Integrins mediate cell adhesion in response to activation signals that trigger conformational changes within their ectodomain. It is thought that a compact bent conformation of the molecule represents its physiological low affinity state and extended conformations its active state. We have determined the structure of two integrin fragments of the β2 subunit. The first structure, consisting of the plexin-semaphorin-integrin domain, hybrid, integrin-epidermal growth factor 1 (I-EGF1), and I-EGF2 domains (PHE2), showed an L-shaped conformation with the bend located between the I-EGF1 and I-EGF2 domains. The second structure, which includes, in addition, the I-EGF3 domain, showed an extended conformation. The major reorientation of I-EGF2 with respect to the other domains in the two structures is accompanied by a change of torsion angle of the disulfide bond between Cys^{361}–Cys^{392} by 180° and the conversion of a short α-helix (residues Ser^{468}–Cys^{475}) into a flexible coil. Based on the PHE2 structure, we introduced a disulfide bond between the plexin-semaphorin-integrin domain and I-EGF2 domains in the β2 subunit. The resultant αLβ2 integrin (leukocyte function-associated antigen-1) variant was locked in the structure of two integrin fragments of the extended conformations its active state. We have determined the molecule represents its physiological low affinity state and main. It is thought that a compact bent conformation of the molecule normally in a resting state of low adhesiveness, but they can rapidly become activated in response to internal "inside-out" or external "outside-in" signals. Integrin molecules are formed by two non-covalently associated α and β subunits, both type 1 membrane glycoproteins, with a globular ligand-binding "head" linked to two rod-like "legs" (3, 4). The domain organization of a typical integrin molecule is schematically depicted in Fig. 1A. The crystal structure of the ectodomain of the αβ3 integrin reveals a compact V-shaped molecule having each leg markedly bent, thus orientating the headpiece toward the plasma membrane (4). Extensive analyses of electron microscopic images of αβ3 suggested that its compact form could represent a resting state, and extension of the legs may be associated with activation (5). In association with activation, it was also suggested that the hybrid domain swings out with respect to the headpiece, a hypothesis later substantiated by x-ray crystallographic studies of a fragment of the αIIbβ3 integrin, consisting of the β-propeller from the α subunit and the PSI, β1, and hybrid domains from the β subunit (6). This interpretation was supported by further electron microscopic image analyses of the αIIbβ3, α5β1, αLβ2, αXβ2, and αIIbβ3 integrins (5, 7–10). However, it is not clear whether extension is absolutely necessary for ligand binding (11).

