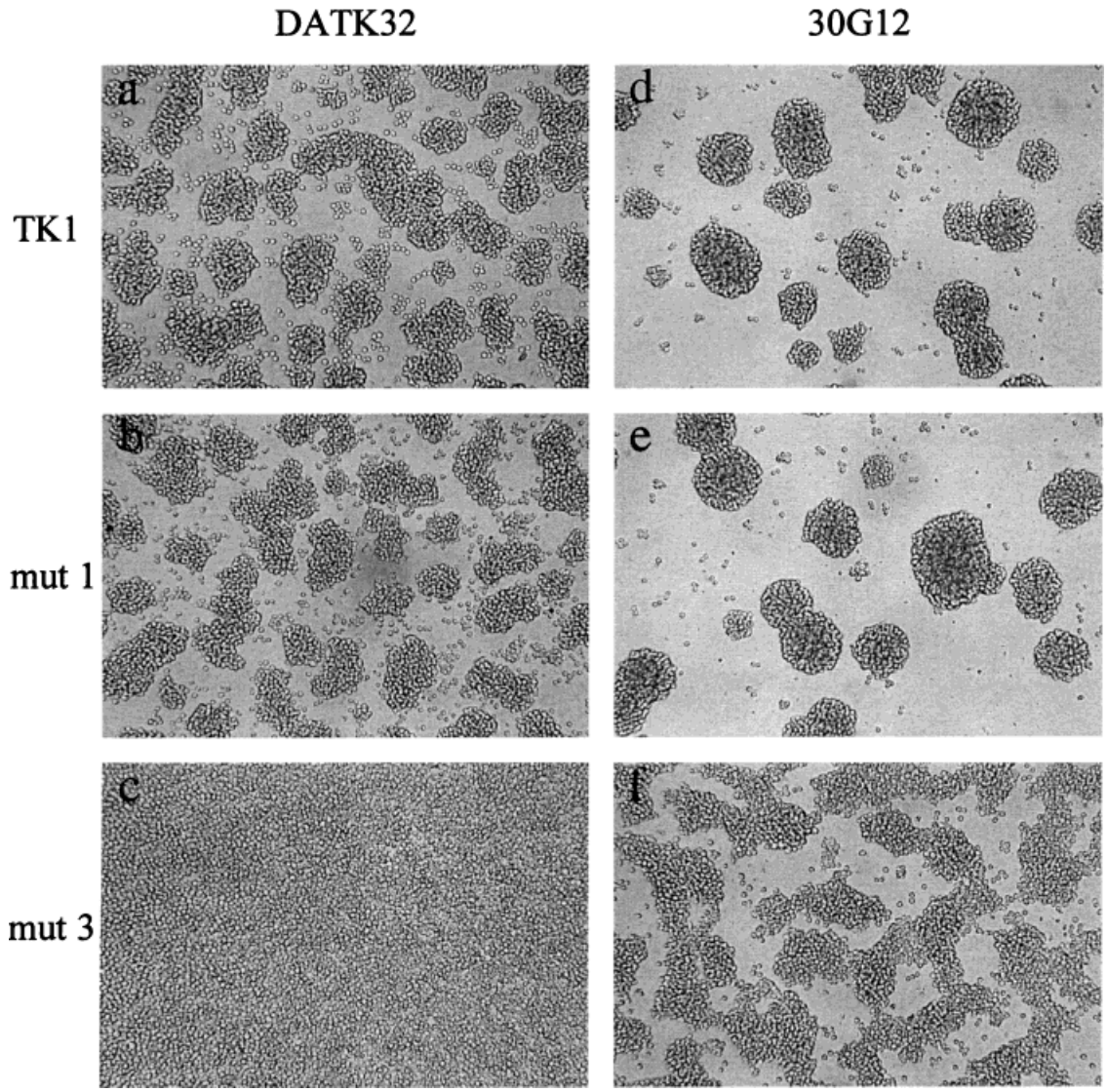


Fig. 5. Antibody-induced aggregation of TK1 cells and P1C2 transfectants. Cells were treated with 0.2 μg/ml of the α4β7-specific mAb DATK32 (**a**, **b**, **c**) or the CD45-specific mAb 30G12 (**d**, **e**, **f**) for 7 h. Aggregates formed by α4 wt transfectants (not shown) are comparable to those of TK1 cells. Data presented are representative from four individual experiments with identical results.

mately six-fold and was comparable to the binding of Mn^{2+}-activated α4 wt and mut 1 transfectants. In contrast to that, mut 3 cells showed a significant reduction in cell binding. Untransfected P1C2 cells did not bind. As shown in Figure 8B, TK1 cells, P1C2 α4 wt, and mut 1 cells bound to FN and the binding was also increased in the presence of 0.5 mM Mn^{2+} ions. P1C2 mut 3 cells showed only minimal binding in the presence of 0.5 mM Mn^{2+} ions whereas P1C2 cells did not bind at all. In addition, the binding of P1C2 transfectants or TK1 cells to FN or MAdCAM-1-Ig was completely abrogated in the presence of the anti-α4 mAb 5/3, indicating that it was specifically mediated by α4 integrins (not shown). Further binding experiments using K562 cells and transfectants on FN40 substrate gave very similar results (data not shown).

