Leukocyte Integrins $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ as Collagen Receptors – Receptor Activation and Recognition of GFOGER Motif

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Footnotes

Abbreviations: CR, complement receptor; ICAM; intercellular cell adhesion molecule; iC3b, inactivated complement component C3b; HL-60, human promyelocytic leukemia cells; O; hydroxyproline in GFOGER.

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ABSTRACT

Integrins $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ are expressed on leukocytes. Their primary ligands are counter transmembrane receptors or plasma proteins, such as intercellular cell adhesion molecule-1 (ICAM-1) or components of complement system (iC3b, iC4b), respectively. Function blocking antibodies for these integrins may also reduce cell adhesion to collagens. To make the first systematical comparison of human $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ as collagen receptors, we produced the corresponding integrin αI domains both in wild-type and activated form and measured their binding to collagens I-VI. In the "closed" (wild-type) conformation, the α_LI and α_MI domains bound with low avidity to their primary ligands, and the interaction with collagens was also very weak. Gain-of-function mutations α_L I306G, α_L K287C/K294C and α_M I316G are considered to mimic "open", activated αI domains. The binding of these activated αI domains to the primary ligands was clearly stronger and they also recognized collagens with moderate avidity (K_d < 400 nM). After activation, the α_LI domain favored collagen I ($K_d \approx$ 80 nM) when compared to collagen IV. The integrin α_XI domain acted in a very different manner since already in native, wild-type form it bound to collagen IV and iC3b ($K_d \approx$ 200-400 nM). Antibodies against $\alpha_X\beta_2$ and $\alpha_M\beta_2$ blocked promyelocytic leukemia cell adhesion to the collagenous GFOGER motif, a binding site for the β_1 integrin containing collagen receptors. In brief, leukocyte β_2 integrins may act as collagen receptors in a heterodimer specific manner.

Keywords: Adhesion, cell trafficking, collagen, complement receptors, extracellular matrix, integrin.

1. Introduction

Collagens are a large family of extracellular matrix molecules present in all multicellular animals (Myllaharju and Kivirikko, 2004). Despite the ancient origin of collagens, their major cellular receptors, namely the collagen binding αI domain ("inserted" domain) integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$), evolved much later and are found only in vertebrates (Johnson et al., 2009). Another subset of the αI domain integrins is often referred to as leukocyte integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$ and $\alpha_E\beta_7$). Their primary ligands include other cell adhesion receptors, for instance intercellular adhesion molecules (ICAM). Integrin–ICAM interaction is essential for leukocyte homing into inflamed tissues through the endothelial cell layer (Evans et al, 2009). In addition to counter receptors, leukocyte integrins $\alpha_M\beta_2$ and $\alpha_X\beta_2$, which are also referred to as complement receptors 3 and 4 (CR3, CR4), bind to plasma proteins, such as fibrinogen and the components of the complement system (iC3b, iC4b) (Humphries et al., 2006). Both subgroups of the αI domain integrins have a common evolutionary origin. Primitive chordates, such as the sea squirt (*Ciona intestinalis*), also have αI domain integrins. The functions of these proteins are incompletely known, but they seem to be clearly distinct from the vertebrate collagen receptors (Tulla et al., 2007).

The ligand binding αI domains in both collagen receptors and leukocyte integrins share the same basic structure. These domains have a classical α/β Rossman fold with a magnesium ion bound into the metal ion dependent adhesion (MIDAS) site on the surface. Based on the similarity with the von Willebrand A domain, the integrin αI domains are frequently referred to as αA domains (Lee et al., 1995).

Integrins are bidirectionally signaling receptors (Hynes, 2002). Integrin activation by inside-out signals is mediated via large conformational changes in the β subunit and subsequently via alterations in the conformation of the αI domain (Luo et al., 2007, Arnaout et al., 2005). The low affinity stage of the αI domain is often described as a "closed" conformation. Inside-out activation and ligand binding to the extracellular region are both considered to induce similar changes in the αI structure. When the αI domain is activated, there is a small shift in the position of the metal in MIDAS and a larger movement ‘downwards’ in the α7 helix. These above-mentioned conformational changes are shared between the leukocyte and collagen αI domains.

A characteristic feature common to the collagen receptor αI domains, but missing in the leukocyte integrins, is a short extra helix, named αC. The exact role of this structure is uncertain but it has been suggested that it could participate in the ligand recognition or the regulation of integrin activation (Käpylä et al., 2000; Tulla et al., 2008; Lahti et al., 2011). In the α_1I domain, as a consequence of a gain-of-function mutation, the αC helix unwinds and moves away from the metal binding site. This activated αI domain binds to the ligands with a higher affinity (Lahti et al., 2011). Activation may also affect the binding specificity and allow the binding of less favored ligands (Tulla et al., 2008).

The physiological roles of the collagen receptors and the leukocyte integrins seem to partially overlap. For example $\alpha_2\beta_1$ integrin can recognize proteins that participate in the regulation of innate immunity (Zutter and Edelson, 2007). Furthermore, the leukocyte integrins may, at least in some circumstances, participate in collagen binding (Garnotel et al., 1995; Walzog et al., 1995; Garnotel et al., 2000). Nevertheless, the ability of the leukocyte integrins to directly bind to various collagen subtypes has never been systematically analyzed or compared to the collagen receptor function. Furthermore, their putative binding sites in collagen have been uncertain.

Here we have designed α_2I, α_LI, α_MI and α_XI domain constructs in a way that they can be compared to each other and the corresponding recombinant proteins can be produced. We have also used a solid phase binding assay to estimate their ability to bind to collagen subtypes I-VI. In general, all three leukocyte integrins could recognize at least some collagen subtypes with moderate avidity. The action of α_LI and α_MI was dependent on the activation, whereas α_XI was a relatively good collagen receptor also in the closed conformation. The highest estimated avidities were measured in the α_LI – collagen I and the α_XI – collagen IV binding. The collagen binding was clearly weaker when compared to the α_2I domain. Still $\alpha_X\beta_2$ and $\alpha_M\beta_2$ could mediate cell adhesion to the triple-helical GFOGER peptide, that represents a high affinity binding motif for the collagen receptor integrins.

2. Materials and methods

2.1. Materials

The ligands used were rat collagen I (from tail tendon; Sigma-Aldrich), collagen IV from a mouse Engelbreth–Holm–Swarm tumor (Becton Dickinson), human collagens I-VI (Biomarket), recombinant human ICAM-1 (R&D Systems) and human inactivated complement fragment 3 (iC3b; Calbiochem/Merck). A short, synthetic triple helical GFOGER peptide, where O is hydroxyproline, was synthetized by Auspep (Australia). Before binding and adhesion assays, fibrillar collagens (I, II, III and V) were kept in acetic acid (0.1 M) to maintain them in triple helical tropocollagen form. Other chemicals used are described in the methods section.