The structures of I-EGF1, I-EGF2, and I-EGF3 however, were missing in these studies. The structure of I-EGF3 of the β2 integrin subunit was determined by NMR (12), which led to the definition of a novel subset of EGF-like domains typically found in integrins, now referred to as integrin-EGF (I-EGF) domains. Using x-ray crystallography, we have determined the structure of I-EGF1 as part of a fragment of the β2 subunit, consisting of the PSI, hybrid, and I-EGF1 domains (13). This fragment is hereafter named PHE1 (Fig. 1B). Superposition of the PHE1 structure onto the bent αβ3 ectodomain crystallographic structure (4) suggested that the bend in the β subunit must lie between the I-EGF1 and I-EGF2 domains, a conclusion also supported by electron microscopic studies of the αLβ2 and αXβ2 integrins (10). However, a clear picture of the modules making up the “knee” (Fig. 1A) in the β subunit has not emerged yet, and the atomic basis for its flexibility is still largely unresolved. Thus, to further understand the molecular mechanism of integrin activation, it is crucial to obtain the structure of the I-EGF2 domain, and to determine how it is connected with the neighboring integrin modules, both in their bent and extended.
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states. Accordingly, we expressed the PHE2 and PHE3 fragments (Fig. 1B), both from the \( \beta^2 \) integrin subunit, and determined their structures by x-ray crystallography. The PHE2 integrin fragment assumes a bent conformation at the junction between the I-EGF1 and I-EGF2 domains, and PHE3 is extended. We propose that structural changes within the I-EGF2 domain may act as a conformational switch associated with integrin activation. To test this hypothesis, we engineered a disulfide bond in the full-length leukocyte function-associated antigen-1 (LFA-1) molecule and showed that it can be locked in a bent conformation.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The construction of PHE2 and PHE3 was similar to that reported for PHE1 (13), except that the I-EGF2 domain (residues Cys461–Glu513) is also included in PHE2, and both I-EGF2 and I-EGF3 domains (residues Cys461–Gln552) in PHE3. The final amplified cDNA constructs of PHE2 and PHE3 were subcloned into the pRES2-EGFP vector (BD Biosciences Clontech) and transfected using the Polyfect reagent (Qiagen) into the HEK293S GnTIII- cell line that is deficient in N-acetylgalactosaminyltransferase-I (GnTIII) (14). Stable cell lines incorporating the expression vector were selected by culturing transfectants in media (Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin) containing G418 (1 mg/ml, Invitrogen), followed by their isolation based on the EGFP expression, using the fluorescence-activated cell sorting Aria flow cytometry-based cell sorter (BD Biosciences). The clones that secreted the highest quantity of recombinant proteins, as determined by the intensity of EGFP signal and sorting Aria flow cytometry-based cell sorter (BD Biosciences). Purified PHE2 and PHE3 were concentrated to 1 ml and subjected to size-exclusion chromatography on a Superdex-75 16/180 column (Amersham Biosciences), mounted on an Akta fast-protein liquid chromatography system (Amersham Biosciences). PHE2 and PHE3 samples were concentrated to 1 ml and subjected to size-exclusion chromatography on a Superdex-75 16/180 column (Amersham Biosciences) in buffer A. Purified PHE2 and PHE3 were concentrated to 1 mg/ml and treated with endoglycosidase H\( _\text{p} \) (4000 units/ml, New England Biolabs) overnight at room temperature. After further purification by size-exclusion chromatography, the proteins were concentrated to 15 mg/ml in 20 mM Tris-HCl, pH 7.2. Molecular masses were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Reagents and Antibodies—The mAbs MHM24 (anti-\( \alpha^L \)) and MHM23 (specific for \( \beta^2 \) integrin heterodimers) were gifts from A. J. McMichael (John Radcliffe Hospital, Oxford, UK); the anti-\( \beta^2 \) mAbs, KIM185, KIM127, KIM89, and KIM202 were obtained from M. K. Robinson (Celltech, Slough, UK); MEM48 from V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic); 7E4 was obtained from the Beckman Coulter Company; and MEM148 was purchased form Serotec, UK. The anti-\( \alpha^X \) mAb KB43 was obtained from Dr. K. Pulford (LRF Diagnostic Unit, Oxford, UK). The anti-\( \alpha^L \) mAb clone 27 for Western blotting was obtained from BD Bioscience. Horseradish peroxidase-conjugated sheep anti-mouse IgG was purchased from Amersham Biosciences.

Crystallization and Data Collection—Crystallization was performed by vapor diffusion using the hanging drop method at 18 °C. PHE2 crystals grew over a week into thin plates of dimensions ~0.05 × 0.3 × 0.4 mm\(^3\) in a precipitating solution containing 0.2 mM magnesium acetate, 0.1 mM sodium acetate (pH 4.6), and 18% polyethylene glycol 3350. PHE3 crystals grow in 0.2 M ammonium sulfate, 15% polyethylene glycol 4000, and 5% isopropanol as hexagonal plates, with dimensions of ~0.1 × 0.4 × 0.5 mm\(^3\). Both crystals contain one monomer per asymmetric unit, with solvent contents of 34 and 48% and \( V_m \) of 1.86 and 2.35, respectively. Before cooling the crystals to 100 K in a nitrogen gas stream (Oxford Cryosystems), three rounds of 12-h increases in polyethylene glycol concentrations (7% each cycle) were carried out in the mother liquors (15), and finally the polyethylene glycol concentrations were 39 and 36% for PHE2 and PHE3, respectively. PHE2 native data were collected on an R-axis IV++ Image Plate detector using Cu\( _\text{K} \alpha \) radiation from a Micromax-007 rotating anode. PHE3 native diffraction data were recorded on an ADSC charge-coupled device detector (ADSC Corp., Powey, CA) on the ID23-1 beamline at the European Synchrotron Radiation Facility (Grenoble, France). A data set was collected at a wavelength of 1.907 Å, and anomalous difference Fourier synthesis was performed to confirm the location of the disulfide bonds. Data were processed using programs MOSFLM and SCALA (16). Crystal parameters and data collection statistics are summarized in Table 1.

