A Minimized Human Integrin \(\alpha_5\beta_1\) That Retains Ligand Recognition

(Received for publication, July 9, 1999, and in revised form, October 27, 1999)

Jean-Louis Bane`res‡, Françoise Roquet§, Aimée Martin, and Joseph Parello
From the Chimie Biomoléculaire et Interactions Biologiques, UPRESA CNRS 5074, Faculté de Pharmacie, 15 Av. Ch. Flahaut, 34060 Montpellier Cédex 2, France

Two isolated recombinant fragments from human integrin \(\alpha_5\beta_1\), encompassing the FG-GAP repeats III to VII of \(\alpha_5\) and the insertion-type domain from \(\beta_1\), respectively, are structurally well defined in solution, based on CD evidence. Divalent cation binding induces a conformational adaptation that is achieved by \(\text{Ca}^{2+}\) or \(\text{Mg}^{2+}\) (or \(\text{Mn}^{2+}\)) with \(\alpha_5\) and only by \(\text{Mg}^{2+}\) (or \(\text{Mn}^{2+}\)) with \(\beta_1\). \(\text{Mn}^{2+}\) bound to \(\beta_1\) is highly hydrated (\(\approx 3\) water molecules), based on water NMR relaxation, in agreement with a metal ion-dependent adhesion site-type metal coordination. Each fragment saturated with \(\text{Mg}^{2+}\) (or \(\text{Mn}^{2+}\)) binds a recombinant fibronectin ligand in an RGD-dependent manner. A conformational rearrangement is induced on the fibronectin ligand upon binding to the \(\alpha_5\), but not to the \(\beta_1\) fragment, based on CD. Ligand binding results in metal ion displacement from \(\beta_1\). Both \(\alpha_5\) and \(\beta_1\) fragments form a stable heterodimer (\(\alpha_5\beta_1\) mini-integrin) that retains ligand recognition to form a 1:1:1 ternary complex, in the presence of \(\text{Mg}^{2+}\), and induces a specific conformational adaptation of the fibronectin ligand. A two-site model for RGD binding to both \(\alpha\) and \(\beta\) integrin components is inferred from our data using low molecular weight RGD mimetics.

Integrins (IN) are a family of structurally and functionally related adhesion receptors that participate in cell-cell and extracellular matrix (EM) interactions (1). All integrins are heterodimers of non-covalently associated \(\alpha\) and \(\beta\) subunits. At the functional level, the interactions between integrins and their EM protein ligands involve the following: (i) the extracellular integrin heteromeric “head” that encompasses the N-terminal halves from both \(\alpha\) and \(\beta\) subunits and hosts the ligand-recognition pocket with a variety of binding sites on each subunit (2); (ii) short specific amino acid sequences (adhesion motifs) from the EM ligands. The prototype for these adhesion motifs is the Arg-Gly-Asp (RGD) sequence that is present in fibronectin, fibrinogen, vitronectin, and other EM-adhesive proteins (3). The exact location of these binding loci in the integrin subunits, as well as their respective role on ligand binding energy and specificity, still remains an open question. The N-terminal half of the integrin \(\alpha\) subunits is characterized by the presence of seven N-terminal repeats of about 60 amino acids each (4, 5). Some of the \(\alpha\) subunits include an insertion domain, or I-domain, about 200 residues in length, between repeats II and III (2). The homologies between repeats I and VII essentially include the FG and GAP consensus sequences, so that these repeats are also referred as FG-GAP repeats (6). Three to four of these repeats (i.e. repeat IV or V to repeat VII) display sequences that resemble the EF-hand consensus sequence found in various divalent cation-binding proteins (7). However, the integrin EF-hand type sequences are systematically devoid of an acidic residue at their relative position 12, a highly invariant Glu residue in the typical EF-hands, that is replaced by a non-polar residue in integrins (8). Isolated recombinant integrin fragments encompassing repeats III to VII of \(\alpha_{1b}\) (9) and IV to VII of \(\alpha_6\) (10) have been shown to mimic the ligand-binding features (divalent cation and RGD dependence) that are observed with native integrin receptors, indicating that the divalent cation-binding domains in the \(\alpha\) subunits are part of the ligand-recognition pocket of integrins. Repeats III and IV in the \(\alpha\) subunits have also been shown to be involved in cell spreading and in assembly of focal contacts at the cell surface (11).