2.2. Cloning and Mutagenesis of the Human Integrin α_LI, α_MI, α_XI, α_1I and α_2I Domains

The α_2I domain, including the amino acids 125-PDGF-EGTV-339, was earlier cloned into the pGEX-2T vector (Amersham Biosciences) (Ivaska et al., 1999), and the point mutation E318W to the α_2I domain was carried out as previously described (Tulla et al., 2008). For cloning the recombinant leukocyte αI domains, α_LI, α_MI, and α_XI, leukocytes were isolated from heparinized (10 units/ml) venous blood collected from healthy adult donors using ammonium chloride (0.83% NH_4Cl, 15 min, + 20 ^oC) to disrupt red blood cells leaving intact white blood cells, which were collected by centrifugation (400g, 10 min, +4^oC). The pellet was washed with ammonium chloride and recentrifugated. Total RNA was isolated from the white blood cells by Illustra RNA spin Mini (GE Healthcare) and converted to total cDNA by RT-PCR [M-MLV RT RNAase H(-) Point Mutant with Random Primers, Promega]. αI domain cDNAs were amplified from total cDNA by PCR (DNA Pol Phusion, Finnzymes). The primers used for the amplification of the αI domain cDNAs are shown in Table 1. The primers were designed to introduce two restriction sites into the product: a *Bam*HI site at the 5' end and either an *Eco*RI (for α_LI) or an *Xho*I site (for α_MI and α_XI) at the 3' end. α_LI cDNA double-digested with *Bam*HI and *Eco*RI was inserted into pGEX2T (Amersham Biosciences) and α_MI and α_XI cDNAs digested with *Bam*HI and *Xho*I were inserted into pGEX-4T-3 (Amersham Biosciences). These recombinant plasmids we transformed into *Escherichia coli Tuner*^{TM} cells (Novagen). The point mutations (α_LI K287C/K294C, α_LI I306G, α_MI I316G and α_XI I314G) to αI domains were carried out by using a QuikChange

site-directed mutagenesis kit (Stratagene). All mutations were verified by DNA sequencing. The primers used in mutagenesis are shown in the Supplemental Data (Table S1).

2.3. Protein Expression and Purification

Proteins were expressed in *Escherichia coli BL21 Tuner*™ and purified as glutathione-S-transferase (GST) fusions to homogeneity by glutathione affinity chromatography, as has been described for the α_2I domain (Lahti et al., 2011). The purity of the proteins was checked by electrophoresis in 8–25% gradient polyacrylamide gels (PAGE) in the presence of 0.55% sodium dodecyl sulphate (SDS) using the Phast System (Amersham Pharmacia Biotech). The protein concentrations were measured by the Bradford method (Bio-Rad Protein Assay, Bio-Rad).

2.4. Solid Phase Binding Assays

In the binding assays, different subtypes of collagens, the synthetic triple helical GFOGER peptide, ICAM-1 and iC3b were used as ligands. Binding assays were carried out as previously described (Tulla et al., 2008), except for bovine serum albumin (BSA) which was not used for blocking the wells but Tween-20 (0.05%) was instead included in the washing buffer (PBS, pH 7.5, 2 mM $MgCl_2$) to reduce non-specific binding. Briefly, amine binding 96-well plates (DNA-bind®, Corning) were coated with 16.4 μg/ml of collagenous ligands (collagens and GFOGER peptide) or 0.2 μg/ml of non-collagen ligands (ICAM-1, cB3i) in PBS (pH 8.5) o/n at + 4 °C. The wells were washed once with PBS (phosphate-buffered saline) buffer including 0.05% Tween-20. The GST-fusion αI domains (4 – 400 nM, if not otherwise stated) were allowed to bind to the ligands for 1 h in the presence of 2 mM $MgCl_2$. The wells were washed three times and Europium labeled anti-GST antibody was added (Delfia®, Wallac PerkinElmer). After 1 h incubation, the unbound antibody was removed by washing three times. To increase the signal, the label was dissociated with enhancement solution (Delfia®, Wallac PerkinElmer). The signal was detected by using a time-resolved fluorescence spectrophotometer (Victor3 multilabel counter, Wallac PerkinElmer). Estimates

for the dissociation constants (K_d) were obtained using an equation: measured binding = maximal binding / (1 + K_d / [αI]).

2.5. Adhesion and Spreading Assays

Attachment and spreading of human promyelocytic leukemia (HL-60) cells (ATCC) were tested with xCELLigence real-time cell analyzer (RTCA; Roche). This technology measures impedance at the bottom of a microtiter plate well and allows the estimation of the progression of cell adhesion. E-plates 96 (Roche) were coated either with rat collagen I, mouse collagen IV (5 μg/cm^2 per well; 16.4 μg/ml) or GFOGER peptide (5 μg/cm^2 per well; 16.4 μg/ml) in Dulbecco's phosphate buffered saline o/n at +4 °C and washed with PBS (Sigma-Aldrich). Function-blocking antibodies for integrin subunits α_L (anti-CD11a [MEM-25], Abcam), α_M (anti-CD11b [ICRF44], Sigma-Aldrich), α_X (anti-CD11c [FK24], Abcam) and β_1 (anti-CD29 [BV7], Abcam) were diluted (5 μg/ml, 5 μg/ml, 1/10, 5 μg/ml, respectively) to RPMI-1640 (Lonza) culture medium without fetal calf serum (FCS) and added to the wells. The background signal was measured and 40000 HL-60 cells/well were added. Anti-mouse IgG (SC-2025, Santa Cruz Biotechnology; 5 μg/ml dilution) served as a negative control. Six replicates were used for cell adhesion experiments. Statistical analysis was performed for 30 min and 2-hour time points by the nonparametric Mann-Whitney U-test with IBM SPSS Statistics software version 16.0. Cell adhesion was followed for two hours (+37 °C, 5% CO_2). The HL-60 cells used in these experiments were grown in an incubator (+37 °C, 5% CO_2) in the RPMI-1640 culture medium (Lonza) with the following supplements, unless otherwise stated: 10 % FCS (PromoCell), 2 mM L-glutamine (Lonza), and antibiotics penicillin (Lonza) and streptomycin (Lonza) (100 U/ml of both). To remove serum from the medium, the cells were pelleted twice by centrifugation (500 x g, 5 min, +37 °C) and resuspended in the RPMI-1640 medium without FCS.