Structure Determination and Refinement—The PHE2 and PHE3 structures were determined by molecular replacement using the program PHASER (17) with PHE1 (1YUK) and PHE2 lacking the I-EGF2 domain, as search models, respectively. Molecular replacement for the I-EGF3 domain was performed using its NMR structure 1L3Y (12) as a search probe. Models were improved by alternating cycles of model building with the program Coot (18) and refinement with REFMAC (16). The relatively high refinement \( R \) values for the PHE3 crystal form are probably caused by the large anisotropy observed in its unit-cell dimensions (\( a = b = 52.3 \text{ Å}, c = 423.9 \text{ Å}, \text{ see Table 1} \)). PHE3 crystals diffract anisotropically, and refinement programs can only partially compensate for such effects. Based on the \( R \) free values, the estimated overall coordinate errors for the PHE2 and PHE3 molecules are 0.16 and 0.26 Å, respectively. Overall, the chain traces are unambiguous with clear electron density, including, for a single \( N \)-acetylgalactosamine residue observed on all three potential \( N \)-glycosylation sites in the PHE2 protein, corresponding to residue Asn\(^{28} \) in the PSI, Asn\(^{94} \) in the hybrid, and Asn\(^{779} \) in the I-EGF2 domains. For the PHE3 protein, electron density for an \( N \)-acetylgalactosamine residue could be observed only on Asn\(^{94} \) in the hybrid domain. Four histidine residues from the hexahistidine tag were visible at the C-terminal end. Three segments are poorly defined in PHE3: residues His\(^{69} \)-Gly\(^{72} \) in the hybrid domain, residues Arg\(^{432} \)-Asp\(^{435} \) in
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the I-EGF1 domain, and residues Ser467 in the I-EGF2 domain. These regions, which are not included in the refined PHE3 structure, are exposed to the solvent and are likely to be flexible. Translation, libration, and screw-rotation displacement introduced in the last refinement step. Solvent-accessible surfaces were calculated using the program AREAIMOL (16) with a 1.7-Å radius sphere as the probe (Table 3) and values rounded to the nearest 5 Å². Conformational differences were analyzed using the DynDom server. Figures were created using PyMOL (19).

Mutagenesis and Transfection of 293T Cells—Plasmids encoding the full-length αL and β2 human integrin subunits in pcDNA3 were described previously (20). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene). All mutations were confirmed by DNA sequencing. 293T cells were transfected using Polyfect reagent (Qiagen), according to the manufacturer’s instructions. 2.0 μg each of the αL and β2 cDNA, wild type or mutant, were used to cotransfect one 6-cm dish of cells at 70–80% confluency.

Surface Labeling and Detection—Free cysteine labeling was performed as described previously (21). Briefly, transfected cells were washed once Tris-buffered saline (TBS) (20 mM Tris- HCl, 200 mM NaCl, pH 7.4) containing 1 mM Ca²⁺, and labeled with 400 μM of biotin-BMCC (1-biotinamido-4-(4'-[maleimidoethylcylohexane]carboxamido)butane, Pierce) for 30 min at room temperature. Cells were washed three times with TBS containing 1% Triton X-100 and 0.1% Nonidet P-40 at 4 °C. LFA-1 was immunoprecipitated with Protein A-Sepharose beads (Amerham) and biotin-labeled LFA-1 bands were detected using streptavidin-horseradish peroxidase, followed by detection with a horseradish peroxidase-conjugated sheep F(ab’)_2 anti-mouse antibody (Sigma) at 1:400 dilution for 45 min at 4 °C. Stained cells were washed once and fixed in 1% (v/v) formaldehyde in phosphate-buffered saline before analysis on a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest software.

Adhesion Assay—Adhesion of 293 transfecants to ICAM-1 was carried out as previously described (24).