The N-terminal regions of the integrin \(\beta\) subunits are characterized by a conserved domain that displays sequence homology with the I-domain found in several integrin \(\alpha\) subunits (12). This I-type domain in the integrin \(\beta\) subunits includes the following: (i) a functional cation-binding site that displays strong similarities at the level of its metal-coordinating residues with the MIDAS site of the \(\alpha\) subunit I-domains (13, 14); (ii) a totally conserved DDL motif, close to the MIDAS-type site that is apparently involved in the recognition of RGD-containing ligands (15); (iii) possibly a specific sequence responsible for heterodimer formation; in the case of the human \(\beta_3\) I-type domain this specific sequence corresponds to the segment \(\beta_3-(275–280)\) with the unique hexapeptide sequence, VGSNDH, that is responsible for species-restricted \(\alpha\) heterodimer assembly (16). As tentatively proposed by McKay et al. (16), the sequences in the \(\beta\) subunits that are topologically related to the

* This work was supported by CNRS Programme Physique et Chimie du Vivant Grant 98N21/0108, Ligue Nationale et Ligue Régionales (Hérault, Drôme) de Lutte Contre le Cancer, Association de la Recherche sur le Cancer, Fondation pour la Recherche Médicale (Ligue Languedoc-Roussillon-Rouergue), and National Institutes of Health Grant 5PO1CA28896–15 (component III to J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: UPRESA CNRS 5074, Faculté de Pharmacie, 15 Av. Ch. Flahaut, 34060 Montpellier Cédex 2, France. Tel: 33 4 67 52 23 01; Fax: 33 4 67 54 71 79; E-mail: baneres@pharma.univ-montp1.fr.

‡ Present address: CNRS UMR 9921, Faculté de Pharmacie, 15 Av. Ch. Flahaut, 34060 Montpellier Cédex 2, France.

§ The abbreviations used are: IN, integrin; EM, extracellular matrix; 3Fn7–10, recombinant fibronectin fragments from human origin containing 3Fn7 to 3Fn10; \(\beta_1-(121–329)\), recombinant fragment encompassing residues 121–329 of the human \(\beta_1\) integrin subunit preceded by the N-terminal dipeptide AM; FN, fibronectin; GdnHCl, guanidine hydrochloride; His-tag, \(\alpha_5\)-(i-j); recombinant fragments encompassing residues i to j of the human \(\alpha_5\) integrin subunit fused with an N-terminal polyhistidine tag; IPTG, isopropyl-\(\beta\)-D-thiogalactosidase; I-domain, metal ion-dependent adhesion site; MIDAS, metal ion-dependent adhesion site; Ni-NTA, nickel-loaded nitritrolactetic acid-agarose; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TntTag-\(\beta_1-(121–329)\), recombinant fragments encompassing residues 121–329 of the human \(\beta_1\) integrin subunit fused with thioredoxin and a hexa-histidine tag at its N terminus; PRE, protein relaxation enhancement.
the β_{1p}(275–280) sequence are likely to participate in heterodimer assembly for the different integrins.