3. **Results**

3.1. HL-60 cells use $\alpha_X\beta_2$ and $\alpha_M\beta_2$ integrins in binding to collagen IV

Previous data have indicated that function blocking antibodies for $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins may reduce cell adhesion to collagen. To confirm these observations, we assayed the attachment and spreading of HL-60 cells using xCELLigence technology (Roche). Importantly, unlike in some previously published reports, we did not use BSA or other putative integrin ligands (Davis, 1992) in blocking the background binding. The integrin subunit α_X function blocking antibody decreased cell adhesion on both rat collagen I (not shown) and mouse collagen IV (Fig. 1A), when compared to the control antibody (mouse IgG) treated ($p=0.002$ on collagen IV) or untreated cells. Similarly antibodies for the integrin α_M subunit of macrophage-1 antigen (Mac-1) blocked adhesion to collagen IV ($p=0.002$; Fig. 1B). Antibodies for the integrin α_L subunit of lymphocyte function-associated antigen 1 (LFA-1) showed some inhibitory effect at an early time point (Fig. 1A; $p=0.009$). Antibodies for β1 integrin were also inhibitory (Fig. 1A). Thus, in the nonactivated cells $\alpha_X\beta_2$ (CR4) and $\alpha M\beta_2$ (Mac-1) were clearly functional receptors for collagen IV. Therefore, we decided to perform a detailed analysis of the integrin function at the αI domain level.

3.2. Specific gain-of-function mutations activate ligand binding by the integrin αI domains

The αI domains of the human leukocyte integrins, namely α_LI, α_MI, and α_XI, were produced as recombinant proteins. The recombinant proteins were carefully designed to cover a comparable sequence in each αI domain. More specifically, the N-terminus of the proteins started 14-15 residues prior to the conserved cystein residue (C125 in α_L, C128 in α_M and C126 in α_X), and their C-terminus ended one residue after the conserved EGT-sequence (EGT-S313 in α_L, EGT-Q323 in α_M and EGT-E321 in α_X). All proteins contained GST-tags. For control purposes a similar α_2I domain was also produced (P125- EGT-V339). In addition to the wild-type proteins, the following variants were produced: I306G and K287C/K294C for α_LI (Huth et al., 2000; Lu et al., 2001), I316G for α_MI (Xiong et al., 2000), I314G for α_XI (Vorup-Jensen et al., 2003), and E318W for α_2I (Aquilina et al., 2002, Tulla et al., 2008) (Fig. 2).

In a solid phase binding assay, the wild-type α_2I domain bound to collagen I with estimated K_d of ≈ 3 nM, α_XI bound to iC3b with K_d of 170 nM, whereas no significant

binding of α_LI and α_MI was correspondingly detected to ICAM-1 and iC3b (Figs. 3 and 4, Table 2). The gain-of-function mutations in α_2I, α_LI and α_MI increased binding to collagen I, ICAM-1 and iC3b, respectively. Mutation I314G in α_XI had, however, no clear activating effect on binding to iC3b (Fig. 3 and 4, Table 2). Thus, it is not clear whether in our study the I314G α_XI protein represents an activated conformation.

3.3. Integrin α_LI, α_MI and α_XI domains favor different collagen subtypes and differ in their requirements for activation

The binding of leukocyte integrin αI domains to human collagens I-VI was first tested at one αI concentration. Collagens I-III and V represent fibril-forming subtypes; collagen IV is a network forming collagen; and collagen VI forms the beaded filaments. Some binding of the wild-type α_LI to collagens IV and VI was detected, but not to collagens I-III or V. However, the activated domain showed more binding to fibril forming collagens (Fig. 5A). Wild-type α_MI recognized all collagen subtypes and the activation increased binding (Fig. 5B). Wild-type α_XI interacted with all collagen subtypes, favoring collagens IV and VI, but I314G mutation in α_XI had very little effect (Fig. 5C). Thus, the results suggest that all three integrin αI domains can bind to collagens. However, the ligand preferences and the dependency on activation seemed to be different for each αI domain.

3.4. Integrin α_LI domain binds to collagen I and integrin α_XI domain to collagen IV with an avidity comparable to their primary ligands

To estimate the avidity of the wild-type and activated leukocyte integrins for collagen I and IV, the binding of each αI domain was tested as a function of ligand concentration in a solid phase binding assay, and the apparent dissociation constants were calculated (Fig. 6; Table 2). These results mostly confirmed the observations shown in Fig. 5. Integrin α_LI required activation before it could recognize collagen I (Fig. 6A). Furthermore, in these measurements, binding of wild-type α_LI to collagen IV was not considered to be significant, whereas activation (I306G, but not K287C/K294C) clearly improved avidity (Fig. 6 A,B). Activation also increased the avidity of α_MI for both collagen I and IV (Fig. 6 C,D). Binding of wild-type α_XI to collagen IV was confirmed in these experiments. After I314G mutation, the avidity was even slightly better (Fig. 5 E,F). The strongest interactions were measured between the α_LI I306G domain and collagen I ($K_d \approx 80$ nM) and between α_XI I314G and

collagen IV ($K_d \approx 180$ nM). Based on these estimated values, collagen binding may be even stronger than to the natural ligands of the α_LI, α_MI and α_XI domains using the same assay (Table 2). Furthermore, integrin α_XI (I314G) bound with high avidity ($K_d \approx 200$ nM) to collagen VI (data not shown).

3.5. Leukocyte integrin α_XI recognizes the GFOGER motif

The binding of the collagen receptor integrins to collagens is based on the specific recognition of triple helical GFOGER-like sequences. Here we tested the adhesion of recombinant αI domains to synthetic peptides containing this motif. As a positive control, we showed that α_2I domains, both wild-type and activated, bind to the GFOGER-peptide with very high affinity (Fig. 7A, Table 2). Both wild-type and I314G α_XI bound with moderate affinity to GFOGER ($K_d \approx$ 200-300 nM), and activated α_MI domain showed weak interaction (Fig. 7B; Table 2). Neither wild-type nor activated α_LI bound to GFOGER-peptides better than GST alone (not shown).

3.6. Leukocyte integrins bind to the GFOGER motif

We used xCelligence technology to measure HL60 cell adhesion to GFOGER peptides and tested the binding mechanism using specific antibodies (Fig. 8). At 30 min time point antibodies against α_M, α_X and β_1 integrin subunits could block adhesion, while after 2 hours' attachment time only α_X antibodies had a significant effect ($p=0.002$; Fig. 8). We concluded that in HL60 cells both $\alpha_M\beta_2$ and $\alpha_X\beta_2$ are functional receptors for collagenous GFOGER peptides.

