Inhibition of β_2 Integrin–mediated Leukocyte Cell Adhesion by Leucine–Leucine–Glycine Motif–containing Peptides

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Abstract. Many integrins mediate cell attachment to the extracellular matrix by recognizing short tripeptide sequences such as arginine–glycine–aspartic acid and leucine–aspartate–valine. Using phage display, we have now found that the leukocyte-specific β_2 integrins bind sequences containing a leucine–leucine–glycine (LLG) tripeptide motif. An LLG motif is present on intercellular adhesion molecule (ICAM)-1, the major β_2 integrin ligand, but also on several matrix proteins, including von Willebrand factor. We developed a novel β_2 integrin antagonist peptide CPCFLLGCC (called LLG-C4), the structure of which was determined by nuclear magnetic resonance. The LLG-C4 peptide inhibited leukocyte adhesion to ICAM-1, and, interestingly, also to von Willebrand factor. When immobilized on plastic, the LLG-C4 sequence supported the β_2 integrin–mediated leukocyte adhesion, but not β_1 or β_3 integrin–mediated cell adhesion. These results suggest that LLG sequences exposed on ICAM-1 and on von Willebrand factor at sites of vascular injury play a role in the binding of leukocytes, and LLG-C4 and peptidomimetics derived from it could provide a therapeutic approach to inflammatory reactions.

Key words: cell adhesion • extracellular matrix • leukocyte • phage display • peptides

Introduction

The migration of leukocytes through the body and the various lymphoid organs is an essential element of the immune system. While circulating in blood or lymphatic vessels, leukocytes are in a resting and low adhesive state. However, when leukocytes are stimulated by signals from the immune system, such as exposure to an immune complex or a chemokine gradient, their integrin adhesion receptors become activated (Hemler, 1990; Hynes, 1992; Springer, 1994; Gahmberg et al., 1997). The activation of the integrins is essential for the many leukocyte functions. Such functions are, for example, binding to antigen-presenting cells, recirculation through lymph nodes, and migration out of the vasculature and through the extracellular matrix to sites of inflammation. The integrin activation needs to be tightly regulated as inappropriate leukocyte adhesion leads to injury of normal tissues.

Leukocytes express a specific subset of the integrin family, the β_2 integrins, of which four members are known. They have a common β_2 chain (CD18), but different α subunits (α_L or CD11a, α_M or CD11b, α_X or CD11c, α_D or CD11d) (Gahmberg et al., 1997). The α subunits contain a conserved 200-residue A or I domain, which is essential for binding of most ligands. The crystal structures of I domains from the α_L and α_M subunits indicate the presence of a cation binding site called the metal-dependent adhesion site (Lee et al., 1995; Qu and Leahy, 1995). Amino acid substitutions in this site abrogate ligand binding (Huang and Springer, 1995; Kamata et al., 1995).

The major ligands of these integrins, the intercellular adhesion molecules (ICAMs),[1] belong to the Ig superfamily, and five ICAMs with slightly different binding specificities have been described (Simmons et al., 1988; Staunton et al., 1989; Fawcett et al., 1992; Bailly et al., 1995; Tian et al., 1997). The expression of ICAM-1 on endothelial cells is subject to stimulation by inflammatory cytokines, which enhances the β_2 integrin–mediated adhesion of leukocytes on endothelial cells (Springer, 1994; Gahmberg et al., 1997). In addition to the ICAMs, fibrinogen (Languino et al., 1993) and the iC3b complement protein (Ueda et al., 1994; Kamata et al., 1995) are known ligands of the β_2 integrins, particularly of $\alpha_M\beta_2$ (Mac-1).

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[1]*Abbreviations used in this paper:* GST, glutathione *S*-transferase; ICAM, intercellular adhesion molecule; LLG, leucine–leucine–glycine; NMR, nuclear magnetic resonance; nOe, nuclear Overhauser enhancement; RGD, arginine–glycine–aspartic acid; TNF, tumor necrosis factor.

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Because of the importance of the β_2 integrins for leukocyte function, antagonists of them are potential antiinflammatory agents. Antibodies to β_2 integrins or ICAMs have a therapeutic effect in animal models of immune system disorders (Clark et al., 1991; Kavanaugh et al., 1994; Miyamoto et al., 1999). Agents targeting the β_2 integrins could also be valuable in the development of therapeutic strategies to human leukemias (Lalancette et al., 2000). However, only a few small molecule antagonists of the β_2 integrins have been described so far (Kallen et al., 1999; Kelly et al., 1999). Lack of such compounds has prevented the detailed examination of the role of each member of the β_2 integrin family in leukemia dissemination as well in inflammatory diseases. In particular, it would be desirable to design compounds that distinguish between the inactive and active state of an integrin. Modeling of such small molecule inhibitors has been hampered by the large size of the peptide ligands developed so far. Linear peptides are often without a well-defined structure when free in solution. Among the few β_2 integrin ligands discovered is the 22–amino acid–long peptide known as P1, which was derived from ICAM-2 (Li et al., 1993). This peptide retains the leukocyte integrin–activating effect that is typical for ICAM-2 (Li et al., 1995; Kotovuori et al., 1999). Complementarity-determining regions of anti-β_2 integrin antibodies have been another source of ligand peptides (Feng et al., 1998).

To develop smaller peptide ligand-leads to the β_2 integrins, we have screened random peptide libraries displayed on filamentous phage. The phage display technique has previously yielded selective peptide ligands to the integrin species $\alpha_5\beta_1$ (Koivunen et al., 1994), $\alpha_V\beta_3/\beta_5$ (Koivunen et al., 1995), and $\alpha_V\beta_6$ (Kraft et al., 1999). Phage library screenings have confirmed the earlier findings that the tripeptide sequence arginine–glycine–aspartic acid (RGD) is a common recognition sequence of a subset of integrins (Pierschbacher and Ruoslahti, 1984). The leukocyte integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are known to have a specificity for peptides containing another type of tripeptide sequence, leucine–aspartate–valine (Komoriya et al., 1991). We have now found that the $\alpha_M\beta_2$ integrin also shares the ability to recognize a motif comprising three amino acids, thus showing a functional similarity to other integrins. The tripeptide favored by $\alpha_M\beta_2$ turned out to be a previously unknown adhesion motif, leucine–leucine–glycine (LLG). Interestingly, such sequences are present on several adhesion proteins, such as ICAM-1 and von Willebrand factor. We developed a nonapeptide ligand LLG-C4, which has a compact disulfide-restrained structure as determined by nuclear magnetic resonance (NMR). This biscyclic peptide is a potent inhibitor of leukocyte cell adhesion and migration, and is a novel lead compound for development of antiinflammatory agents.

Materials and Methods

Monoclonal Antibodies

Antibodies against the integrin β_2 subunit were 7E4, 11D3, 3F9, 2E7, 1D10, and 2F3 (Nortamo et al., 1988). The anti-α_L subunit antibodies were TS2/4 and MEM-83 (Monosan). The antibodies OKM1, OKM10, and MEM-170 were used against the anti-α_M subunit, and the antibody 3.9 was used against the α_X subunit (Li et al., 1993, 1995). The $\alpha_{IIb}\beta_3$ integrin antibody P2 was purchased from Immunotech, and the $\alpha_V\beta_3$ integrin antibody LM609 and the β_1 subunit antibody 6S6 were from Chemicon.

Peptide Synthesis

Peptide synthesis was carried out using Fmoc chemistry (model 433A; Applied Biosystems). Disulfides were formed by oxidation in 10 mM ammonium bicarbonate buffer, pH 9, overnight. Peptides were then purified by HPLC on an acetonitrile gradient. Generation of disulfides was confirmed by mass spectrometry analysis. The C(1-8;3-9) and C(1-9;3-8) peptides with the guided disulfide bridges were custom-made by Anaspec. The ACDCRGDCFCG (RGD-4C) peptide (Koivunen et al., 1995) was obtained from Dr. E. Ruoslahti (The Burnham Institute, San Diego, CA).