RESULTS

Expression of the PHE2 and PHE3 Fragments—The PHE2 fragment comprises the PSI (residues Gln¹–Asp¹⁵⁸ and Glu¹²⁴–Arg⁴²⁶), hybrid (residues Pro⁵⁹–Ala¹⁰⁰ and Lys³⁴⁰–Cys⁴²³), I-EGF1 (residues Cys⁴²⁷–Glu⁶⁰⁶) and I-EGF2 domains (residues Cys⁴⁶¹–Glu⁵¹³). In addition to the domains that make up PHE2, the PHE3 fragment also includes the I-EGF3 domain at its C-terminal end (residues Cys⁵¹⁴–Gln⁵⁰⁵) (Fig. 1B). Typical preparations of PHE2 and PHE3 proteins before and after treatment with endoglycosidase H were shown in Fig. 1C. The recombinant PHE2 protein recovered from the cultured media was shown to express conformational epitopes of mAbs H52, 7E4, KIM202, and MEM148 (20, 23, 24) and KIM894 (Fig. 1D) indicating that it is in a native conformation. In addition, the PHE3 fragment, but not PHE2, is recognized by mAbs MEM48 and KIM127, in agreement with previous findings that the expression of the epitopes of these two antibodies requires both I-EGF2 and I-EGF3 domains (25, 26). MHM24, whose epitope is in the αL subunit, is included as a negative control.

Structural Determination of PHE2 and PHE3—The structures of PHE2 and PHE3 recombinant integrin β2 chain fragments were determined by x-ray crystallography. A summary of the data collection and refinement statistics for crystals of the PHE2 and PHE3 is shown in Table 1. Both structures were determined by molecular replacement using individual components from the PHE1 fragment and the I-EGF3 domain as search probes (12, 13). As independent confirmations of the correctness of the polypeptide chain traces, we used the anomalous signal from the sulfur atoms to confirm the positions of the 13 disulfide bridges present in the PHE2 structure, using CuKα radiation (at 1.5418 Å; see Table 1 and supplementary Fig. S1A). In addition, an anomalous Fourier map was computed from a low energy data set (collected at 1.907 Å, see Table 1) using the same PHE3 crystal (Table 1), confirming the location of the disulfide bridges present in the PHE3 fragment (Fig. S1B). The overall structures of PHE2 and PHE3 were drastically different: whereas PHE2 assumed a compact L-shaped configuration, PHE3 was extended (Fig. 2).

The Bent Structure of PHE2—Each of the three domains found in both PHE1 (13) and PHE2 (this study), i.e. the PSI, hybrid, and I-EGF1 domains, retained similar structures with
r.m.s. deviations in atomic positions < 0.9 Å (Table 2). Moreover, their relative orientation was essentially preserved between these two fragments. The largest movement observed among these three domains occurred in a long loop (Thr³⁰ to Arg³⁹) in the PSI domain, which moved toward the I-EGF1 domain by ~2.0 Å in PHE2, thereby bringing several of its residues into contact with Lys⁴⁵⁷ of I-EGF1 and Asp⁴⁸⁹ of I-EGF2 (Figs. 2A and 4A). I-EGF2 formed an acute angle with I-EGF1, giving PHE2 an L-shaped conformation (Fig. 2A). The associated interfaces, however, were modest with values between the I-EGF2 domain and the PSI and I-EGF1 domains of 405 Å² and 740 Å², respectively (Table 3).

The I-EGF2 Domain—The overall structure of I-EGF2 is similar to other I-EGF domains. The eight conserved cysteine residues are arranged in the C₁–C₅, C₂–C₄, C₃–C₆, and C₇–C₈ pattern (Fig. 3A) as shown previously in the I-EGF3 domain (Ref. 12; see also PHE3 structure) and in the I-EGF4 domain of the integrin β₃ subunit (4). A central core, formed by the four disulfide bridges and two β-strands, was superimposable with other I-EGF structures (see supplementary Fig. S3). Compared with other I-EGF domains, I-EGF2 has a long stretch of 13 residues (Gln⁴⁶² to Ser⁴⁷⁴) between its first two cysteines. This polypeptide segment folds into an α-helix spanning