4. Discussion

Collagen receptor and leukocyte integrins have a common evolutionary history and they share structural features, such as the ligand binding αI domain. A major difference between the two subgroups is the presence of an extra αC helix in the collagen binding αI domains. In general, in the leukocyte integrin αI domains, the ligand binding site in located in a quite "flat" environment when compared to the "groove" on collagen receptor αI domains. The exact function of the αC helix is not known but it has been speculated that the αC could be responsible for ligand recognition since it positions itself on one edge of the ligand binding site and partly covers it (Käpylä et al., 2000; Tulla et al., 2008; Lahti et al., 2011). To find out whether the αC helix is critical for collagen recognition, we have deleted it from the α_2I domain (Käpylä et al., 2000). Measurements by IASYS surface plasmon resonance technology suggested that the variant ΔαC α_2I domain may have diminished avidity to collagen I (Käpylä et al., 2000). When αI domains and solid phase binding assay conditions comparable to the present study were used, the αC deletion variant could bind even better than wild-type α_2I (Lahti, M., unpublished results). This effect might be due to the fact that αC deletion breaks the Arg^{288}-Glu^{318} ion pair and promotes the open fold. Thus, the conclusion is that the lack of the αC helix in leukocyte αI domains is hardly a problem in collagen binding.

Based on the number of their potential ligands the integrins in both leukocyte and collagen receptor subgroups seem to be promiscuous. The collagen receptor integrin $\alpha_2\beta_1$ may bind to various collagen subtypes and additionally to laminins (Elices and Hemler, 1989; Languino et al., 1989; Tulla et al., 2008), tenascin C (Sriramarao et al., 1993), chondroadherin (Camper et al., 1997), proteoglycans (Guidetti et al., 2002; Bix et al., 2004), matrix metalloproteinase 1 (Dumin et al., 2001), adhesion receptor E-cadherin (Whittard et al., 2002) and collectin family members (Zutter and Edelson, 2007). On the other hand, there is some experimental evidence that leukocyte integrins may also bind to collagens (Garnotel et al., 1995; Walzog et al., 1995; Garnotel et al., 2000). More precisely, the function blocking antibodies for $\alpha_L\beta_2$ have been reported to inhibit the binding of human polymorphonuclear leukocytes (PMN) to the telopeptide area of collagen I (Garnotel et al., 1995). However, despite the fact that $\alpha_L\beta_2$ is not a receptor for the RGD recognition motif, this interaction could also be inhibited by RGD peptides (Garnotel et al., 1995). Thus, the mechanism may

also require other receptors. In another study, PMNs were reported to bind to collagens II and VI in an $\alpha_M\beta_2$ integrin dependent manner (Walzog et al., 1995). Furthermore, monocytes have been reported to recognize collagen I by $\alpha_X\beta_2$ related mechanism (Garnotel et al., 2000). However, the fact that $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins bind to albumin and many denatured proteins frequently used to block unspecific background makes the interpretation of the adhesion assays very challenging (Davis, 1992). In general, the binding of the leukocyte integrins to collagens has never been systematically studied. To obtain more detailed information about the ability of leukocyte integrins to serve as collagen receptors, we cloned the αI domains of α_L, α_M, α_X integrins and analyzed them using collagen binding assays. Based on our previous experiments, the length of the αI domain construct is critical for the affinity (Käpylä, J., unpublished results). Therefore, we carefully designed the produced recombinant proteins to be comparable to each other and to the collagen binding α_2I domain. Since integrin αI domains can have at least two different conformations, open and closed, it was also necessary to test the activated gain-of-function variants of all αI domains.

The purpose of the variants was to mimic the open conformations of αI domains. The mutations introduced into the leukocyte integrin αI domains were I306G and K287C/K294C (α_LI), I316G (α_MI), and I314G (α_XI) (Huth et al., 2000; Lu et al., 2001; Xiong et al, 2000; Vorup-Jensen et al., 2003, respectively). The α_2I gain-of-function variant was α_2E318W (Aquilina et al, 2002; Tulla et al., 2008). Mutation E318W in the α_2I domain breaks an interchain salt bridge (Aquilina et al, 2002), whereas mutation I306A in α_LI, I316G in α_MI and I314G in α_X opens the αI domain structure by inhibiting isoleucine side-chain binding to a specific hydrophobic pocket (Huth et al., 2000; Xiong et al, 2000; Vorup-Jensen et al., 2003, respectively). The integrin α_LI domain was also opened by creating a novel interchain disulphide bond (K287C/K294C) (Lu et al, 2001). In most cases, activated variants increased their binding to primary ligands – α_2I to collagen I, α_LI to ICAM-1 and α_MI to iC3b – but, in the case of the α_XI variant, there was no increase in the binding of iC3b. Therefore, we cannot be sure that in our assays this variant represents an open, activated αI domain. Notable wild-type α_LI and α_MI domains bound their primary ligands very weakly, whereas the wild-type α_2I and α_XI domains interacted with their primary ligands with higher avidity. This is in agreement with the $\alpha_X\beta_2$ ectodomain structure in which αI domain is inserted into the α_X

subunit by a flexible linker (Xie et al., 2010) suggesting that ligand binding might be possible also for the closed form of αI domain.

The human collagen family contains at 28 different triple helical collagen subtypes (Myllyharju and Kivirikko, 2004). Six collagen subtypes representing three subgroups were used to analyze the leukocyte integrin αI domain ligand binding preference: collagen subtypes I-III and V (fibril-forming collagens), network-forming collagen IV and beaded filament-forming collagen VI. Each leukocyte integrin αI domain showed a unique recognition pattern in collagen binding. The α_LI needed to be activated after which it bound to the fibril-forming collagens. The α_MI seemed to recognize all collagen subtypes very weakly but activation improved somewhat this recognition. The α_XI bound with moderate avidity to collagen IV even before activation. Generally, it can be stated that α_LI, α_MI, and α_XI domains are capable of recognizing and binding to collagen subtypes, but all αI domains have individual features. The estimated K_d for the strongest leukocyte αI domain–collagen interactions were within the same range as the interactions of the same αI domains with their primary ligands. We also confirmed the role of $\alpha_X\beta_2$ and $\alpha_M\beta_2$ as a collagen receptor by measuring human promyelocytic leukemia cells (HL-60) adhesion with xCelligence technology (Roche). In these experiments, the blocking of the α_X or the α_M function markedly decreased cell adhesion to collagen IV.

The recognition of GFOGER-like motifs is thought to be the main mechanism in the collagen binding by the collagen receptor integrin αI domains (Knight et al., 1998; Emsley et al., 2000). Other similar motifs include GROGER and GLOGEN (Kim et al., 2005; Raynal et al., 2006). To test whether leukocyte integrins can bind to collagen with a similar mechanism than the collagen receptors, we performed more solid phase binding assays. The α_LI domain showed no significant binding to triple helical GFOGER peptides when compared to binding of GST-protein alone. By contrast, wild-type and I314G α_XI interacted with GFOGER showing moderate affinity. The binding of activated (I316G) α_MI was weaker than α_XI. Furthermore, our cell adhesion assays suggested that the action of $\alpha_X\beta_2$ and $\alpha_M\beta_2$ as low affinity collagen receptors can be based on the recognition of the GFOGER motif.