Altered Cell Spreading on FN

We investigated the spreading ability of P1C2 and K562 transfectants on FN. Neither P1C2 transfectants nor TK1 cells were able to spread

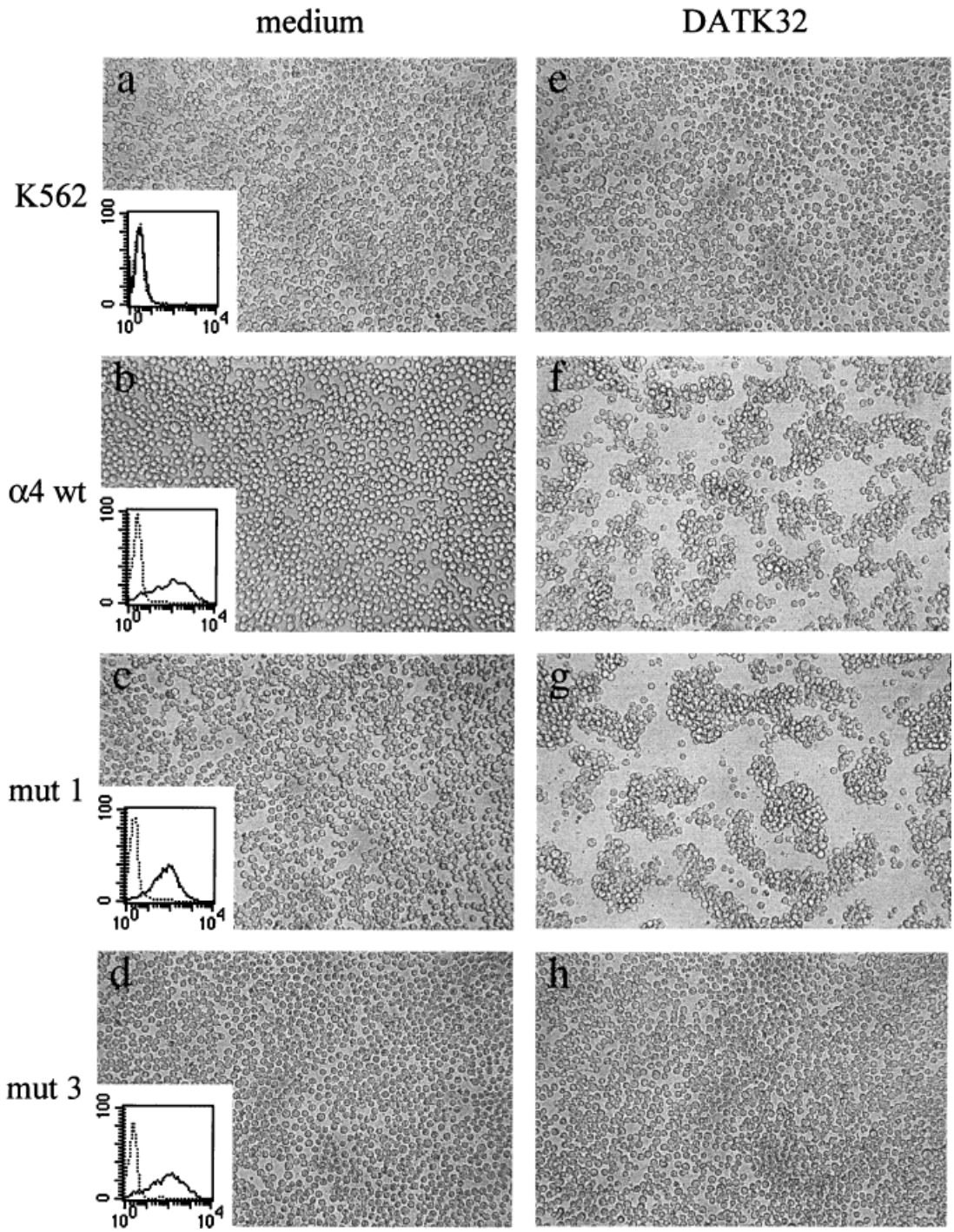


Fig. 6. DATK32-induced aggregation of α4β7 expressing K562 transfectants. K562 cells and α4β7-transfectants were incubated in culture medium (**a**, **b**, **c**, **d**) or in medium containing 0.2 μg/ml of the anti-α4β7 mAb DATK32. Cells were photographed after 2 h (n = 4). The expression of α4β7 was tested by indirect immunofluorescence using mAb DATK32 and PE-conjugated goat anti-rat IgG (embedded histograms).