Phage Display

The $\alpha_M\beta_2$ integrin was purified by antibody affinity chromatography from buffy coats obtained from the Finnish Red Cross blood transfusion service (Li et al., 1995). Integrin was diluted in TBS/1 mM $MnCl_2$ and coated onto microtiter wells using 1 μg/well for the first biopanning and 100, 10, and 1 ng for subsequent pannings. Biopanning was performed using CX_7C and CX_9C phage libraries essentially as described (Koivunen et al., 1994). For construction of the libraries, the single-stranded DNA encoding degenerate sequences was converted into a double-stranded form using 5 cycles of PCR with only the reverse primer, followed by 11 cycles in the presence of both the reverse and forward primers. 6 μg of the double-stranded oligonucleotide was purified using a PCR purification kit (QIAGEN) and ligated with 42 μg of the Fuse5 phage vector. The number of recombinants in the libraries was $>10^9$. Phage binding, elution, and subsequent amplification in *Escherichia coli* were repeated five times, and after each panning bacterial colonies were picked up and stored in a 10-μl vol of TBS in microtiter wells at −20°C. For direct colony sequencing, a 1-μl aliquot of the thawed samples was subjected to PCR with 10 pmol each of the forward primer 5′-TAATACGACTCACTATAGGGCAAGCTGATAAACCGATACA-ATT-3′ and the reverse primer 5′-CCCTCATAGTTAGCGTAAC-GATCT-3′. The PCR conditions were 92°C for 30 s, 60°C for 30 s, and 72°C for 60 s, and the cycle number was 35. A 1-μl aliquot of the PCR reaction was taken for sequencing using 15 pmol of either one of the primers and analyzed on an ABI 310 apparatus (PE Biosystems).

Preparation of Glutathione S-transferase and Fc Fusion Proteins

The nucleotide sequence coding for LLG-C4 was PCR amplified from phage DNA with the primers containing a BamHI 5′-AGGCTCGAG-GATCCTCGGCCGACGGGGCT-3′ and an EcoRI site 5′-AGGTCTA-GAATTCGCCCCAGCGGCCCC-3′. The PCR product was purified on an agarose gel, digested with the two restriction enzymes, and ligated into the PGEX-2TK vector (Amersham Pharmacia Biotech). Recombinants expressing LLG-C4-Glutathione S-transferase (GST) were verified by DNA sequencing. LLG-C4-GST was produced in *E. coli* strain BL 21 and purified by glutathione affinity chromatography followed by dialysis. ICAM-1-Fc fusion protein containing the five ICAM-1 Ig domains was produced in CHO cells and purified by protein A affinity chromatography (Hedman et al., 1992) α_M I domain was expressed as a GST fusion protein in *E. coli* and purified by affinity chromatography on glutathione-coupled beads followed by cleavage with thrombin to release the recombinant I domain (Ueda et al., 1994).

Integrin Binding Assays

Integrins were immunocaptured on microtiter wells that were coated with nonspecific IgG or the subunit antibodies OKM1, MEM170, TS2/4, 2E7, or 7E4. A 200-μl aliquot of the buffy coat lysate in 1% octylglucoside/1 mM $MnCl_2$/TBS was allowed to incubate for 2 h at 4°C. The wells were then washed five times with the octylglucoside-containing buffer. LLG-C4-GST or GST (10 μg/ml) was incubated in the integrin-coated or the α_M I domain–coated wells in 25 mM octylglucoside/TBS/1 mM $MnCl_2$ for 1 h. After washing of the wells, the bound GST was determined with anti-GST antibodies (Amersham Pharmacia Biotech), which were labeled with an Eu^{3+} chelate according to the instructions of the manufacturer (Wallac). The Eu^{3+} fluorescence was measured with a fluorometer (1230 Arcus; Wallac).

Cell Culture

The leukocytic cell lines THP-1, Jurkat, U-937, and K562 were maintained as described (Li et al., 1995). The nonleukocytic cell lines Eahy926, HT1080, KS6717, and SKOV-3 were as described previously (Koivunen et al., 1999). T cells were isolated from blood buffy coats by Ficoll-Hypaque centrifugation, followed by passage through nylon wool columns (Valmu and Gahmberg, 1995). Wild-type mouse L929 cells and the $\alpha_X\beta_2$ integrin–transfected L cell line were obtained from Dr. Y. van Kooyk (University Hospital, Nijmegen, Netherlands).

Cell Adhesion

Fibrinogen (Calbiochem), fibronectin (Boehringer), von Willebrand factor (Calbiochem), GST fusion proteins, Fc fusion proteins, or synthetic peptides were coated on microtiter wells at a concentration of 2 μg in 50 μl TBS unless otherwise indicated. The wild-type and A2 domain–deleted recombinant von Willebrand factors (Lankhof et al., 1997) and a capturing anti-von Willebrand factor antibody D′-D3 used for coating were provided by Drs. J.J. Sixma and Ph.G. de Groot (University Medical Center, Utrecht, Netherlands). To prepare polymerized peptides, glutaraldehyde (Merck) was added at a final concentration of 0.25%. The wells were saturated with 5% BSA and then washed five times with PBS. Before adhesion assays, cells were treated with 50 nM 4β-phorbol 12,13-dibutyrate (Sigma-Aldrich) or with 200 μM P1 peptide (Kotovuori et al., 1999) in serum-free medium for 30 min at room temperature to activate the integrins. Alternatively, cells were stimulated for 60 min at 37°C with the phorbol ester (50 nM) and the C(1-8;3-9) and RGD-4C peptides, each at a 2.5 μM concentration, after which the peptides were removed by washing with PBS/2.5 mM EDTA. Cells (100,000 per well) were incubated in the microtiter wells for 60 min at 37°C in the absence or presence of competing peptides, antibodies, or EDTA. Unbound cells were removed by gently washing with PBS and pressing the plate against paper towels. The bound cells were determined by an assay measuring cellular phosphatase activity (Li et al., 1995). Alternatively, the attached cells were stained with Crystal Violet (Sigma-Aldrich) essentially as described (Mould et al., 1995). To study T cell binding to an endothelial cell monolayer, Eahy926 endothelial cells were plated on microtiter plates at a density of 5×10^4 cells per well and grown for 3 d. To stimulate the production of ICAM-1, the cells were further grown for 16 h in the presence of tumor necrosis factor (TNF)-α (10 ng/ml; Roche). T cells (1.5×10^5 per well) were allowed to bind to Eahy926 cells for 30 min at 4°C, and then 15 min at 37°C. The unbound T cells were removed by immersing the microtiter plate upside down in PBS. The bound cells were determined by the phosphatase assay.

Cell Migration

Cell migration was studied using 8-μm pore size Transwell filters (Costar). Both the upper and lower filter surfaces were coated with fibrinogen, LLG-C4-GST, or GST at a concentration of 40 μg/ml. Free binding sites were blocked with 5% BSA. THP-1 cells (5×10^4 in 100 μl) were plated on the upper compartment in 10% serum-containing medium in the absence or presence of C(1-8;3-9) or C(1-9;3-8) (200 μM). The lower compartment was filled with 750 μl of the same medium. After a culture for 18 h at 37°C, the filters were immersed in methanol for 15 min, in water for 10 s, and in 0.1% toluidine blue (Sigma-Aldrich) for 5 min. The filters were then washed three to five times with water until cell staining was clear. Cells were removed from the upper surface of the filter with a cotton swab, and cells migrated on the lower surface were counted microscopically. A Student's *t* test was used for statistical analysis.

NMR Analysis of Peptides

For NMR structure determination, the C(1-8;3-9) peptide was dissolved in DMSO/H_2O (90/10) and C(1-9;3-8) in H_2O at the concentrations of 1–3 mM. Two-dimensional spectra, acquired with spectrometers operating at 600- and 800-MHz ^1H frequency, allowed us to identify 114 nuclear Overhauser enhancements (nOes) for C(1-8;3-9) and 85 for C(1-9;3-8) peptide. 40 structures with no restraint violations above 0.2 Å were selected from families of 200 structures generated by simulated annealing (DYANA program; Güntert et al., 1997).