FIGURE 1. A, schematic representation of the domain organization within an integrin αβ heterodimer. The α-leg contains three β-sandwich domains, referred to as Thigh, Calfl, and Calfl. The β-leg comprises an N-terminal plecin, semaphorin, and integrin (PSL) domain, a unique immunoglobulin-like hybrid domain, four I-EGF cysteine-rich repeats, and a β-tail domain (β-TD). B, illustrations of protein constructs used for this study. Both PHE2 and PHE3 are devoid of the inserted βl domain. The sequence numbering scheme is indicated. The same color codes for the domains are adopted throughout the figures depicting PHE2 and PHE3: green, PSI domain; yellow, hybrid domain; red, I-EGF1 domain; purple, I-EGF2; pink, I-EGF3. β-TD, β-tail domain; TM, transmembrane helix; Cyt, cytoplasmic domains are not colored. The PHE1 construct (13) is also included for comparison. The numbers in parentheses refer to the last (highest) resolution shell.}

TABLE 1

| Data collection and refinement statistics | PHE2 | PHE3|
|----------------------------------------|------|------|
| Space group                           | P2₁ | P6₆,2₂ |
| Cell parameters (a, b, c) (Å)          | 58.5, 30.8, 65.2 | 52.3, 52.3, 423.9 |
| (αβγ) (°)                             | 90, 94.3, 90 | 90, 90, 120 |
| Wavelength (Å)                        | 1.5418 | 0.976 |
| X-ray source                         | Rigaku Micromax-007 | ESRF ID23-1 |
| Resolution (Å)                       | 1.75 | 2.2 |
| Number of reflections (total/unique)  | 144,663/21,822 | 177,586/18,886 |
| Completeness (%)                      | 91.3 (55.5) | 99.5 (99.3) |
| Multiplicity                         | 6.6 (4.5) | 9.4 (8.7) |
| Rmerge (%)                           | 23.4 (2.0) | 22.2 (2.5) |
| Rmerge (%)                           | 6.5 (52.0) | 8.0 (60.7) |
| r.m.s. deviation bond lengths (Å)     | 0.007 | 0.009 |
| r.m.s. deviation bond angles (°)      | 1.20 | 1.22 |
| Rwork (%)                            | 20.4 (40.4) | 26.1 (30.2) |
| Rfree (%)                             | 25.4 (59.2) | 30.8 (40.5) |
| Ramachandran plot Residues in most favored and additional allowed regions (%) | 86.4/13.6 | 85.2/14.8 |

Average temperature factors (Å²)

| Main chain                           | 18.0 | 22.3 |
| Side chains                           | 19.5 | 23.4 |
| Water (total number)                  | 33.7 (268) | 76.1 (200) |

* The same crystal of PHE3 was used for both data sets, which were collected successively at high (left) and low (right) energy. The second PHE3 data set was collected to maximize the anomalous signal from the sulfur atoms. An anomalous difference Fourier map was calculated to confirm the trace of the polypeptide chain (see supplementary Fig. S1).

The PHE3 structure was refined using the high energy data set only.

* The numbers in parentheses refer to the last (highest) resolution shell.

* Rmerge = 1/NΣř|Fo| ÅFoc|, where Fh is the rih observation of the reflection h, whereas (Fo) is its mean intensity.

* N/A, not applicable.

* Rwork = Σ|Fo| ÅFoc| / Σ|Fo| ÅFobs|.

* Rfree was calculated with 5% of reflections excluded from the whole refinement procedure.

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Ser\textsuperscript{468} to Cys\textsuperscript{475} in the bent (PHE2) conformation, but becomes flexible in the elongated (PHE3) form (see below).