Divalent cations play a central role in ligand recognition by the integrins (17). Both α and β subunits are responsible for divalent-cation binding (2). In the α subunit, the divalent cation-binding sites are located at the level of the EF-hand type sequences. By using isolated recombinant EF-hand domains from α_{Ib} (9) and α_{3} (10), a stoichiometric ratio of four cations bound per protein has been inferred, in agreement with the occurrence of four EF-hand-type sequences in both integrin α subunits (2). These four divalent cation-binding sites are distributed in two classes of sites (each class with two sites), differing by their affinities with the low affinity sites regulating ligand recognition (9, 10). Divalent cation binding to the integrin β subunits is also intimately linked to integrin function. Apparently, a single cation-binding site occurs in the β subunits at the level of their MIDAS-type sequences (13, 14). Based on model studies, D’Souza et al. (18) proposed that divalent cation binding to the β_{3} subunit promotes a ligand-competent conformation and that the initially protein-bound cation is released after formation of the ligand-receptor complex. This mechanism has not yet been fully assessed with a native receptor or with an isolated β I-type domain. Evaluation by equilibrium dialysis of the number of divalent cation-binding sites in an intact integrin receptor, α_{Ib}β_{3}, amounts to a total of 4–5 (19), in agreement with the aforementioned distribution of 4 sites in the α subunits and 1 site in the β subunits.

So far the respective role of these divergent cation-binding sites, and their possible synergy, on ligand recognition by the integrins have not been fully elucidated. McKay et al. (16) have reported a recombinant αβ heterodimer formed of both segments α_{Ib} (1–233) and β_{3} (111–318) that binds Arg-Gly-Asp-Trp (RGDW), as a peptide ligand, in the presence of Ca^{2+}. Interestingly, this minimized α_{Ib}β_{3} integrin does not contain any of the α_{Ib} EF-hand cation-binding sites; its α component is represented exclusively by repeats I to III. It is likely that this mini-integrin only contains part of the ligand recognition pocket in the absence of the ligand-competent EF-hand domain from its α_{Ib} subunit. A model has been recently proposed (10) in which the RGD tripeptide sequence from the protein ligand acts as a dual motif with two oppositely charged groups, a negative one (the carboxylate from Asp) and a positive one (the guanidinium from Arg), that interact with distinct structural elements on each of both α and β subunits. Such a model would account for the binding of the RGDW tetrapeptide by the mini-integrin α_{Ib}β_{3}, as reported by McKay et al. (16). One possibility for retaining ligand-binding capacity in an RGD-dependent manner could be related to the fact that this α_{Ib}β_{3} mini-integrin includes the RGD-binding sequence DLG conserved in all integrin β subunit I-type domains (15). However, the three N-terminal FG-GAP repeats I to III, that are present in the α component of the α_{Ib}β_{3} mini-integrin could also contain ligand-binding sites unidentified so far. As emphasized by Weber (20), protein interactions need to viewed as the convergence of cooperative effects of many inputs rather than a sum of fixed independent influences. In this respect, the RGD adhesive sequence displays two complex charged groups, a guanidinium and a carboxylate (which will give rise to electrostatic as well as hydrogen bonding) and several hydrogen bond acceptors and donors from the tripeptide main chain that will elicit a variety of contacts with elements from the integrin receptor. These elements can be rather interspersed in the amino acid sequences from both α and β subunits. Since McKay et al. (16) expressed their mini-integrin directly as a heterodimeric assembly, a strict delineation of the ligand-binding properties of each subunit fragment, taken separately, as well as of their respective cation requirements, could not be achieved.