Results

The LLG Peptide Motif Binds to a β_2 Integrin

We used the CX_7C and CX_9C phage libraries to search for peptide ligands to purified $\alpha_M\beta_2$ integrin. After the fifth round of selection, the CX_7C library gave a 600-fold enrichment and CX_9C a 1,000-fold enrichment of phage bound to the integrin in comparison to background. Sequencing of the bound phage revealed altogether only seven different sequences, indicating selection of specific peptides by the integrin (Table I). Four of them contained the LLG tripeptide motif. The two sequences most strongly enriched were CPCFLLGCC (LLG-C4) and CWKLLGSEEEC, and these were the only clones remaining after searching for high affinity binders by using low integrin-coating concentrations. Screening protein databases indicated that the LLG tripeptide sequence is present on several adhesion proteins. Most interestingly, it is located on the first Ig domain of ICAM-1, just preceding the Glu-34 residue, which is critical for ICAM-1 binding to the $\alpha_L\beta_2$ integrin (Staunton et al., 1990; Stanley and Hogg, 1998). The CWKLLGSEEEC peptide showed the highest similarity, five out of six consecutive residues being identical to the ICAM-1 sequence (Table I). The LLG tripeptide sequence is also contained in domains A2 and D3 of von Willebrand factor. These LLG-containing sequences, except that of ICAM-1, have not been reported previously to contain potential cell attachment sites.

We focused our studies on the LLG-C4 nonapeptide because it showed higher affinity to $\alpha_M\beta_2$ in phage-binding experiments in comparison to the other clones (data not shown). Due to the presence of four cysteine residues, the peptide appeared to be structurally constrained by two disulfide bonds. We first examined whether an integrin-binding peptide could be obtained by bacterial expression of LLG-C4 tethered to GST. The LLG-C4-GST fusion protein, but not GST alone, had a potent activity and bound to the $\alpha_M\beta_2$ integrin in a divalent cation–sensitive manner like a typical integrin ligand. The cation chelator EDTA inhibited the binding of LLG-C4-GST to the integrin, which was immunocaptured on microtiter wells with the α_M subunit antibodies MEM170 or OKM1 (Fig. 1 A). Similar EDTA-inhibitable binding of LLG-C4-GST was detected with the $\alpha_L\beta_2$ integrin, which was captured with the TS2/4 antibody. Surprisingly, EDTA only partially inhibited LLG-C4-GST binding when the β_2 subunit antibody 2E7 was used. We have found this antibody to stimulate leukocyte adhesion to various matrix proteins. LLG-C4-GST binding did not differ from GST control and was not inhibitable by EDTA, when a nonspecific IgG was used for immunocapture (not shown).

We next studied whether the peptide can directly interact with the I domain of the $\alpha_M\beta_2$ integrin, the known ligand binding site. LLG-C4-GST, examined at the concentrations of 0.01–100 μg/ml, showed a concentration-dependent binding to the isolated I domain of the α_M subunit (Fig. 1 B). GST at the same concentrations did not bind. The ability of the I domain to bind LLG-C4-GST was dependent on the Mn^{2+} cations added to the binding medium, and chelating Mn^{2+} with EDTA blocked the binding (Fig. 1 C). Initially, we encountered difficulties in chemical synthesis of an active and water-soluble LLG-C4 peptide, apparently because mixed disulfides easily formed during air oxidation. One LLG-C4 (1) preparation was highly active and blocked the ability of the I domain to bind the LLG-C4-GST (Fig. 1 C). The same peptide was also active in cell culture experiments. Another preparation, LLG-C4 (2), was inactive apparently due to disad-

Table I. Seven Phage Sequences Bound to the $\alpha_M\beta_2$ Integrin (Mac-1) and their Alignment with LLG-containing Sequences Present in Cell Adhesion Proteins

	CPCFLLGCC	(15)
	CWKLLGSEEEC	(15)
	CWHKDLLGC	(4)
	CWSMELLGC	
	CPPDLFWYC	(4)
	CPEDLYFFC	(3)
	CPEDFIFFC	
ICAM-1	CDQPKLLGIETPL	
von Willebrand factor A2	TVGPGLLGVSTLG	
von Willebrand factor D3	GRYIILLGKALSV	
Type I collagen-α2	PGPQGLLGAPGIL	
Type IV collagen-α4	PGPPGLLGRPGEA	

The amino acids that are identical to the phage peptides are shown in bold. The ICAM-1 sequence is from the first Ig domain (Simmons et al., 1988). The von Willebrand factor sequences are from A2 and D3 domains (Lynch et al., 1985) and the type I and IV collagen sequences are from α chains (De Wet et al., 1987; Leinonen et al., 1994). The number of isolated nucleotide sequences encoding each peptide is indicated in parentheses.

vantageous disulfide bonding and did not inhibit LLG-C4-GST binding to the I domain.

Immobilized LLG-C4 Nonapeptide Selectively Supports β2 Integrin–mediated Cell Adhesion

We examined the integrin-binding specificity of LLG-C4 in cell adhesion assays. Phorbol ester–activated THP-1 monocytic cells efficiently bound to LLG-C4-GST, but not to GST or peptide-GST controls (CLRSGRGC-GST, CP-PWWSQC-GST) coated on microtiter wells (Fig. 2 A). EDTA at a concentration of 2.5 mM abolished the binding. Screening with a panel of antiintegrin antibodies indicated that the cell adhesion on LLG-C4-GST was completely inhibited by the blocking antibody to the β_2 chain, 7E4 (Fig. 2 B). Antibodies to the β_1 (6S6) and β_3 integrins (LM609, P2) had no effect. Partial inhibition was obtained with the β_2 chain antibodies 11D3 and 3F9. The order of the potency of the three β_2 antibodies is the same as that obtained previously in other assays (Nortamo et al., 1988). We also studied the β_2 chain antibodies 2E7, 1D10, and 2F3 that activate the β_2 integrin–mediated cell adhesion. In accordance, each of these antibodies stimulated THP-1 adhesion on LLG-C4-GST (data not shown).

Studies with antibodies against the integrin α subunits showed that the α_X subunit antibody 3.9 effectively inhibited the THP-1 adhesion to LLG-C4-GST. The α_M subunit antibodies OKM10, MEM170, and 60.1 were weakly inhibitory, whereas the α_L-directed antibodies TS1/22 and TS2/4 had hardly any effect. Furthermore, we found that the α_X antibody 3.9 and the α_M antibody OKM10 had a synergistic effect when added together, causing a complete inhibition of the cell adhesion.

THP-1 cells similarly bound strongly to the synthetic air-oxidized LLG-C4 nonapeptide coated on plastic, and the antibodies against the $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins (3.9, OKM10, and 7E4) prevented the binding (data not shown). To determine the arrangement of the disulfide bonds in the active form of LLG-C4, we prepared synthetic peptides with different disulfide configurations. The most active peptide, C(1-8;3-9), was obtained by directing one disulfide