Leukocyte–collagen interaction plays an important role during inflammation (Evans et al., 2009). The participation of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins has been extensively studied, while the

role of two other collagen receptors, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$ is not known. Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were originally described as lymphocyte very late activation antigens 1 and 2 (VLA-1 and -2) (Hemler et al., 1985). The use of specific antibodies against the $\alpha_1\beta_1$ integrin in animal models as well as experiments with α_1 deficient mice have indicated the involvement of this receptor in graft-versus-host disease, arthritis, colitis, allergen-induced bronchoconstriction, glomerulonephritis and Alport disease (Ben-Horin and Bank, 2004; Dennis et al, 2010). Integrin $\alpha_2\beta_1$ is also expressed on various inflammatory cells, including Th17 T lymphocytes (Boisvert et al., 2010; Immunological genome project, Hang and Painter, 2008) and granulocytes (Werr et al., 2000). It is involved in processes related to both acquired and innate immunity (McCall-Culbreath et al., 2008).

Our results propose that the functions of the leukocyte integrins and the collagen receptor integrins are overlapping, since $\alpha_X\beta_2$ and $\alpha_M\beta_2$ seem to bind to GFOGER motif and collagen IV. It can be hypothesized that leukocyte integrins may act as inflammatory cell collagen receptors prior to the appearance of the "professional" collagen receptors and after that they may continue in an assisting role. For example, the ameboid locomotion of leukocytes in tissues may require low affinity, rather than high affinity adhesion mechanisms. This may also be true for some cancers, such as leukemias, having elevated levels of $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ as seen from the Cancer Cell Line Encyclopedia (Barretina et al., 2012).

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Figure Legends

Fig. 1. Effect of anti-integrin mAbs on HL60 adhesion to collagen IV. Cell adhesion was measured on collagen IV for HL-60 cells using xCELLigence real-time cell analyzer (Roche). "Cell index" means the change in impedance and it reflects the attachment and the spreading of cells. The 96-well E-plates were coated with mouse collagen IV in PBS (pH 7.5). The coated wells were washed with PBS. PBS was removed and antibodies (anti-α_L, anti-α_X, anti-β_1 and anti-mouse IgG in panel A; anti-α_M and anti-mouse IgG in panel B) in RPMI-1640 (without FCS) were added to the wells. The background signal was measured, and 40,000 cells/well were added. Six replicates were used for each sample. Data at 30 min and 2 h time points were taken from the corresponding data sets (not shown). The p values showed in the panels were estimated according to the Mann-Whitney U test. The p values were less than 0.01 where indicated **.

Fig. 2. The structures of the integrin α_LI, α_MI, α_XI and α_2I domains (A, B, C and D, respectively) in closed conformation (Protein Data Bank ID: 1ZON, 1JLM, 1N3Y, 1AOX, respectively). The αI domains are shown as cartoon representations; highlighted residues are drawn as sticks; and a MIDAS (metal ion-dependent adhesion site) coordinated divalent metal ion is drawn as a sphere (Mn^{2+} in α_MI and Mg^{2+} in α_2I). Gain-of-function point mutations of specific isoleucines to glycines (I306G in α_LI, I316G in α_MI and I314G in α_XI) can be used to open the αI domain structure of leukocyte integrins and experimentally mimic the activated αI domains. Furthermore, a disulfide bond can be created by a double-mutation (K287C/K294C) to stabilize the activated, open conformation of the α_LI domain. E318W mutation of the collagen receptor α_2I domain disrupts the salt bridges between Glu^{318} and Arg^{288} of the αC helix present in the closed form of the α_2I domain, subsequently promoting the adoption of the open form. The α subunits of leukocyte integrins, α_L, α_M and α_X, lack the αC helix, which is typical of the collagen receptor α subunits, α_1, α_2, α_{10} and α_{11}. All αI domains have a MIDAS site, although in these crystal structures of α_LI and α_XI there is no metal ion present.

Fig. 3. Binding of the collagen receptor α_2I domain and its variant (E318W) to collagens. Binding to human collagen types I-VI (A). The gain-of-function variant of α_2I (E318W) has increased avidity to rat collagen I (B) and to mouse collagen IV (C) compared to the α_2I WT

domain. Ligand recognition of the recombinant GST fusion collagen receptor integrin α_2I domain and its variants was studied in a solid phase binding assay. 400 nM of GST fusion α_2I domain and E318W variant were used in (A) and 4-400 nM in (B-C). GST served as a negative control in the binding assay. Each sample was measured in three parallel wells. Estimates for the dissociation constants (B and C) were obtained by using the equation: measured binding = maximal binding/(1 + K_d/[αI]).

Fig. 4. Activating mutations increased the binding of primary ligands to α_LI (A), and α_MI (B), but not to α_XI (C). Ligand recognition of the recombinant GST fusion leukocyte integrin αI domains was studied in a solid phase binding assay. GST served as a negative control in the binding assay. Each sample was measured in three parallel wells. Estimates for the dissociation constants were obtained by using the equation: measured binding = maximal binding/(1 + K_d/[αI]).

Fig. 5. Binding of the leukocyte integrin α_LI (A), α_MI (B) and α_XI (C) domains and their activated variants to human collagen types I-VI. Ligand recognition of recombinant GST fusion leukocyte integrin αI domains (400 nM) was studied in a solid phase binding assay. GST served as a negative control in the binding assay. Each sample was measured in three parallel wells.

Fig. 6. Activated leukocyte integrin α_LI, α_MI and α_XI domains recognize rat collagen type I (A, B and C, respectively) and mouse collagen type IV (D, E and F, respectively). Ligand recognition of recombinant GST fusion leukocyte integrin αI domains was studied in a solid phase binding assay. GST served as a negative control in the binding assay. Each sample was measured in three parallel wells. Estimates for the dissociation constants were obtained by using the equation: measured binding = maximal binding/(1 + K_d/[αI]).

Fig. 7. Binding of α_2I, α_MI and α_XI domains and their activated variants to the GFOGER peptide ligand (A, B and C, respectively). Ligand recognition of recombinant GST fusion integrin αI domains was studied in a solid phase binding assay. GST served as a negative control in the binding assay. Each sample was measured in three parallel wells. Estimates for the dissociation constants were obtained by using the equation: measured binding = maximal binding/(1 + K_d/[αI]).

Fig. 8. Effect of anti-integrin mAbs on HL60 adhesion to GFOGER peptides. Cell adhesion was measured on GFOGER peptides for HL-60 cells using xCELLigence real-time cell analyzer (Roche). "Cell index" means the change in impedance and it reflects the attachment and the spreading of cells. The 96-well E-plates were coated with peptides in PBS (pH 7.5). The coated wells were washed with PBS. PBS was removed and antibodies (anti-α_M, anti-α_X, anti-β_1 and anti-mouse IgG) in RPMI-1640 (without FCS) were added to the wells. The background signal was measured, and 40,000 cells/well were added. Six replicates were used for each sample. Data at 30 min and 2 h time points were taken from the corresponding data sets (not shown). The p values showed in the panels were estimated according to the Mann-Whitney U test. The p values were less than 0.01 where indicated **.