To carry out such an analysis, we cloned and expressed in Escherichia coli two recombinant proteins from the α and β subunits of human integrin α_{Ib}β_{3}, i.e. α_{5}(160–448) and β_{3}- (121–329). Both fragments form a stable and soluble αβ mini-heterodimer that is ligand-competent in an RGD- and cation-dependent manner. Based on previous results with a minimal EF-hand domain of α_{5} (10), we expressed here a novel α_{5} fragment as a more integrated structural unit with regard to its capacity of combining ligand and cation binding. In doing so, we were guided by previous results indicating that the FG-GAP repeat III in α_{5} converges toward the ligand-binding pocket of α_{Ib}β_{3} (21). Moreover, the β subunit I-domain is likely to lie close to repeat III of the α subunits in native integrin receptors, indicating that repeat III participates to heterodimer stability (22). Besides the whole EF-hand domain (repeats IV through VII), for which cation-dependent ligand binding has been previously demonstrated (10), the α_{5} component of our minimized human integrin α_{Ib}β_{3} also includes the totality of repeat III, whereas the β_{3} component of this mini-integrin corresponds to the totality of the I-type domain. We establish here through a detailed study of the binding to α_{5}(160–448) and β_{3}- (121–329) of (i) divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}), (ii) RGD-containing ligands (fibronectin fragments and synthetic peptides), and (iii) simple compounds mimicking the RGD motif (n-butylate and guanidinium chloride) that these isolated integrin fragments taken separately display cation-dependent ligand-binding properties that are closely related to those observed with the α_{5}(160–448)β_{3} (121–329) mini-integrin assembly itself.

**EXPERIMENTAL PROCEDURES**

**Materials and Buffers**—Buffer A contained 12.5 mM sodium borate, 50 mM KCl at pH 8.3. Buffer B contained 20 mM potassium cacodylate, 50 mM KCl at pH 6.0. Buffer C contained 20 mM HEPES, 50 mM KCl, 1 mM MgCl₂ at pH 6.0. The RGD-containing fibronectin ligands of human origin include the following recombinant proteins: 3Fn7–10 (residues 1150–1508; Ref. 23) and 3Fn10–11 (residues 1423–1691; Ref. 24). The α_{5}β_{3} integrin fragments from human origin used in this work are described below.

**Cloning, Expression, and Purification of α_{5}(160–448)**—The DNA sequence coding for residues 160–448 of human α_{5} was produced by PCR using the full-length α_{5} cDNA (25) as a template. The following oligonucleotides were used as primers: 5′-GCC CGT CAG ATG GGC CAC CAT CTG TCT GCC-3′ (22). Besides the whole EF-hand domain (repeats IV through VII), three mini-integrin fragments were designed to generate an in-frame stop codon, as well as a BamHI end. Amplification was performed in a Perkin-Elmer thermal cycler using Taq polymerase (Perkin-Elmer) and proceeded through a cycle of denaturation at 94 °C (1 min), annealing at 55 °C (1 min), and extension at 72 °C (2 min), for a total of 30 cycles. The PCR fragment was purified using the Qiapquick gel extraction kit (Qiagen, Chatsworth, CA), digested with NdeI and BamHI, and inserted into the pET15b vector (Novagen, Madison, WI). This vector allows expression of the corresponding sequence fused with a HisTag N-terminal segment that includes the hexahistidinyl segment, as well as the thrombin cleavage site. The clone obtained was confirmed by dideoxy sequencing (26). *E. coli* BLR(DE3) host strain (Novagen) was transformed with the pET15b-α_{5}(160–448) vector. The corresponding recombinant protein was purified from the insoluble fraction obtained after bacterial lysis, as described previously for α_{5}(229–448) (10). The His-tagged protein was digested with thrombin under conditions similar to those described for α_{5}(229–448) (10) to remove the oligohistidine N-terminal appendix. The protein obtained includes the extraneous N-terminal tetrapeptide GSHH resulting from thrombin cleavage.