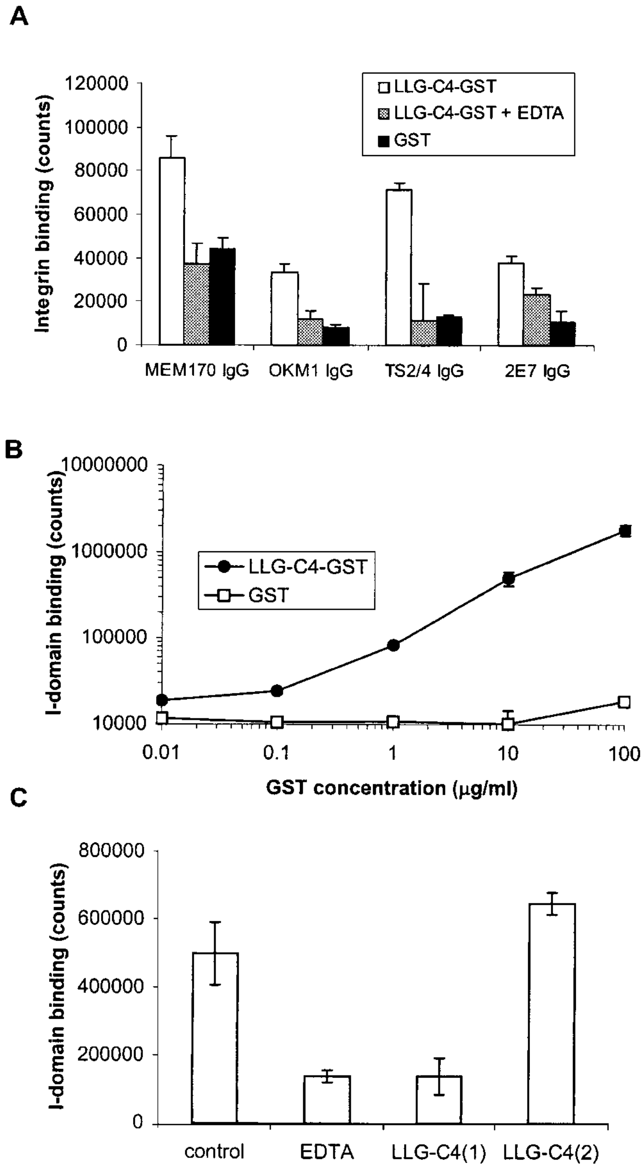

Figure 1. Divalent cation–dependent binding of LLG-C4 nonapeptide to leukocyte β_2 integrin and its I domain. (A) Integrin from a blood cell lysate was immunocaptured on microtiter wells using the α_M subunit antibody MEM170 or OKM1, the α_X subunit antibody TS2/4, or the β_2 subunit antibody 2E7. Purified LLG-C4-GST or GST control (2 μg/well) was allowed to bind for 60 min in the absence or presence of EDTA. The bound GST protein was determined by using anti-GST antibodies. The results show the means ± SD from triplicate wells. The experiment was repeated three times with similar results. (B) LLG-C4-GST or GST was incubated in microtiter wells coated with purified α_M subunit I domain. The concentrations of GST proteins were as indicated. The bound GST was determined with anti-GST antibodies. The results are means ± SD from triplicate wells. The results were similar in two other experiments. (C) LLG-C4-GST (10 μg/ml) was incubated in I domain–coated wells in the absence or presence of EDTA (2.5 mM), the LLG-C4 (1) peptide (100 μM), or the inactive LLG-C4(2) peptide (100 μM). The binding was determined with anti-GST antibodies. The results are the means ± SD from triplicate wells.

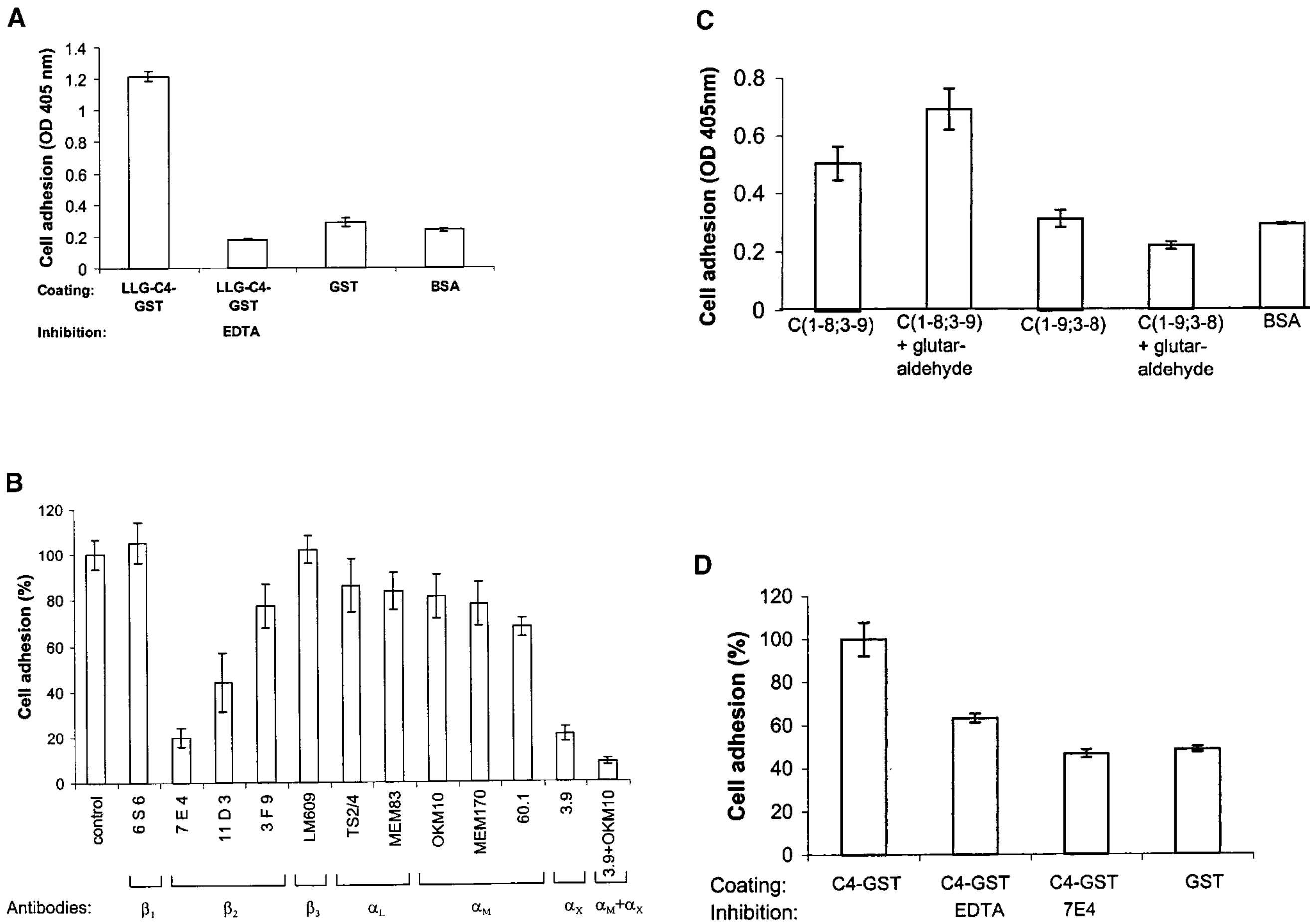

Figure 2. Immobilized LLG-C4 supports β_2 integrin–directed cell adhesion. (A) Phorbol ester–activated THP-1 cells were allowed to bind for 60 min to microtiter wells coated with LLG-C4-GST, GST, or albumin. EDTA was included at a 2.5-mM concentration. The bound cells were determined by the assay measuring cellular phosphatase activity as described in Materials and Methods. The data are the means ± SD from triplicate wells. Similar results were obtained in six other experiments. (B) THP-1 cells were mixed with each antibody against the β_1, β_2, β_3, α_X, α_M, or α_L integrin subunit as indicated. An aliquot of cells was then transferred to wells coated with LLG-C4-GST and incubated for 60 min. The bound cells were determined by the phosphatase assay. The results are the mean percentage of adhesion ± SD of two to four independent experiments, each done in triplicate wells. (C) The C(1-8;3-9) and C(1-9;3-8) peptides were coated on microtiter wells in the absence or presence of glutaraldehyde. THP-1 cells (10^5 per well) were allowed to bind for 60 min and the bound cells were determined. The results show the mean ± SD of triplicate wells. The experiment was repeated twice. (D) The $\alpha_X\beta_2$ integrin–transfected L cells were allowed to bind to LLG-G4-GST or GST. The 7E4 antibody and EDTA were used as competitors. The results, mean percentage of adhesion ± SD, are representative of three experiments conducted in triplicate wells. The difference in the binding to LLG-C4-GST versus GST is statistically significant ($P = 0.016$).

bond between the C1 and C8 cysteines and a second one between the C3 and C9 cysteines. Cells bound to the C(1-8;3-9) disulfide–containing peptide but failed to bind to the conformer with C(1-9;3-8) disulfides (Fig. 2 C). Cross-linking of the C(1-8;3-9) peptide with glutaraldehyde further enhanced cell binding, apparently due to better coating of the multimeric peptide. C(1-9;3-8) was inactive even after the cross-linking. In general, the C(1-8;3-9) peptide specifically supported the binding of β_2 integrin–expressing cells lines such β_2 integrin–transfected L cells and the leukocytic cell lines THP-1, U-937, and Jurkat. The binding of $\alpha_X\beta_2$-transfected L cells to LLG-C4-GST was inhibited by EDTA and the β_2 integrin–blocking antibody 7E4 (Fig. 2 D). Nonleukocytic cell lines L929, K562, SKOV-3, KS6717, and Eahy96, which do not express β_2 integrins, showed no binding to the peptide or LLG-C4-GST, whether the cells were pretreated with phorbol ester or not (data not shown).