on FN during a period of 24 h. Therefore, only spreading of K562 transfectants could be analyzed. Three hours after plating on FN a substantial portion (between 40 and 60%) of α4 wt and mut 1 transfectants had spread. The spreading could be prevented in the presence of the α4-specific mAb 5/3. Mut 3 cells did not spread and were round shaped as

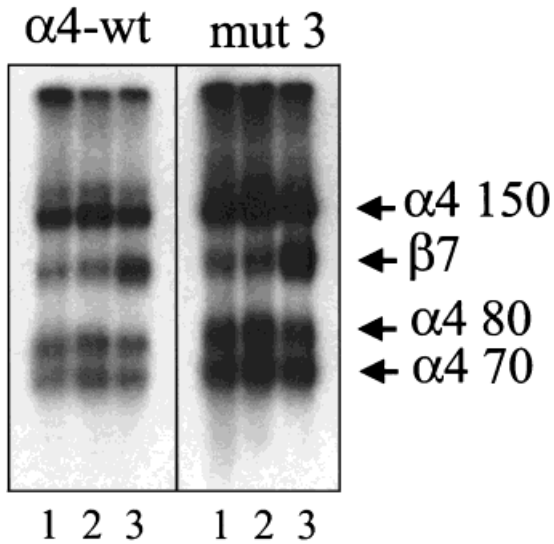


Fig. 7. Biochemical analysis of α4β7 stability. Cells were labeled with ^{125}[I] and lysed in the presence of (1) 0.3% CHAPS; (2) 1% IPEGAL CA-630; (3) 1% IPEGAL CA-630 + 2 mM Ca^{2+} and Mg^{2+} ions. Lysates were incubated with mAb 5/3 to α4 coupled to sepharose. The intact α4 chain, the 70 and 80 kDa fragments and the β7 chain are indicated. All samples were analyzed by SDS–PAGE under non-reducing conditions.

parental K562 cells even after 24 h (data not shown).

DATK32-Induced Clustering of α4β7 at the Cell Surface

Previous studies have shown that changes in avidity via integrin redistribution within the cell membrane, independent of alterations in affinity of an individual integrin receptor, can regulate integrin adhesive functions [Yauch et al., 1997; van Kooyk et al., 1999]. To study α4 integrin rearrangement, the distribution of α4β7 after addition of Cy3-labeled mAb DATK32 was analyzed by fluorescence microscopy. As illustrated in Figure 9A, mAb DATK32 induced pronounced clustering of α4β7 integrins on TK1 cells, whereas α4β7 integrins on mut 3 cells were much less clustered, despite being expressed on the cell surface at levels nearly equivalent to TK1 cells (see Fig. 3). The distribution of α4β7 into large clusters was dependent upon the addition of DATK32, because staining of prefixed cells revealed no accumulation in clusters. Similar results were obtained using K562 transfectants (Fig. 9B). α4-wt transfectants formed large clusters after addition of mAb DATK32, which were much smaller in size in K562 mut 3 cells.

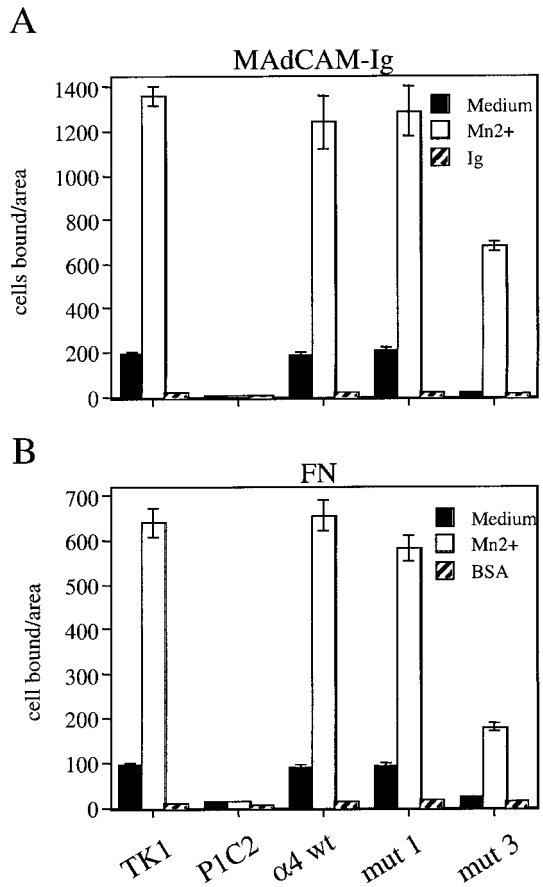


Fig. 8. Adhesion of cells to MAdCAM-1-Ig and fibronectin. Adhesion of TK1 cells and P1C2 transfectants to MAdCAM-1-Ig (**A**) and FN (**B**) each coated at 10 μg/ml is shown. The effect of Mn^{2+} ions on the adhesion of cells was tested in the presence of 0.5 mM Mn^{2+}.