The Extended Conformation of PHE3—In contrast to PHE2, the PHE3 fragment adopted an extended conformation with overall dimensions of \( \sim 128 \times 30 \times 30 \) Å (Fig. 2B). Superposition of individual domains from PHE3 with the corresponding segments in the PHE1 and PHE2 showed higher r.m.s. deviations compared with those observed between PHE1 and PHE2 (Table 2). The PSI-hybrid tandem remains invariant in all structures reported for \( \beta_2 \) and \( \beta_3 \) fragments (6, 13, 27), including PHE2 and PHE3 reported here. However, the I-EGF domains in these two structures show remarkable differences in their relative orientation to the PSI-hybrid tandem. With reference to the invariant PSI-hybrid tandem, two maneuvers are required to bring the I-EGF1 and I-EGF2 domains from the PHE2 conformation to the PHE3 conformation: first a rotation of 40° about an axis passing through residues Lys\textsuperscript{427}–Ser\textsuperscript{431} and a 0.2-Å shift (a value that falls within the experimental errors), followed by a rotation of 69° of the I-EGF2 domain about an axis passing through residues Ser\textsuperscript{468}–Glu\textsuperscript{472} with a translation of 0.8 Å (supplementary Fig S2). As a result, the interface between I-EGF1 and I-EGF2 domains is reduced from 740 Å\textsuperscript{2} in PHE2 to 535 Å\textsuperscript{2} in PHE3, and the contact area of 405 Å\textsuperscript{2} between the PSI and I-EGF2 domains in PHE2 no longer exists in PHE3. Accordingly, the interactions between Lys\textsuperscript{457} of I-EGF1, Asp\textsuperscript{489} of I-EGF2, and the main-chain amide groups projecting from the loop of the PSI domain in PHE2 were disrupted in PHE3 (Fig. 4A). Perhaps the most interesting difference between the two structures lies in the I-EGF2 domain. The disulfide bond between Cys\textsuperscript{461} and Cys\textsuperscript{492} (C1 to C5 within the I-EGF2 domain) assumes different conformations in PHE2 and PHE3. The dihedral angle about this S–S disulfide bond (\( \xi \) torsion angle) switches from the right-handed (\( \xi \sim 100^\circ \)) in PHE2 to the left-handed (\( \xi \sim 80^\circ \)) stable conformer (Fig. 3B). Concomitantly, the short \( \alpha \)-helix (residues Ser\textsuperscript{468}–Cys\textsuperscript{475}) in PHE2 became disordered in the elongated integrin structure.

The Bent Conformation Observed in the PHE2 Crystal Structure Can Be Adopted by a Complete Integrin Heterodimer—The PHE2 and PHE3 molecules are only fragments of a complete integrin \( \beta_2 \) subunit. To rule out possible artifacts introduced by crystal packing forces and to assess whether the bent conformation displayed by the PHE2 molecule could also be adopted by a complete integrin receptor, we used site-directed
mutagenesis to introduce an artificial disulfide bond that would constrain the integrin in the bent conformation observed in PHE2. In the PHE2 crystal structure, the α-carbon atoms of residues Gly33 from the PSI and Gly486 and Leu487 from the I-EGF2 domains are separated by distances of 4.2 and 5.0 Å, respectively, which are compatible with the distance spanned by disulfide bonds (Fig. 4A). Two double substitutions were made in the full-length β2 integrin subunit, with cysteines replacing Gly33 and Gly486 in one (β2G33C/G486C) and Gly33 and Leu487 in the other (β2G33C/L487C). In addition, a single mutant having Gly33 substituted by a cysteine residue (β2G33C) was also constructed. The αL and β2 cDNA plasmids were transfected into 293T cells. All three β2 variants supported LFA-1 (leukocyte function-associated antigen-1, αLβ2 integrin) surface expression, as detected by flow cytometry using mAb MHM23, which is specific for

FIGURE 3. A, topology of the I-EGF2 domain. The four evolutionary conserved disulfide bridges are indicated. The C1–C5 disulfide bridge, which adopts different torsion angles in the bent and extended forms, is labeled with a star. The N-glycosylation site of Asn479 is indicated with a triangle. B, superposition of the I-EGF2 domain in the bent (cyan) and extended (dark blue) conformations, highlighting the variation in the torsion angle occurring within the C1–C5 disulfide bond that links Cys461 and Cys492. The segment corresponding to the α-helix becomes very mobile in the extended conformation. The N-linked glycan at position Asn479 is also shown.