LLG-C4 Nonapeptide Specifically Blocks β_2 Integrin–mediated Adhesion of Leukocytes

We examined the ability of LLG-containing peptides to block leukocyte binding to adhesion proteins containing or lacking an LLG tripeptide sequence. THP-1 cell adhesion on LLG-C4-GST was inhibited by the C(1-8;3-9) peptide with an IC_{50} of 20 μM (Fig. 3 A). The other conformer, C(1-9;3-8), was 20-fold less active than C(1-8;3-9). To study whether the LLG tripeptide sequence is sufficient for recognition by the β_2 integrins, we prepared the minimal cyclic CLLGC peptide. In a control peptide the leucines were replaced by alanines. THP-1 cell adhesion experiments using the LLG-C4-GST substratum indicated that CAAGC was only a weak competitor of cell adhesion, whereas CLLGC readily inhibited cell adhesion at concentrations of ≥1 mM, indicating a specific recognition of the LLG motif by the β_2 integrins (Fig. 3 B).

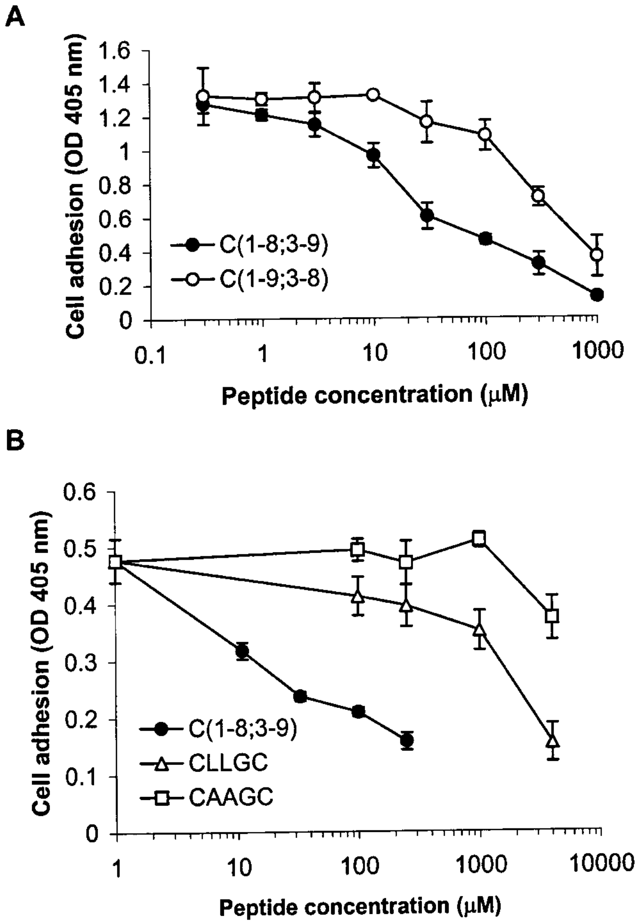

Figure 3. Effect of guided disulfide bridges on activity of LLG-C4. (A) THP-1 cells were mixed in suspension with peptides containing the C(1-8;3-9) or C(1-9;3-8) disulfides. The final peptide concentrations are indicated. Cells were then incubated for 60 min in microtiter wells coated with LLG-C4-GST. The bound cells were quantitated by the phosphatase assay. The results represent the mean ± SD of triplicate wells with similar results obtained in two other experiments. (B) THP-1 cell binding to LLG-C4-GST was examined in the presence of C(1-8;3-9), CLLGC, or CAAGC. The bound cells were determined by the phosphatase assay. The data show the means ± SD from triplicate wells and were similar in two other experiments.

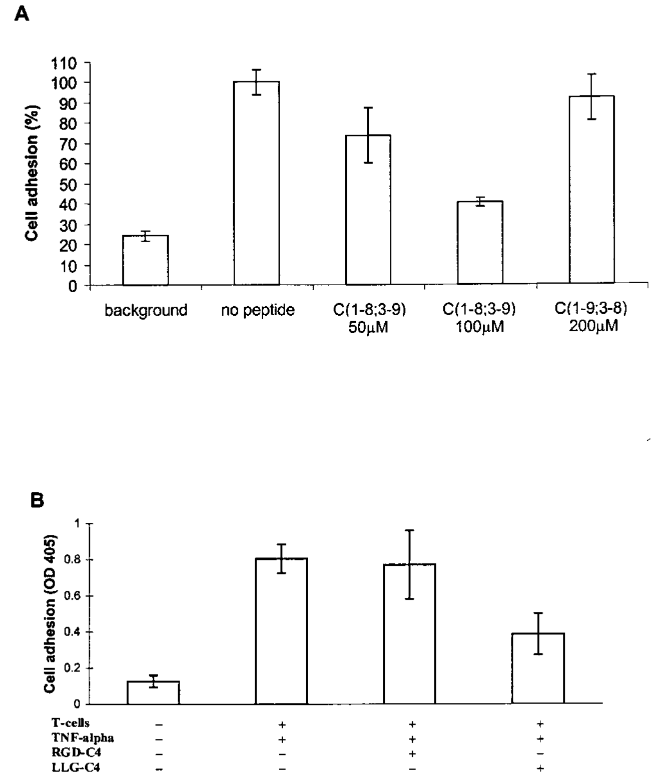

Figure 4. Inhibition of leukocyte cell adhesion to ICAM-1 by LLG-C4. (A) Jurkat cells were allowed to attach to immobilized ICAM-1-Fc in microtiter wells in the absence or presence of the LLG-C4 peptides. After a 45-min incubation, the unbound cells were removed by immersing the microtiter plate upside down on a decanter containing PBS. The attached cells were stained with Crystal Violet. The results show the percentage of cell adhesion ± SD derived from two experiments, each with triplicate or quadruplicate wells. (B) T cells were allowed to bind to TNF-α–stimulated EaHy926 endothelial cell monolayers that were grown on microtiter wells. LLG-C4 or RGD-4C was included at a concentration of 50 μM. After a 45-min incubation, the unbound cells were removed by immersing the microtiter plate in a PBS solution, and the bound T cells were determined by the phosphatase assay. The data are the mean ± SD of triplicate wells.

We next examined the ability of LLG-containing peptides to inhibit the $\alpha_L\beta_2$ integrin–mediated binding of Jurkat cells to ICAM-1-Fc recombinant protein, which contains the LLG sequence of the first Ig domain. ICAM-1-Fc was directly coated on microtiter wells or captured via protein A. In both cases we found concentration-dependent inhibition by C(1-8;3-9) on Jurkat cell adhesion and the IC_{50} was ~80 μM (Fig. 4 A). The C(1-9;3-8) conformer was severalfold less active and had hardly any effect. C(1-8;3-9) similarly inhibited the binding of freshly isolated T cells to cultured endothelial cells which were stimulated to express ICAM-1 by treatment with TNF-α (Fig. 4 B). T cells did not bind to unstimulated endothelial cells. As a control, the RGD-C4 peptide had no effect on T cell binding to endothelial ICAM-1.

As von Willebrand factor contains LLG peptide motifs, we were interested in the capability of the protein to function as a substratum for leukocytes. We found that phorbol ester–activated THP-1 cells strongly bound. The β_2 integrin antibody 7E4 blocked the THP-1 cell binding to von Willebrand factor (Fig. 5 A) and was nearly as efficient an inhibitor as the cation chelator EDTA (data not shown). The β_3 integrin antibodies LM609 and P2 were without effect. C(1-8;3-9) was a potent inhibitor of THP-1 cell binding to von Willebrand factor. The peptide inhibited with an IC_{50} of ~20 μM (Fig. 5 B). In addition, CLLGC but not CAAGC inhibited at a 500 μM concentration (data not shown). Similar C(1-8;3-9) peptide–mediated inhibition was observed on Jurkat cell binding to von Willebrand factor (not shown). Importantly, THP-1 showed weaker binding (35% of wild-type) to a mutated von Willebrand factor, from which the A2 domain, including the LLG sequence, was deleted (Fig. 5 C). Furthermore, THP-1 adhesion to the A2-deleted von Willebrand factor was not blocked by C(1-8;3-9), but by the RGD-4C peptide. To further study the specificity of the LLG peptides, we examined THP-1