Cell surface staining was specific for α4β7, because untransfected K562 cells showed no fluorescence (Fig. 9B).

DISCUSSION

The α4 subunit in both human and mouse contains three conserved LDV sequence motifs. These motifs are identical to the minimal binding site for α4 integrins in FN and homologous to the ligand binding sites in VCAM-1 and MAdCAM-1. This surprising similarity has raised questions with respect to the possible function of these motifs as cation binding sites as well as its role in α4-triggered homotypic cell aggregation. We proposed before that α4β1 and α4β7 integrins may be able to bind to α4 subunits on adjacent cells and that a LDV site might act as ligand providing a structural basis for α4-integrin-mediated interactions [Altevogt

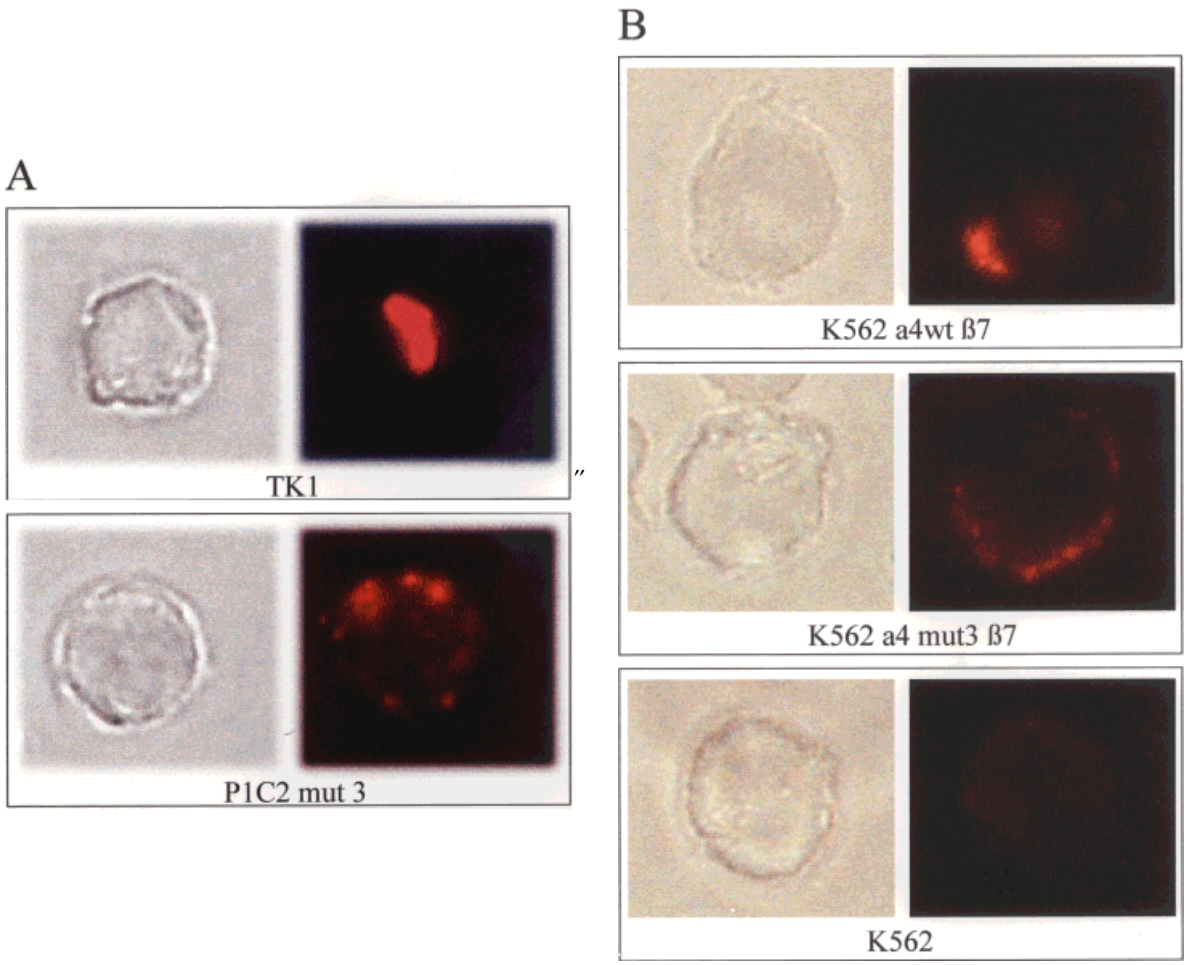


Fig. 9. Cell surface clustering of α4β7 integrins. TK1 and P1C2 mut 3 cells (**A**) or K562 transfectants (**B**) were treated with Cy3-conjugated anti-α4β7 mAb DATK32 for 2 h. Cells were washed, fixed, and analyzed using immunofluorescence microscopy. Presented data are representative of four individual experiments. The same cell is shown using fluorescence or phase contrast. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1995]. There is suggestive evidence that homotypic α4 interactions can play a role in memory B lymphocyte activation [Silvy et al., 1997]. The present study was performed (i) to test the hypothesis that LDVs are involved in α4-triggered homotypic aggregation and (ii) to analyze the effect of specific mutations in the LDVs on α4β7 integrin function and conformation.

Role of LDVs in α4β7 Heterodimer Formation and Integrin Clustering

Two mutations, D698N and D811N, showed a significant effect on α4 function. The D698N mutation led to the complete lack of α4β7 heterodimer expression on the cell surface in Balb3T3 fibroblasts, K562, and P1C2 cells. Surface expression was not affected by the mutation D811N, but the α4β7 integrin was functional inactive in adhesion and spreading on different α4 integrin ligands. Cell adhesion to MAdCAM-1 or FN40 could be partially restored by addition of manganese. Manganese is known to artificially increase integrin affinity via induction or stabilization of a heterodimer conformation highly accessible to ligand binding. The increase in cell adhesion after addition of Mn^{2+} ions indicated