FIGURE 4. Introduction of a disulfide bond that covalently links the PSI and I-EGF2 domains in the full-length LFA-1, in a cell-based system. A, the locations of residues Gly33, Gly486, and Leu487 in the PHE2 structure are shown. Gly33 and Gly486 (marked with a green sphere and a purple sphere, respectively), when substituted by cysteines, form a disulfide bond. The KIM127 epitope residues Gly504, Leu506, and Tyr508 are shown to lie on the other side of the central β-sheet, distal to the engineered Cys33–Cys486 disulfide bond. Interactions between the PSI, I-EGF1, and I-EGF2 domains through residues Gly31, Gly33, and Asp34 in the PSI domain, Lys457 in the I-EGF1 domain, and Asp489 in the I-EGF2 domains are also shown. B, cell-surface expression of LFA-1 and variants on 293T transfectants were detected by flow cytometry using MHM23 (αL integrin heterodimer-specific mAb). The shaded histogram represents staining with MHM23, and the unshaded histogram represents staining by the irrelevant mAb KB43 (integrin αX-specific mAb). C, formation of the disulfide bond in LFA-1 variants was monitored using the free sulfhydryl-labeling reagent BMCC conjugated to biotin. LFA-1 was immunoprecipitated with mAb MHM23, resolved on a non-reducing 7.5% SDS-PAGE gel, followed by detection of biotin-BMCC-labeled LFA-1 (upper panel). The reducing agent DTT-treated wild-type LFA-1 was included as control. The amount of LFA-1 and variants immunoprecipitated with MHM23 was comparable, as indicated on the Western blot with the αL-specific antibody clone 27 (lower panel). D, reactivity of KIM127 with LFA-1 and variants. Biotinylated surface-expressed LFA-1 and variants on 293T transfectants were immunoprecipitated with MEM48 and KIM185 in the presence of Mg2+/EGTA. Same results are obtained in the absence of Mg2+/EGTA (data not shown). The subunits of LFA-1 were resolved on a 7.5% SDS-PAGE gel followed by detection. E, immunoprecipitation of LFA-1 in Mg2+/EGTA and at different concentrations of DTT by KIM127. Wild-type immunoprecipitation is not affected up to 30 mM DTT. No precipitation was observed for the variant. The introduced disulfide is not reduced up to 30 mM DTT.
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The transfectants were surface-labeled with biotin-BMCC, a reagent containing maleimide, which binds to free sulfhydryl groups. LFA-1 was immunoprecipitated from solubilized membranes of the transfectants with MHM23. After SDS-PAGE and blotting, the proteins were analyzed for biotin labeling. Minimal signal was detected from the bands of the wild-type αLβ2. In contrast, the β2G33C and β2G33C/L487C bands were labeled, indicating the presence of free sulfhydryl in these variant β2 subunits. The intensity of the β2G33C/G486C band was significantly lower than those of the other two mutants, suggesting that, for the majority of the molecules, a disulfide bond was formed between the two introduced cysteine residues. The same samples were also analyzed for the presence of the αL subunit by Western blot using the anti-αL mAb clone 27. The results showed that the αL subunits are present in equal amounts in the four transfectants, suggesting that the different levels of free cysteines in the four samples were not due to differences in immunoprecipitation.

Expression of the KIM127 epitope in LFA-1 is associated with an active extended conformation (10, 25). To test the reactivity of KIM127 with the LFA-1 variants, transfectants were surface-labeled with biotin (22), and the labeled cells were incubated at 37 °C with KIM127 and Mg2+/EGTA. It should be noted that wild-type LFA-1 under physiological divalent cation concentration in culture medium is not capable of full extension in the presence of activating Mg2+/EGTA. In contrast, the KIM127 reactivity with the LFA-1 variants, transfectants expressing wild-type LFA-1 or the αLβ2G33C/G486C variants were assayed for the ICAM-1-coated surface in the presence or absence of Mg2+/EGTA. Forward bars represent results obtained in the presence of the LFA-1-specific blocking mAb MHM24.

DISCUSSION

A number of reports have described conformational changes accounting for the allosteric mechanism of integrin activation (for reviews, see Refs. 29–31). The αVβ3 integrin crystal structure revealed an overall V-shaped compact molecule, but only the bend in the αV subunit could be resolved (4). The junction between I-EGF1 and I-EGF2 of the β3 subunit was not visible in the electron density map, but the location of the bend could be deduced from the positions of other domains, namely the PSI and the hybrid domain on one side, and the I-EGF3 domain on the other (4, 13). No atomic structure is available for either the α or the β subunit in their extended conformation. In this study, we determined the structures of the PHE2 and PHE3 fragments of the integrin β2 subunit. An acute bend is seen in the PHE2 structure between the I-EGF1 and I-EGF2 domain, whereas PHE3 assumes an extended structure. How do these structures relate to the bent and extended conformations of a complete integrin LFA-1 molecule?