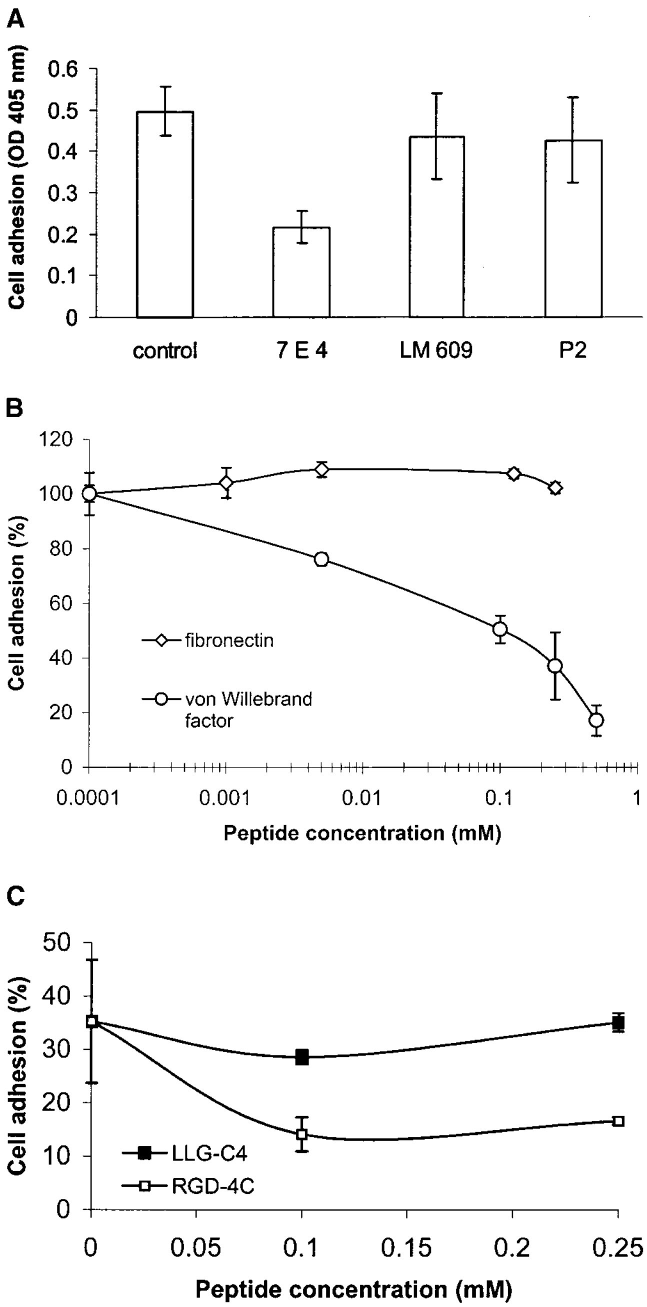

Figure 5. THP-1 cell adhesion to von Willebrand factor is inhibited by the LLG-C4 peptide. (A) THP-1 cell binding to von Willebrand factor was examined in the presence of antibodies against the β_2 (7E4), $\alpha_V\beta_3$ (LM609), or $\alpha_{IIb}\beta_3$ (P2) integrins. After a 60-min incubation in von Willebrand factor–coated wells, the bound cells were determined. The data are the mean ± SD of triplicate wells. The experiment was repeated three times. (B) THP-1 cell binding to wild-type von Willebrand factor or fibronectin in the presence of the indicated concentrations of C(1-8;3-9). (C) Binding of THP-1 cells to domain A2–deleted von Willebrand factor. The binding to wild-type von Willebrand factor was given as 100%. C(1-8;3-9) or RGD-4C was included as competitor at the concentrations described. The bound cells were determined by the phosphatase assay. The data are the mean ± SD of triplicate wells and were similar in two other experiments.

adhesion to fibronectin, a known ligand of several β_1 and β_3 integrins. C(1-8;3-9) showed no significant inhibition of fibronectin binding by THP-1 cells. C(1-8;3-9) also had no effect on binding of nonleukocytic cell lines such as HT1080 on fibronectin or fibrinogen (data not shown).

Finally, we examined THP-1 adhesion to fibrinogen, which is predominantly mediated via the $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins (Li et al., 1995). C(1-8;3-9) readily inhibited the binding, whereas C(1-9;3-8) did not (Fig. 6 A). Similar results were obtained with U937 cells, which also express the $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins (data not shown). As RGD-directed integrins can also mediate cell attachment on fibrinogen, we compared C(1-8;3-9) to the RGD-4C peptide, the selective ligand of $\alpha_V\beta_3/\beta_5$ integrins. We prestimulated THP-1 cells with low concentrations of C(1-8;3-9) and RGD-4C to fully activate both the β_2 and RGD-dependent integrins. After the peptide prestimulation, RGD-4C inhibited THP-1 cell adhesion on fibrinogen more effectively than C(1-8;3-9) (Fig. 6 B). To study whether C(1-8;3-9) and RGD-4C target different integrins, the peptides were given together to cells. The effects of C(1-8;3-9) and RGD-4C were additive and the peptide combination blocked cell adhesion efficiently.

As a model of monocyte rolling and extravasation, we examined in vitro migration of THP-1 cells on fibrinogen immobilized on Transwell filters. Cells effectively migrated in the presence of 10% serum. C(1-8;3-9) at a concentration of 200 μM completely abolished the ability of the cells to traverse the filter and bind to its lower surface (Fig. 6 C; $P = 0.005$, $n = 6$). The C(1-9;3-8) conformer was less active than C(1-8;3-9) and inhibited only partially ($P = 0.01$, $n = 6$). The activity difference between C(1-8;3-9) and C(1-9;3-8) was significant ($P = 0.003$). In a reverse strategy, when the filter was coated with LLG-C4, cell migration was strongly enhanced. Approximately 10-fold more cells migrated on the LLG-C4-GST substratum than on control GST substratum (Fig. 6 D). Cell migration on LLG-C4-GST was also more efficient when compared with fibronectin and fibrinogen coatings. C(1-8;3-9) at the 200 μM concentration completely suppressed the cell migration on LLG-C4-GST ($P = 0.0026$, $n = 6$; data not shown).

NMR Structures of Nonapeptide Conformers

We analyzed the C(1-8;3-9) and C(1-9;3-8) peptides by NMR spectroscopy to determine whether there are differences in peptide conformations due to the directed arrangement of the disulfide bonds. The structure determinations resulted in well-defined backbone conformations. The root mean square of deviation of the main chain atoms was 0.4 ± 0.2 Å for C(1-8;3-9) and 0.3 ± 0.2 Å for C(1-9;3-8) calculated from ensembles of 40 structures. For both peptides, all main chain dihedrals ϕ and ψ are in the favorable and allowed regions of Ramachandran plot. There are only a few nOes to define the side chain orientation, and therefore the side chain dihedrals of F4, L5, and L6, in particular, are dispersed (Fig. 7 A).

The pairing of the disulfides in the two ways influenced the structure of the nonapeptide considerably. The "crossing arrangement of disulfides" of C(1-8;3-9) constrains the overall structure tighter than the "parallel arrangement of disulfides" of C(1-9;3-8). This is reflected by the larger