that the individual integrin receptor was not completely non-functional and suggested that other regulatory mechanisms had been influenced by a mutation in Asp-811. Next we investigated the spreading behavior of the transfectants on α4 specific substrates. Analogous to adhesion, D811N mutants were also shown to be defective in spreading. Further analysis of D811N mutants

revealed an impaired ability of α4 integrins to redistribute within the cell membrane as shown by a diminished cluster formation of α4β7 integrins in response to the α4β7 specific mAb DATK32. It has been demonstrated previously that integrin-mediated cell spreading is regulated by affinity independent mechanisms including modulation of cytoskeletal interactions. Cytoskeletal rearrangements lead to integrin clustering and focal adhesion formation, a prerequisite for cell spreading [Peter and O'Toole, 1995]. Furthermore, the importance of integrin cluster formation was corroborated by showing that diffusion-regulated clustering of integrins is essential and sufficient to induce cell adhesion independent of changes in integrin affinity [Yauch et al., 1997; Grabovsky et al., 2000]. At present the reason for the observed defect of the D811N mutant in lateral clustering remains unclear. Reports about cytoplasmic tail deletion mutants imply that regulation of integrin diffusion/clustering through cytoplasmic tail interactions with components of the cytoskeleton might be a key step in controlling cell adhesion. Interactions of cytoskeletal components with integrin β chains are known for a long time and recently the α4 subunit was demonstrated as first α chain to bind paxillin [Liu et al., 1999]. We do not think that cytoskeletal interactions are directly affected by an α4 mutation in Asp-811, because the residue is located in the extracellular part of α4 proximal to the cell membrane. This location rather enables interactions with other membrane bound proteins. There is an increasing number of transmembrane proteins found to be associated with α4 integrins, including members of the transmembrane-4 superfamily like CD151 [Fitter et al., 1999], TAPA-1, CD82, CD63, and CD53 [Mannion et al., 1996]. The functional relevance of these integrin–protein interactions is under extensive study and data exist, suggesting a direct or indirect role for cell adhesion [Mannion et al., 1996].