Table 1: The primers used for amplification of leukocyte integrin αI domain cDNAs.

Cloning primers

Forward	N-terminal	Sequence	Restriction
αLI	L111	5′-CAGAGGATCCCTGCAGGGTCCCATGC-3′	*Bam*HI
αMI	N113	5′-CAGAGGATCCAACCTACGGCAG-3′	*Bam*HI
αXI	P112	5′-GAAAGGATCCCCCACCCAGCTCACC-3′	*Bam*HI
Reverse	C-terminal	Sequence	Restriction
αLI	S313	5′-GCAAGAATTCGCTTGTGCCCTCAATGACATA-3′	*Eco*RI
αMI	Q323	5′-AGATCTCGAGTCACTGAGTACCCTCGATCGCAAA-3′	*Xho*I
αXI	E321	5′-AGATCTCGAGTCACTCCGTACCCTCAATGGC-3′	*Xho*I

Restriction sites are underlined.

Table 2: Dissociation constants (nM) of integrin αI domains and their variants for rat collagen I, mouse collagen IV, GFOGER peptide, human ICAM-1 and iC3b.

Ligand	Collagen I	Collagen IV	GFOGER	ICAM-1	iC3b
αLI WT	>>500	>500	N.B.	N.B.	N.D.
αLI I306G	80	200	N.B.	400	N.D.
αLI K287C/K294C	120	>500	N.B.	200	N.D.
αMI WT	>500	>>500	>>>500	N.D.	>500
αMI I316G	350	360	>500	N.D.	280
αXI WT	>>500	370	270	N.D.	170
αXI I314G	240	180	220	N.D.	180
α2I WT	3	20	4	N.D.	N.B.
α2I E318W	1	10	2	N.B.	290

Not determined (N.D.). No binding (N.B.).

Figure 1

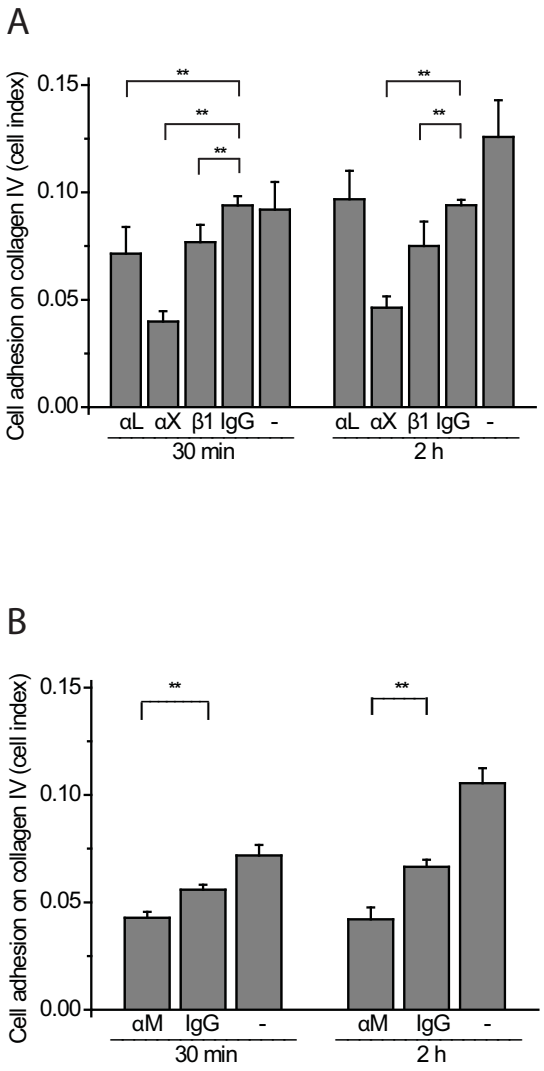

A
Cell adhesion on collagen IV (cell index)
0.15
0.10
0.05
0.00
**
**
**
**
**
**
αL αX β1 IgG -
αL αX β1 IgG -
30 min
2 h
B
Cell adhesion on collagen IV (cell index)
0.15
0.10
0.05
0.00
**
**
αM IgG -
αM IgG -
30 min
2 h