**Cloning, Expression, and Purification of β_{3}-(121–229)**—The DNA sequence coding for residues 121–329 of human β_{3} (25) was produced by PCR using a human cDNA library (CLONTECH, Palo Alto, CA) as a template. The following oligonucleotides were used as primers: 5′-GCA CGT CGA TGG TAT CCC ATT GAC CTC TAC CTT-3′ and 5′-CGG GAG TCC CTA GGA AAG GGA ATT GTA TGC ATT AAT-3′. These primers were designed to generate an NdeI restriction site at the 5′ end and an in-frame stop codon, as well as a BamHI restriction site, at the 3′ end.
were pelleted by centrifugation, resuspended in 25 mM Tris-HCl, 500 mM imidazole, loaded forms using trichloroacetic acid 3% (v/v) as a precipitating agent, protein was eluted at a substrate ratio of 10 units of enterokinase (Sigma) per mg of cleavable protein. Molar absorptivities were determined by the procedure of Gill and von Hippel (29). Molar absorptivities at 276 nm (in contrast with the CD effects reported under hydration of the paramagnetic cation in the protein-bound state (derived from the previous study with parvalbumin, the paramagnetic cation to the macromolecule, the number of water molecules surrounding the paramagnetic cation (38). Upon binding of the paramagnetic cation with the integrin apoforms with regard to their UV molar absorptivities at 276 nm (spectra in Fig. 1, A and C, only display data down to 195 nm). Although the signal-to-noise ratio in the 190–195 nm region is less favorable than at higher wavelengths, a complete set of the far-UV data down to 190 nm was systematically included in the deconvolution procedure. It is known that the shape of the CD spectra of proteins in the low wavelength range is important for correctly predicting the content of α-helical residues (31). Besides the strong double minimum at 222 and 208–210 nm, the α-helix displays a stronger maximum at 191–193 nm (31).

Computer-assisted predictions based on the amino acid sequences were performed using the following numerical methods: Geno2 (33), Gro4 (34), SOPMA (35), Hierarchical Neural Network, and Double Prediction Method (36) methods.

**PRE Measurements**—Water proton T1 relaxation times were measured at 100 MHz (Bruker spectrometer AC100) and 23 °C by the standard inversion-recovery (180°–90°) method with β1 (121–329) solutions in buffer B at constant protein concentration (0.1 mM) and at Mn5+ concentrations ranging from 1 to 100 mM. To avoid isotope effects on the T1 measurements, no D2O was added to the NMR samples so that the T1 measurements were carried out in the absence of frequency field lock. The reproducibility of our measurements was checked by comparing the curves R1 (see definition below) versus [Mn5+] determined at different times with standard Mn2+ solutions. The relative variations in slope of these straight lines did not exceed a few percent. No attempt was made to deoxidize the solutions (according to a previous study with parvalbumin, the paramagnetic effects of dissolved oxygen are small, although not totally negligible (37)). Relaxation rates, R1 (in s⁻¹), are defined as the inverse of the longitudinal relaxation times T1. The dimensionless enhancement factor ε corresponds to the ratio (R1(ε) – R1)/R1, where the exponents ε and R denote the presence of protein and the absence of metal cation, respectively (38). For the aquation itself, ε is identical to 1. The characteristic enhancement factor of the metal-macromolecule complex is defined by εf = lim ε at [P] = x and directly depends on the hydration of the paramagnetic cation in the protein-bound state (defined by q7, i.e. the number of water molecules surrounding the protein-bound cation) as well as on the dynamics and kinetics of the water molecules bound to the paramagnetic cation of the macromolecule, the number of water molecules surrounding the cation decreases, i.e. q7 < q (for aqueous Mn2+ q = 6 (39)). If this were the only parameter to change, then there would be a decrease in the relaxation rate of the water protons in the presence of protein, resulting in εf < 1. However, due to the increase in the dipolar correlation times t of the water protons upon binding to the
macromolecule and/or a decrease in the chemical exchange lifetimes \( \tau_{c}^{*} \) of these protein-bound water molecules, it is usually found that \( \tau_{c}^{*} > 1 \) (enhancement). Under fast exchange conditions on the NMR time scale, i.e., \( \tau_{c}^{*} << T_{2}^{*} \), \( \tau_{c}^{*} \) is independent on \( \tau_{a}^{*} \). The intrinsic exchange

factor \( \epsilon_{c} \), then corresponds to the ratio \( \frac{\langle q_{c}^{2} \rangle}{\langle q_{c}^{2} \rangle_{e}} \). Assuming that the metal–cation to water–proton distance remains unchanged from that in aqueous solution (38). Since \( \frac{\langle q_{c}^{2} \rangle}{\langle q_{c}^{2} \rangle_{e}} > \langle q_{c}^{2} \rangle_{e} \) with macromolecules, then \( \epsilon_{c} > 1 \).