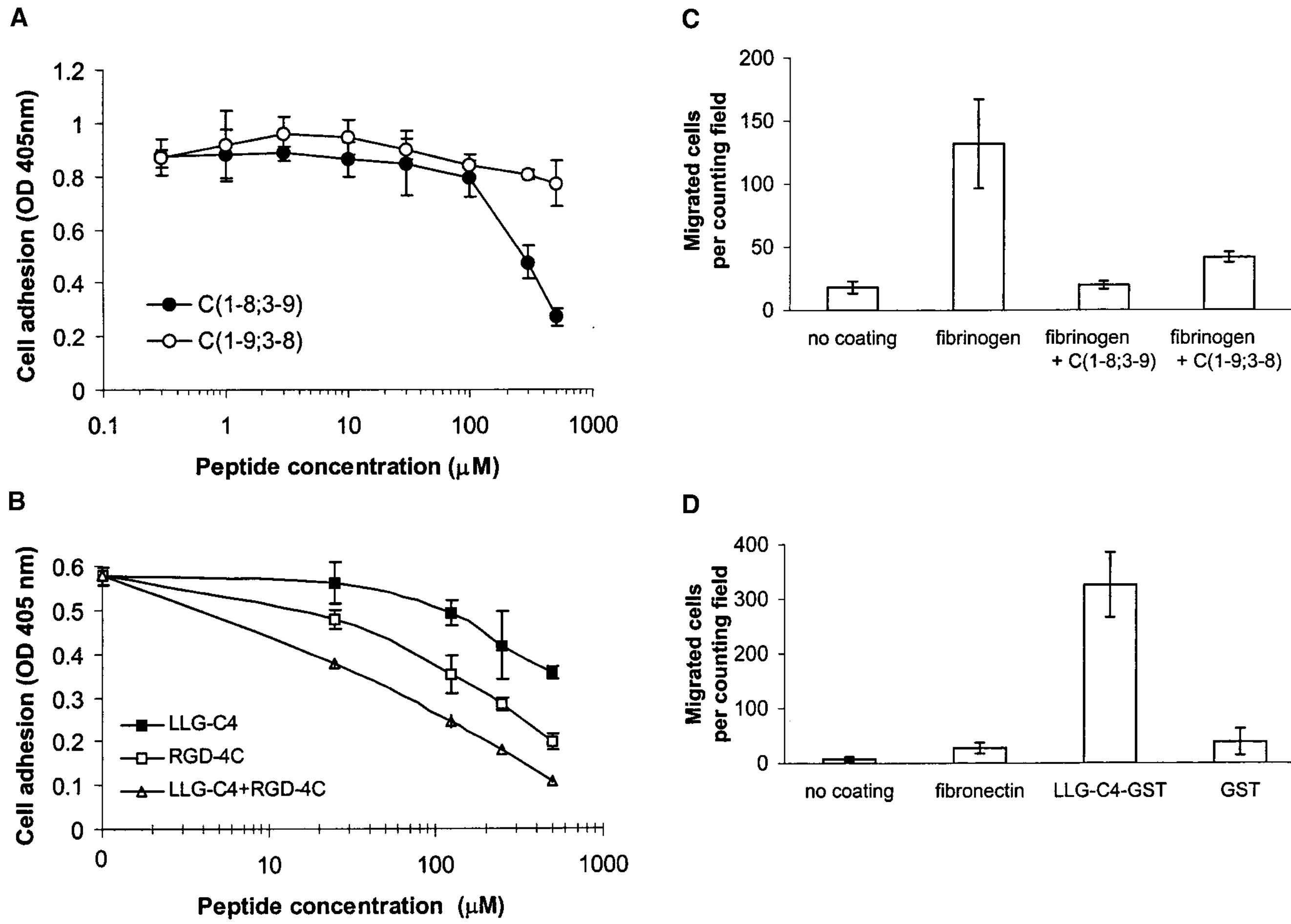

Figure 6. Synthetic LLG-C4 peptide prevents adhesion and migration of THP-1 cells on fibrinogen substratum. (A) Phorbol ester–activated THP-1 cells were administered together with C(1-8;3-9) or C(1-9;3-8), or in the absence of peptides, in microtiter wells coated with fibrinogen. After a 60-min incubation the bound cells were determined by the phosphatase assay. The results are the mean ± SD of triplicate wells and were similar in two other experiments. (B) To activate integrins, THP-1 were stimulated both with phorbol ester (50 nM) and the RGD-4C and C(1-8;3-9) peptides (each 2.5 μM) for 1 h. After washing, the cells were allowed to bind to fibrinogen-coated wells in the presence of C(1-8;3-9) or RGD-4C or both peptides at the concentrations indicated. After a 30-min incubation the bound cells were determined. The results are the mean ± SD of triplicate wells and were similar in two other experiments. At some data points the SD values are too small to be seen. (C) Transwell filters were coated both on the upper and lower surface with fibrinogen, or left uncoated, and then saturated with BSA. THP-1 cells were plated on the upper surface of the filter in the presence of 10% serum-containing medium. The concentrations of C(1-8;3-9) and C(1-9;3-8) were 200 μM. After an 18-h culture, the cells migrated underneath the filter were determined. The cells were fixed, stained, and counted under a microscope. The results show means ± SD of at least three experiments. (D) Both sides of the Transwell filters were coated with LLG-C4-GST, GST, fibrinogen, or BSA. A total of 5 × 10^4 THP-1 cells were administered per filter in 10% serum-containing medium and cultured for 18 h. The number of cells migrated to the lower surface of filter was counted microscopically. The results show means ± SD of at least three experiments.

number of nOes observed for C(1-8;3-9) (114) than for C(1-9;3-8) (85). There is no bias towards shorter distance restraints in C(1-8;3-9) compared with those of C(1-9;3-8). As a result of the different disulfide configurations, there are interresidue nOes found exclusively in one of the structures, 37 in C(1-8;3-9) and 20 in C(1-9;3-8). The crossing arrangement of disulfides in C(1-8;3-9) is topologically more complicated than the parallel bridging in C(1-9;3-8). In the short nonapeptide the adjacent disulfides with large van der Waals radii of sulphur atoms give rise to numerous steric restraints. The residue P2 also limits conformational freedom, whereas G7 contributes to it. The impact of mere topology on the steric restraints is apparent from the representative structures (Fig. 7 B). C(1-8;3-9) is more compact than C(1-9;3-8). Furthermore, there is a continuous hydrophobic surface patch composed of aliphatic groups of P2, F4, and L5 in the C(1-8;3-9) peptide. Overall, the disulfide bridges and the F4-L6 strand are buckled in C(1-8;3-9), whereas in C(1-9;3-8) they are extended. This likely accounts for the poorer water solubility of C(1-8;3-9) and may contribute to its higher activity.

Discussion

We have developed highly specific peptide antagonists of the leukocyte β_2 integrins using phage display. The most active antagonist, LLG-C4, is a biscyclic nonapeptide that is structurally restrained by two disulfide bonds and con-