Figure 2

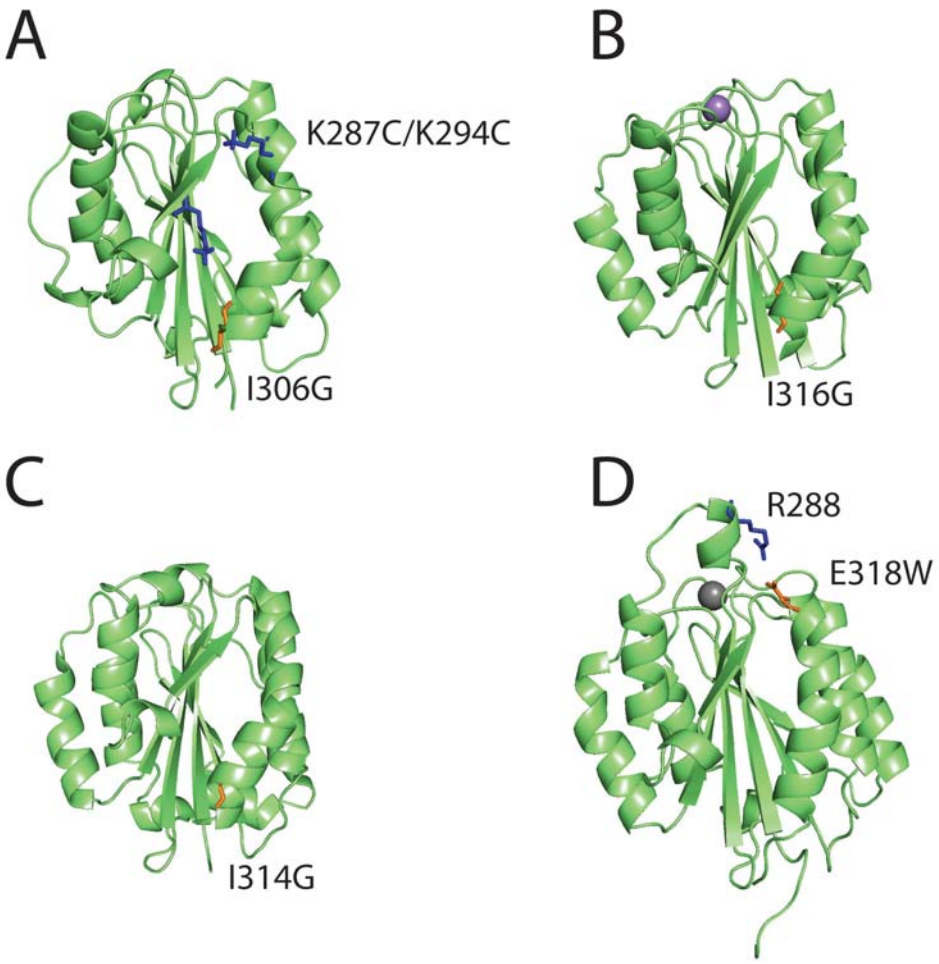


Figure 3

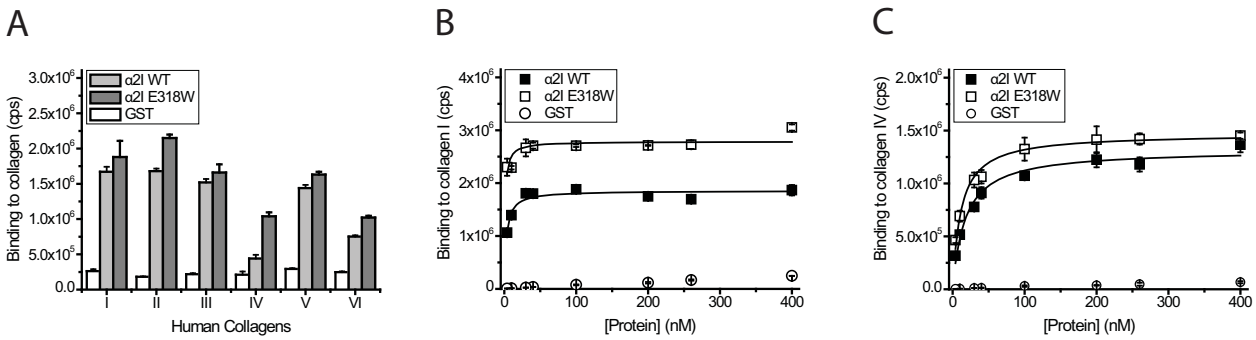

A
B
C
α2I WT
α2I E318W
GST
Binding to collagen (cps)
Human Collagens
I
II
III
IV
V
VI
Binding to collagen I (cps)
Binding to collagen IV (cps)
[Protein] (nM)

Figure 4

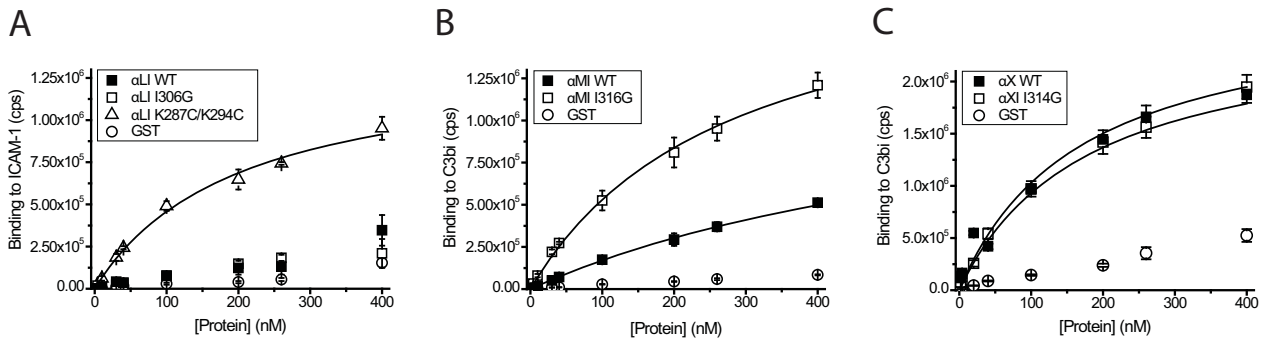

A
αLI WT
αLI I306G
αLI K287C/K294C
GST
Binding to ICAM-1 (cps)
[Protein] (nM)
B
αMI WT
αMI I316G
GST
Binding to C3bi (cps)
[Protein] (nM)
C
αX WT
αXI I314G
GST
Binding to C3bi (cps)
[Protein] (nM)

Figure 5

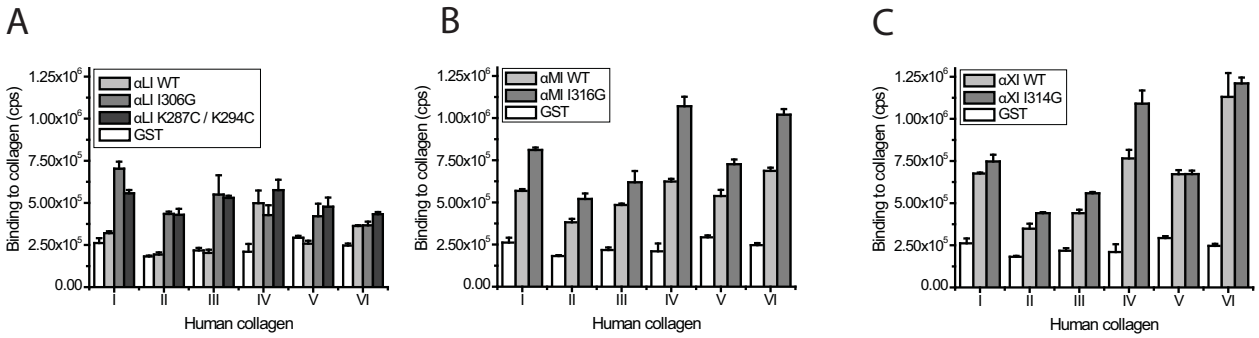

A
B
C
αLI WT
αLI I306G
αLI K287C / K294C
GST
αMI WT
αMI I316G
GST
αXI WT
αXI I314G
GST
Binding to collagen (cps)
1.25x10^6
1.00x10^6
7.50x10^5
5.00x10^5
2.50x10^5
0.00
I
II
III
IV
V
VI
Human collagen