**Protein Interaction Assays**—A protein affinity chromatography assay was used to evaluate the binding of the integrin recombinant fragments to the fibronectin ligand. The assay includes two steps: a complex formation step and an immobilization step on a Ni-NTA-agarose support (see Fig. 5A). In the first step, HisTag-\( \alpha_{c} \)-1-(121–329), TrxTag-\( \beta_{1} \)-121–329, or the HisTag-\( \alpha_{c} \)-1-(160–448)\( \beta_{1} \)-121–329 binary complex was mixed with the different recombinant fibronectin fragments (3Fn7–10 or 3Fn10–11) at a 1:1 molar ratio (for buffer conditions, see legends to Figs. 5, 6, and 8) with protein concentrations in the 10 \( \mu \text{m} \) range in a final volume of 2 ml. In the second step, the protein mixture was deposited on a column of Ni-NTA (usually 1 ml of wet support corresponding to 10–20 mg of immobilized Ni-NTA) washing of the column after the immobilization step was carried out with three column volumes of incubation buffer to remove all unbound material. In all cases, a control assay was carried out in parallel with a standard protein (thioredoxin fused with a C-terminal oligohistidine sequence) with no capacity of IN or Fn binding (complete elution of unbound IN or Fn proteins occurred during the first two eluting volumes). The proteins that remained bound to the agarose support were identified by SDS-PAGE analysis on a 10–20% polyacrylamide gel. This immobilization assay was also used to analyze the interactions between the \( \alpha_{c} \) and \( \beta_{1} \) recombinant fragments. In this case, the immobilization assay was carried out, as described above, using an equimolar mixture of HisTag-\( \alpha_{c} \)-1-(160–448) and \( \beta_{1} \)-121–329 (for buffer conditions, see legend to Fig. 8).

**Chemical Cross-linking**—Before complex formation, the IN and Fn molecules were cross-linked with the AD494(DE3) host strain (43), production of soluble proteins containing disulfide bonds is facilitated in the E. coli cytoplasm. We used this expression system since Cys-241 and Cys-281 in \( \beta_{1} \) are likely linked by an intramolecular disulfide bond, based on previous results obtained with the \( \beta \) subunit of \( \alpha_{c} \beta_{1} \) (44). To improve the production of properly folded \( \beta_{1} \)-121–329, we also used a co-expression system in which the target protein is expressed along with chaperones GroES and GroEL. The role of the GroESL complex in catalyzing the correct folding of heterologous proteins synthesized in E. coli is well documented (45). Under the conditions used, about 20% of the total protein was found in the soluble fraction of the bacterial lysate. Soluble \( \beta_{1} \)-121–329 was subsequently purified and the Trx tag removed through enterokinase cleavage. Under these conditions, about 5 mg of purified protein were obtained from 1 liter of bacterial culture. A mass of 23,544.0 ± 0.5 daltons was determined by electrospray ionization-mass spectrometry, but its molar ellipticity value as in the far-UV (wavelength 208 nm) of this protein in the buffer containing only the IN and Fn molecules was 23,544.6. Partial N-terminal amino acid sequencing, including the 10 first residues, gave the expected sequence, GSHMGGQILSAR, with the four N-terminal residues representing the appendix left after thrombin cleavage of HisTag-\( \alpha_{c} \)-1-(160–448).