Beside Asp-811, we could identify Asp-698 as an important amino acid residue for α4β7 integrins. Asp-698 of the α4 subunit has been previously demonstrated to be functional relevant for α4 integrins [Ma et al., 1995; Bazzoni et al., 1998; Zeller et al., 1998]. In a previous study we demonstrated that α4 mut 2 containing double and triple mutants expressed in Balb/3T3 fibroblasts could reach the cell surface in combination with the β1 but not with the β7 integrin chain [Zeller et al., 1998]. In the present communication we confirmed these results using the single mut 2 subunit. Also in K562 cells and P1C2 cells the α4 mut 2 chain did not reach the surface in combination with β7 as staining with the heterodimer specific mAb DATK32 was not detected whereas staining with mAb 5/3 was positive. This clearly indicated, that the D698N mutation in the α4 chain perturbed heterodimer formation or transport to the cell surface. In this context it is interesting to note, that the cellular effects of a mutation in Asp-698 seems to depend on the type of amino acid substitution. A replacement with glutamic acid impaired α4β1 expressing transfectants to adhere to VCAM-1 or FN40 without affecting integrin heterodimer formation or the stability of the complex [Ma et al., 1995]. Substitution of Asp-698 with asparagine caused a destabilization of the α4β1 heterodimer leading to a cellular defect in adhesion and spreading on α4 specific substrates [Zeller et al., 1998]. In light of our previous results, it seems most likely that the D698N mutation affected the association of α4 with β7 more drastically than with β1. This implies that Asp-698 contributes to a different degree to the heterodimer stability of the two α4 integrins or, alternatively, both α4 integrins differ in terms of their heterodimer stability, namely α4β1 being more stable than α4β7. A hierarchy of integrin stability has already been proposed for β1 integrins [Bazzoni and Hemler, 1998]. Among five β1 integrins, α4β1 has been described to undergo most extensive conformational changes in response to either ligand or manganese as detected by highest staining with the anti-β1 mAb 9EG7 which is indicative for integrin conformational unfolding [Bazzoni et al., 1998]. Regarding the different effects caused by the D698N mutation on α4β7 compared to α4β1, it becomes obvious, that the investigation of a defined amino acid residue of the α4 chain in the context of both α4 integrins might yield different, but complementary informations helpful to elucidate its functional relevance.

LDVs and α4-Triggered Homotypic Aggregation

For homotypic aggregation α4-integrins appeared to be necessary at the cell surface since P1C2 cells did not aggregate. This is explained by their α4-integrin-negative phenotype. Interestingly, these cells also did not form

co-aggregates with TK-1 cells indicating that they may have lost a structure X that is required to serve as ligand. However, at present we can not rule out the possibility that the activating antibody DATK32 has to bind to the cells in order to induce the expression of the ligand structure. Of the two mutants that could be tested in homotypic aggregation only D811N but not D489N showed a substantial effect on α4β7 integrin-triggered homotypic cell aggregation as well as co-aggregation. We previously speculated that LDV-1 including Asp-489 might be a candidate to serve as ligand in homotypic aggregation as this site is most distant from the membrane and might become accessible due to sterical changes induced by an aggregation stimulating mAb [Altevogt et al., 1995]. We failed to demonstrate any role for this LDV site. In contrast, the LDV-3 (Asp-811) is proximal to the membrane and may not qualify for a putative ligand structure. Different functional roles for α4β7 integrins have been proposed in homotypic aggregation vs. heterotypic adhesion, because mAb DATK32 on the one hand induces TK1 cell aggregation and on the other hand inhibits TK1 cell binding to the α4β7 ligands MAdCAM-1, FN, and VCAM-1 [Andrew et al., 1994]. It is remarkable that a D811N mutation affected both types of integrin functions, emphasizing the essential role of Asp-811 within the α4 subunit.

CONCLUSIONS

Most structure–function analyses of the α4 subunit done so far were based on the study of the α4β1 integrin heterodimer. However, very little information is available on regions or amino acid residues that are critical for α4β7 integrins and their interaction with ligands. Epitope mapping studies using function-blocking α4 and β7 mAbs [Andrew et al., 1994] and ligand binding experiments using β7/β1 chimeric integrins [Tidswell et al., 1997] demonstrated that distinct, although partially overlapping binding sites on α4β7 exist for its different ligands. There are two reports that describe loss-of-function mutants of α4β7 integrins. Using truncated forms of the β7 subunit, it has been shown that an α4β7 transfectant, lacking the 34 C-terminal residues of the β7 subunit, displayed a diminished ligand binding activity [Crowe et al., 1994], and very recently Tyr-187 of the α4 chain has been demonstrated to play a key role in α4β7 triggered cell adhesion [Ruiz-Velasco et al., 2000]. It was suggested that this amino acid residue is located on the upper part of the proposed β-propeller structure which carries the ligand binding site [Ruiz-Velasco et al., 2000]. Here we have identified two additional residues, Asp-698 and Asp-811, respectively, within the α4 subunit critical for the function of α4β7 integrins. These residues are part of LDV sites and are localized on the stalk region of the α4-subunit. Due to their location they are not directly involved in ligand binding but seem to be important for the association of integrin subunits and for membrane clustering. The presence of LDV sites within ligands and the α4 subunit emphasizes the important role of putative metal coordination sites for the functional competence of the α4 integrins.

ACKNOWLEDGMENTS

We thank Volker Schirrmacher for support and stimulating discussions. We are grateful to Christine Veit for help with the mutagenesis.