The extended structure of PHE3 may faithfully reflect the extended conformer of LFA-1 because of its reactivity with a panel of conformation-sensitive mAbs, in particular, with KIM127, which only detects an extended LFA-1 conformer (10, 25). By contrast, it is not immediately apparent from the lack of KIM127 reactivity with PHE2 that this structure depicts a bent conformer of LFA-1, because the expression of the KIM127 epitope requires, in addition to I-EGF2, I-EGF3, which is absent in PHE2 (26). To verify that the L-shaped conformation in the PHE2 structure also occurs in an intact integrin, we introduced two cysteine residues, one to replace Gly33 in the PSI domain and the other to replace Gly486 in the I-EGF2 domain of the β2 subunit. The distance between the Cα atoms of these two residues was 16 Å in the extended (PHE3) form but only 4.2 Å in the bent (PHE2) structure. Thus, we expect that this disulfide bond would only be formed if these two cysteine residues are brought...
in close proximity, as seen in the compact PHE2 structure. Formation of this engineered disulfide bond in this LFA-1 variant is supported by data shown in Fig. 4. In addition, the epitope of KIM127 was not expressed in the presence of Mg²⁺/EGTA, which is also expected if the LFA-1 variant is locked by the engineered disulfide into a bent conformation. Thus, these data provide direct evidence for the presence of a bent conformation adopted by LFA-1 integrins at the cell surface that mirrors the PHE2 structure. On the other hand, in the absence of a disulfide bond such as in the LFA-1 variants having αLβ2G33C and αLβ2G33C/L487C substitutions, the extended conformations can be detected by the KIM127 monoclonal antibody.

The MEM48 epitope, which also requires both I-EGF2 and I-EGF3 for expression in wild-type LFA-1 and is not sensitive to Mg²⁺/EGTA, is not expressed in the αLβ2G33C/G486C variant. This is not unexpected, because the introduced disulfide bond would lock I-EGF2 into a conformation that does not allow the I-EGF2 and I-EGF3 to bind MEM48 by an induced-fit mechanism. On the other hand, the expression of the epitope of KIM185, which maps to the I-EGF4/β-TD domains (25), is not affected.

Our attempt to reduce the introduced disulfide bond on the αLβ2G33C/G486C variant was not successful. The expression of the KIM127 epitope was not affected in 30 mM DTT in the wild-type LFA-1. Under these conditions, re-expression of the KIM127 epitope was not detected on the αLβ2G33C/G486C variant, suggesting that the introduced disulfide between the PSI and I-EGF2 domains is not accessible for reduction. At 100 mM DTT, expression of the epitope was abolished in the wild-type LFA-1, presumably by the reduction of other disulfide bonds in the β2 subunit.

The αLβ2G33C/G486C variant can mediate adhesion to ICAM-1, suggesting that the ligand binding site is intact. Furthermore, it also suggests that leg extension is not an absolute requirement for ligand binding, at least under this particular assay system.

In the PHE2 and PHE3 structures, the I-EGF2 domain assumes two different conformations involving alternative dihedral torsion angles of a disulfide bond between Cys⁴⁶¹ and Cys⁴⁹² and the presence or absence of a short α-helix (Fig. 3B). This difference may represent the conformational switch within the leg of the integrin β2 subunit associated with a large reorientation of the I-EGF1 and I-EGF2 domains. An activation energy barrier of ~7 kcal/mol has to be overcome to switch the disulfide bond from one favorable conformer to the other (32). However, this kinetic energy barrier would not contribute to affinity regulation when the system is at thermodynamic equilibrium. Whether an exogenous disulfide isomerase is involved in the transition remains to be clarified (33). It is also possible that the integrin may have intrinsic disulfide isomerase activity (34).

The two different structures of PHE2 and PHE3 reported here clearly bear relevance to the transition between the bent and the extended conformation of the LFA-1 integrin molecules. Conformational changes within the I-EGF2 domain may be a key feature for this transition. How this conformational change is coupled with others described, including leg separation (35), removal of a dead-bolt (36), swing-out of the hybrid domain (6), and conformational changes in the I-domains of the α and β subunits (37, 38, and for reviews see refs. 29–31) will require further examination. Further work is also necessary to assess whether comparable transitions occur in the β1 and β3 subunits, whose family members undergo similar conformational changes during activation.

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