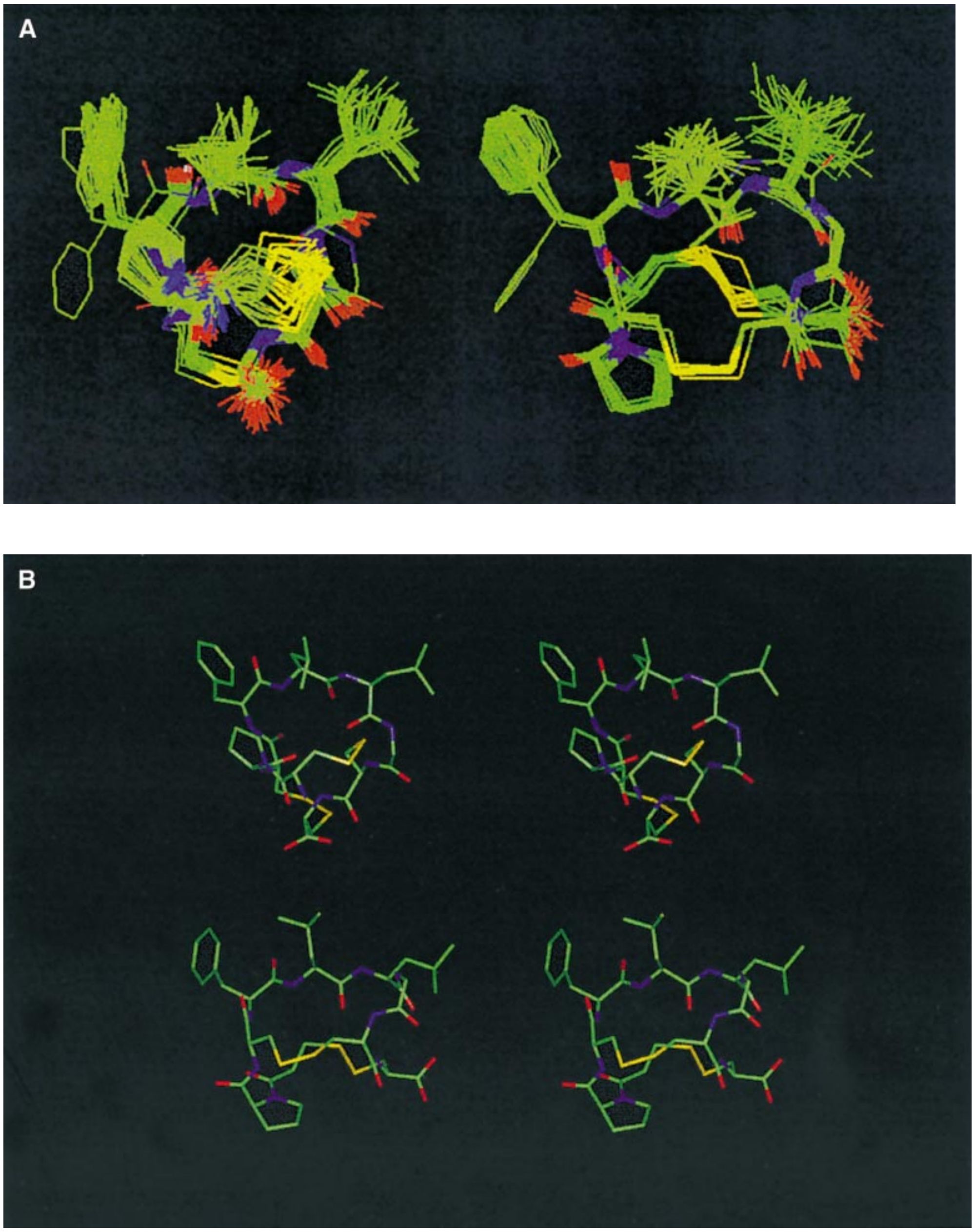

Figure 7. Comparison of structures of cyclic LLG-C4 peptide conformers by NMR. (A) Families of 40 conformations of C(1-8;3-9) (left) and C(1-9;3-8) (right) are shown. The heavy atoms of the disulfide-closed backbones are superimposed in each family and then the two families are translated apart for viewing. (B) Stereo views of representative solution structures C(1-8;3-9) (top) and C(1-9;3-8) (bottom) are shown. For this presentation the two structures were initially superimposed on the main chain atoms of F4, L5, and L6, and then translated apart for viewing. In the C(1-8;3-9) peptide C1 pairs with C8 above and C3 with C9 below the cyclic structure. Likewise, in the C(1-9;3-8) peptide C1 pairs with C9 above and C3 with C8 below the ring.

tains a novel LLG tripeptide adhesion motif. The LLG-C4 peptide specifically blocked the β_2 integrin–mediated leukocyte adhesion and inhibited leukocyte binding to their major ligand ICAM-1. Furthermore, like a typical integrin ligand, the peptide supported cell adhesion when immobilized on plastic and bound leukocytic cell lines, but not cells lacking β_2 integrins. The effectiveness and leukocyte specificity of the peptide are explained by its ability to interact with the I domain, which is a known active site in the leukocyte integrins. Interestingly, not only ICAM-1 but also several other adhesion proteins, including von Willebrand factor, contain the consensus PP/XXLLG sequence identified by phage display.

The activity of the LLG-C4 nonapeptide was strictly dependent on the correct formation of two disulfide bridges. There was a 20-fold difference in the activities of two biscyclic conformers that differed only in the configuration of the disulfide bridges. The more active peptide had a very compact structure due to a "crossing" arrangement of the disulfide bonds as shown by NMR. Interestingly, the leucine side chains protrude from the cyclic structure like antennae, suggesting that they can directly interact with the integrin. The small glycine residue may adjust a correct distance between the leucine side chains. The biscyclic RGD-4C peptide can also exist in two different isomers, depending on internal disulfide bonding, and the two structures have clearly different integrin-binding activities (Assa-Munt et al., 2001).

LLG-C4-GST is a highly efficient adhesion substratum for phorbol ester–activated THP-1 leukemia cells. We also detected cell binding to the immobilized nonapeptide, but the overall binding was weaker, apparently because the short peptide coats less efficiently on microtiter plates. We were not able to detect a similar strong binding of the $\alpha_X\beta_2$

integrin–transfected L cells to LLG-C4-GST as with THP-1. This is likely due to the fact that the integrin expression was limited only to a subset of L cells as determined by FACS® analysis.

Immunocapture experiments with different β_2 integrin antibodies showed that LLG-C4 is able to bind to each of the three integrin species, $\alpha_L\beta_2$, $\alpha_M\beta_2$, and $\alpha_X\beta_2$. EDTA inhibition showed that the binding of LLG-C4 to the integrins as well as to purified I domain is cation dependent. However, one of the antibodies used for integrin immunocapture gave an exceptional result in that EDTA could not completely inhibit the binding of LLG-C4-GST fusion protein. This antibody, 2E7, which recognizes the common β_2 subunit, shows an integrin-activating effect in cell culture and stimulates leukocyte cell adhesion to LLG-C4-GST and various matrix proteins. Thus, it is possible that this antibody changes the conformation of integrin, resulting in stronger binding. The antibody may expose secondary binding sites for ligands in integrins, and GST protein itself may then contribute to the cation-independent binding.

Previous studies have indicated that synthetic peptides spanning the LLG region of ICAM-1 (Ross et al., 1992; Li et al., 1993) or the corresponding region of ICAM-2 (Li et al., 1993) support leukocyte cell adhesion when the peptides are immobilized on plastic. In soluble forms, the peptides block binding of leukocytic cells to ICAM-1 expressed on an endothelial cell monolayer (Ross et al., 1992; Li et al., 1995). The LLG-C4 nonapeptide is significantly smaller than the peptide ligands described previously for the β_2 integrins, and showed high activity, though lacking a negatively charged amino acid residue such as glutamate. Also, the pentapeptide CLLGC inhibited cell adhesion. Thus, β_2 integrin–targeting ligands can be constructed based on the noncharged LLG motif. This is in accordance with the crystal structures and structural models of the first Ig domain of ICAM-1, where the LLG sequence is seen as part of a short β strand apparently capable of directly contacting with an integrin I domain (Bella et al., 1998; Casasnovas et al., 1998). Alanine-scanning mutagenesis studies of individual amino acids within the first Ig domain of ICAM-1 have shown that the LLG region is important for the integrin binding of ICAM-1. Mutation of one of the leucine's residues decreases ICAM-1 binding activity partially and mutation of the glycine completely (Fisher et al., 1997). Because of the inactivity of the glycine-mutated ICAM-1, it has been suggested that the glycine residue does not play a structural role, but rather directly interacts with the integrin (Fisher et al., 1997). Mutations of the corresponding valine and glycine amino acids to alanines in ICAM-2 also give proteins with impaired integrin-binding activity (Casasnovas et al., 1999). Mutation of leucine to alanine can be considered a conservative substitution, which could explain the only marginal, though significant, decrease in the activities this substitution causes in ICAM-1 and the synthetic peptides.

von Willebrand factor contains two LLG sequences, but an ability of these sequences to interact with integrins has not been reported. von Willebrand factor is a multifunctional adhesive ligand binding several proteins, and it prevents bleeding during vascular injury by mediating platelet adhesion to exposed subendothelium (Savage et al., 1998). It contains two RGD sequences, at least one of which is important in binding the platelet integrin $\alpha_{IIb}\beta_3$ (Weiss et al., 1993; Savage et al., 1996). We found that phorbol ester–activated leukocytic cells can bind to von Willebrand factor in an RGD-independent manner. Under these circumstances, leukocyte binding to wild-type von Willebrand factor was inhibited by the β_2 integrin–targeting LLG peptides and by the β_2 integrin–blocking antibody 7E4, but not by antibodies against the β_3 integrins. When the whole A2 domain of von Willebrand factor, including the LLG motif, was deleted, leukocytic cells showed much weaker binding and the LLG-C4 peptide was not inhibitory. It is notable that, besides the LLG sequences, von Willebrand factor contains I domains (Colombatti et al., 1993; Perkins et al., 1994) similar to those present in the α subunits of the β_2 integrins (Li et al., 1995; Qu and Leahy, 1995). Thus, it is possible that there are intra- or intermolecular interactions between the LLG sequences and adjacent I domains, affecting the folding of the protein. If such interactions occur, they could in part explain the inactivity of the plasma form of von Willebrand factor. Our results suggest that leukocytes can bind to the immobilized form of von Willebrand factor, such as that present in vascular subendothelium or other surfaces, and these interactions could play a role in the initial phases of inflammation.

As the β_2 integrins exist in an inactive state and become activated only after physiologic stimuli, such as by chemokines or through contact with antigen-presenting cells, it would be desirable to develop compounds binding preferentially to cells bearing the activated integrins. We found that LLG-C4 exhibits such properties and reacts with cells after integrin activation. Furthermore, LLG-C4 is a promising β_2 integrin–targeting agent, as the sequence can specifically direct phage binding to β_2 integrin–expressing cell lines, and low concentrations of the soluble peptide inhibit the binding (Koivunen, E., R. Pasqualini, and W. Arap, manuscript in preparation). Finally, the presence of LLG sequences in von Willebrand factor suggests a novel function for the protein in mediating not only platelet but also leukocyte adhesion.

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