Figure 6

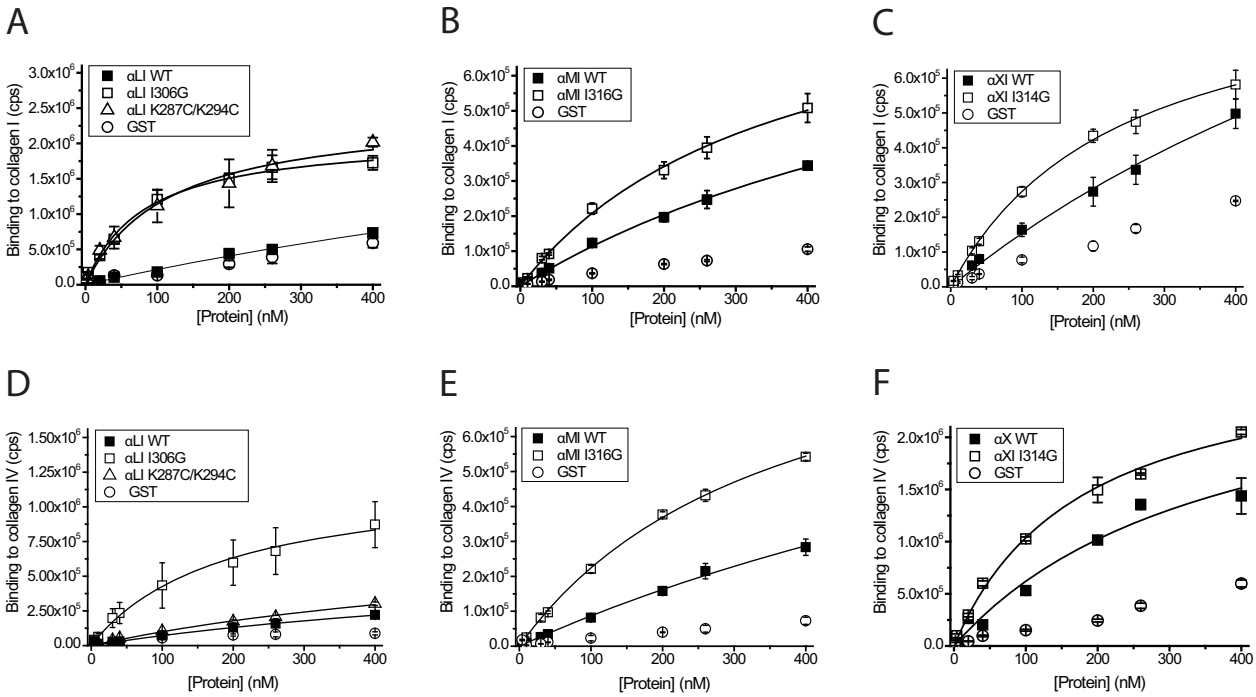

A
αLI WT
αLI I306G
αLI K287C/K294C
GST
Binding to collagen I (cps)
[Protein] (nM)
B
αMI WT
αMI I316G
GST
Binding to collagen I (cps)
[Protein] (nM)
C
αXI WT
αXI I314G
GST
Binding to collagen I (cps)
[Protein] (nM)
D
αLI WT
αLI I306G
αLI K287C/K294C
GST
Binding to collagen IV (cps)
[Protein] (nM)
E
αMI WT
αMI I316G
GST
Binding to collagen IV (cps)
[Protein] (nM)
F
αX WT
αXI I314G
GST
Binding to collagen IV (cps)
[Protein] (nM)

Figure 7

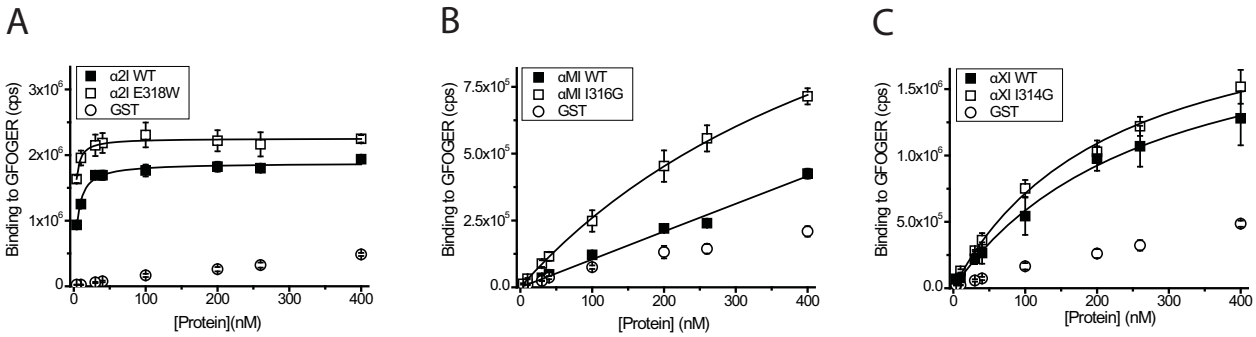
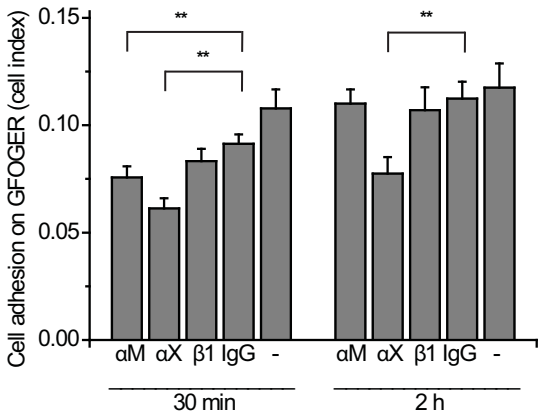

A
α2I WT
α2I E318W
GST
Binding to GFOGER (cps)
[Protein](nM)
B
αMI WT
αMI I316G
GST
Binding to GFOGER (cps)
[Protein] (nM)
C
αXI WT
αXI I314G
GST
Binding to GFOGER (cps)
[Protein] (nM)

Figure 8


Cell adhesion on GFOGER (cell index)
0.15
0.10
0.05
0.00
**
**
**
αM αX β1 IgG -
αM αX β1 IgG -
30 min
2 h

SUPPLEMENTAL DATA [Lahti et al., (2012)]

TABLE S1. Primers used in mutagenesis of leukocyte integrin αI domains.

Mutegenesis primers		
Forward	Mutation	Sequence
αLI	K287C	5′-CGAGTTTGTGTGCATTCTGGACACATTT-3′
αLI	K294C	5′-CTGGACACATTTGAG TGCCTGAAAGATCTATTC-3′
αLI	I306G	5′-CTGCAGAAGAAGGGCTATGTCATTGAG-3′
αMI	I316G	5′- CTTCGGGAGAAGGGCTTTGCGATCG-3′
αXI	I314G	5′-CTGAAGGAGAAGGGCTTTGCCATTGAG-3′
Reverse	Mutation	Sequence
αLI	K287C	5′-AAATGTGTCCAGAATGCACACAAACTCG-3′
αLI	K294C	5′-GAATAGATCTTTCAGGCACTCAAATGTGTCCAG-3′
αLI	I306G	5′-CTCAATGACATAGCCCTTCTTCTGCAG-3′
αMI	I316G	5′-CGATCGCAAAGCCCTTCTCCCGAAG-3′
αXI	I314G	5′-CTCAATGGCAAAGCCCTTCTCCTTCAG-3′

Mutated codons are underlined.