REFERENCES

Altevogt P, Hubbe M, Ruppert M, Lohr J, von Hoegen P, Sammar M, Andrew DP, McEvoy L, Humphries MJ, Butcher EC. 1995. The α4 integrin chain is a ligand for α4β7 and α4β1. J Exp Med 182:345–355.

Andrew DP, Berlin C, Honda S, Yoshino T, Hamann A, Holzmann B, Kilshaw PJ, Butcher EC. 1994. Distinct but overlapping epitopes are involved in α4β7-triggered adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. J Immunol 153:3847–3861.

Arroyo AG, Yang JT, Rayburn H, Hynes RO. 1996. Differential requirements for α4 integrins during fetal and adult hematopoiesis. Cell 85:997–1008.

Arroyo AG, Yang JT, Rayburn H, Hynes RO. 1999. α4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. Immunity 11:555–566.

Barrett SP, Riordon A, Toh BH, Gleeson PA, van Driel IR. 2000. Homing and adhesion molecules in autoimmune gastritis. J Leukoc Biol 67:169–173.

Bazzoni G, Hemler ME. 1998. Are changes in integrin affinity and conformation overemphasized? TIBS 23:30–34.

Bazzoni G, Ma L, Blue ML, Hemler ME. 1998. Divalent cations and ligands induce conformational changes that are highly divergent among β1 integrins. J Biol Chem 273:6670–6678.

Briskin MJ, Rott L, Butcher EC. 1996. Structural requirements for mucosal vascular addressin binding to its lymphocyte receptor α4β7. J Immunol 156:719–726.

Butcher EC, Weissman IL. 1980. Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate.

I. Technical aspects. J Immunol Methods 37:97–108.

Campanero MR, Pulido R, Ursa MA, Rodriguez-Moya M, deLandazuri MO, Sanchez-Madrid F. 1990. An alternative leukocyte homotypic adhesion mechanism, LFA-1/ICAM-1-independent, triggered through the human VLA-4 integrin. J Cell Biol 110:2157–2165.

Clements JM, Newham P, Shepherd M, Gilbert R, Dudgeon TJ, Needham LA, Edwards RM, Berry L, Brass A, Humphries MJ. 1994. Identification of a key integrin-binding sequence in VCAM-1 homologous to the LDV active site in fibronectin. J Cell Sci 107:2127–2135.

Crowe DT, Chiu H, Fong S, Weissman IL. 1994. Regulation of the avidity of integrin α4β7 by the β7 cytoplasmic domain. J Biol Chem 269:14411–14418.

Ebeling O, Duczmal A, Aigner S, Geiger C, Schöllhammer S, Kemshead JT, Möller P, Schwarz-Albiez R, Altevogt P. 1996. L1 adhesion molecule on human lymphocytes and monocytes: expression and involvement in binding to αv β 3 integrin. Eur J Immunol 26:2508–2516.

Fitter S, Sincock PM, Jolliffe CN, Ashman LK. 1999. Transmembrane 4 superfamily protein CD151 (PETA-3) associates with β_1 and $a_{IIb}\beta_3$ integrins in haemopoietic cell lines and modulates cell–cell adhesion. Biochem J 338:61–70.

Grabovsky V, Feigelson S, Chen C, Bleijs DA, Peled A, Cinamon G, Baleux F, Arenzana-Seisdedos F, Lapidot T, vanKooyk Y, Lobb RR, Alon R. 2000. Subsecond induction of α 4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. J Exp Med 192:495–506.

Guerrero-Esteo M, Ruiz-Velasco N, Munoz M, Teixido J. 1998. Role of two conserved glycine residues in the β-propeller domain of the integrin α 4 subunit in VLA-4 conformation and function. FEBS Lett 429:123–128.

Holzmann B, Gosslar U, Bittner M. 1998. α 4 integrins and tumor metastasis. Curr Top Microbiol Immunol 231:125–141.

Irie A, Kamata T, Puzon-McLaughlin W, Takada Y. 1995. Critical amino acid residues for ligand binding are clustered in a predicted β-turn of the third N-terminal repeat in the integrin α4 and α5 subunits. EMBO J 14:5550–5556.

Irie A, Kamata T, Takada Y. 1997. Multiple loop structures critical for ligand binding of the integrin α4 subunit in the upper face of the β-propeller mode 1. Proc Natl Acad Sci USA 94:7198–7203.

Komoriya A, Green LJ, Mervic M, Yamada SS, Yamada KM, Humphries MJ. 1991. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. J Biol Chem 266:15075–15079.

Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, Pfaff M, Ginsberg MH. 1999. Binding of paxillin to α4 integrins modifies integrin-dependent biological responses. Nature 402:676–681.

Ma L, Conrad PJ, Webb DL, Blue ML. 1995. Aspartate 698 within a novel cation binding motif in α4 integrin is required for cell adhesion. J Biol Chem 270:18401–18407.

Mannion BA, Berditchevski F, Kraeft SK, Chen LB, Hemler ME. 1996. Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associate with integrin α4β1 (CD49d/CD29). J Immunol 157:2039–2047.

Masumoto A, Hemler ME. 1993. Mutation of putative divalent cation sites in the α4 subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and invasin. J Cell Biol 123:245–253.

Miyake K, Weissman IL, Greenberger JS, Kincade PW. 1991. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J Exp Med 173:599–607.

Morales-Ducret J, Wayner E, Elices MJ, Alvaro-Gracia JM, Zvaifler NJ, Firestein GS. 1992. α 4/β 1 integrin (VLA-4) ligands in arthritis. Vascular cell adhesion molecule-1 expression in synovium and on fibroblast-like synoviocytes. J Immunol 149:1424–1431.

Munoz M, Serrador J, Sanchez-Madrid F, Teixido J. 1996. A region of the integrin VLAα4 subunit involved in homotypic cell aggregation and in fibronectin but not vascular cell adhesion molecule-1 binding. J Biol Chem 271:2696–2702.

Munoz M, Serrador J, Nieto M, Luque A, Sanchez-Madrid F, Teixido J. 1997. A novel region of the α4 integrin subunit with a modulatory role in VLA-4-triggered cell adhesion to fibronectin. Biochem J 327:727–733.

Osborn L, Vassallo C, Griffiths-Browning B, Tizard R, Haskard DO, Benjamin CD, Dougas I, Kirchhausen T. 1994. Arrangement of domains, and amino acid residues required for binding of vascular cell adhesion molecule-1 to its counter-receptor VLA-4 (α4β1). J Cell Biol 124:601–608.

Oxvig C, Springer TA. 1998. Experimental support for a β-propeller domain in integrin α-subunits and a calcium binding site on its lower surface. Proc Natl Acad Sci USA 95:4870–4875.

Peter K, O'Toole TE. 1995. Modulation of cell adhesion by changes in α L β 2 (LFA-1, CD11a/CD18) cytoplasmic domain/cytoskeleton interaction. J Exp Med 181:315–326.

Ruiz-Velasco N, Guerrero-Esteo M, Briskin MJ, Teixido J. 2000. The α4 integrin subunit Tyr^{187} has a key role in α4β7-dependent cell adhesion. J Biol Chem 275:7052–7059.

Rüegg C, Postigo AA, Sikorski EE, Butcher EC, Pytela R, Erle DL. 1992. Role of integrin α4β7/α4βP in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. J Cell Biol 117:179–189.

Silvy A, Altevogt P, Mondiere P, Bella C, Defrance T. 1997. A role for the VLA-4 integrin in the activation of human memory B cells. Eur J Immunol 27:2757–2764.

Springer TA. 1997. Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. Proc Natl Acad Sci USA 94:65–72.

Tidswell M, Pachynski R, Wu SW, Qiu SQ, Dunham E, Cochran N, Briskin MJ, Kilshaw PJ, Lazarovits AI, Andrew DP, Butcher EC, Yednock TA, Erle DJ. 1997. Structure-function analysis of the integrin β_7 subunit. J Immunol 159:1497–1505.

Tuckwell DS, Brass A, Humphries MJ. 1992. Homology modelling of integrin EF-hands. Evidence for widespread use of a conserved cation-binding site. Biochem J 285:325–331.

Van Kooyk Y, van Vliet SJ, Figdor CG. 1999. The actin cytoskeleton regulates LFA-1 ligand binding through avidity rather than affinity changes. J Biol Chem 274:26869–26877.

Vonderheide RH, Tedder TF, Springer TA, Staunton DE. 1994. Residues within a conserved amino acid motif of domains 1 and 4 of VCAM-1 are required for binding to VLA-4. J Cell Biol 125:215–222.

Wagner N, Müller W. 1998. Functions of α4- and β7-integrins in hematopoiesis, lymphocyte trafficking and organ development. Curr Top Microbiol Immunol 231:23–32.

Yauch RL, Felsenfeld DP, Kraeft SK, Chen LB, Sheetz MP, Hemler ME. 1997. Mutational evidence for control of cell adhesion through integrin diffusion/clustering, independent of ligand binding. J Exp Med 186:1347–1355.

Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against α4β1 integrin. Nature 356:63–66.

Zeller Y, Lohr J, Sammar M, Butcher EC, Altevogt P. 1998. Asp-698 and Asp-811 of the integrin α4-subunit are critical for the formation of a functional heterodimer. J Biol Chem 273:6786–6795.