To maximize the production of soluble protein, the Cys-containing \( \beta_{1} \)-121–329 protein was expressed as a fusion protein with thioredoxin (42). Many target proteins that are normally produced in an insoluble form in E. coli tend to become more soluble when fused with this protein. Moreover, by combining expression of the target protein fused with thioredoxin with the AD494(DE3) host strain (43), production of soluble proteins containing disulfide bonds is facilitated in the E. coli cytoplasm. We thus used this expression system since Cys-241 and Cys-281 in \( \beta_{1} \) are likely linked by an intramolecular disulfide bond, based on previous results obtained with the \( \beta \) subunit of \( \alpha_{c} \beta_{1} \) (44). To improve the production of properly folded \( \beta_{1} \)-121–329, we also used a co-expression system in which the target protein is expressed along with chaperones GroES and GroEL. The role of the GroESL complex in catalyzing the correct folding of heterologous proteins synthesized in E. coli is well documented (45). Under the conditions used, about 20% of the total protein was found in the soluble fraction of the bacterial lysate. Soluble \( \beta_{1} \)-121–329 was subsequently purified and the Trx tag removed through enterokinase cleavage. Under these conditions, about 5 mg of purified protein were obtained from 1 liter of bacterial culture. A mass of 23,544.0 ± 0.5 daltons was determined by electrospray ionization-mass spectrometry, thus correctly matching the calculated value of 23,544.6. Partial N-terminal amino acid sequencing (10 first residues) gave the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues, giving the expected sequence, AMYPIDLYYL, with the 10 first residues.
FIG. 1. Conformational dependence of α5(160–448) (A and B) and β3(121–329) (C and D) on Mg2+ binding. A and C, far-UV region; B and D, near-UV region. Profiles 1 and 2, in all cases, correspond to the cation-depleted and to the Mg2+-saturated forms, respectively. Profile 3 in C and D correspond to Mg2+-saturated β3(121–329) in the presence of 1 mM 2-mercaptoethanol. The CD measurements were carried out with the recombinant proteins at concentration in the 10 µM range in borate buffer A, in the absence (profile 1) or in the presence (profile 2) of 2 mM MgCl2.

**TABLE I**

Secondary structure content, in percentage and in number of residues (res), of the isolated recombinant fragments α5(160–448) and β3(121–329) from the α and β subunits of the human integrin α5β3, as determined from the far UV regions of their CD spectra (Fig. 1, A and C, respectively)

| Protein | Residues (total) | α-Helix | β-Strand | α-Helix residues | β-Strand residues |
|---------|------------------|---------|----------|------------------|-------------------|
| α5(160–448) | 293a | ~30 | ~25 | ~88 | ~73 |
| β3(121–329) | 211b | ~35 | ~30 | ~74 | ~63 |

a GSHM.

b Recombinant protein contains N-terminal appendix AM.

As shown in Fig. 2, titration of the α5(160–448) aapoform was carried out using three different divalent cations, Ca2+, Mg2+, and Mn2+. All titration profiles give the variation of the intensity of the negative Phe band at 268 nm as a function of cation concentration. The CD data of Fig. 1A and B, in agreement with the occurrence of a typical EF-hand protein, such as calmodulin, for which Ca2+ binding affects both Tyr and Phe chromophores. 

Conformational Dependence of the Recombinant Integrin Fragments upon Divalent Cation Binding

As shown in Fig. 1A, both the apoform and the fully Mg2+-loaded form of α5(160–448) display superimposable far-UV CD spectra (profiles 1 and 2, respectively), indicating that the secondary structure is independent of the binding of divalent cations. In contrast, a marked difference is observed in the near-UV between the apoprotein and the fully Mg2+-loaded form (Fig. 1B). The observed increase in absolute rotational strength of both Phe and Trp CD bands upon cation binding likely translates a rearrangement of the hydrophobic interior of the protein leading to a more compact structure in comparison to the apoform, in agreement with a previous observation with α5(229–448) (10). In contrast the Tyr CD bands remain invariant upon divalent cation binding. The minimum of this composite band (16 Tyr residues in total) lies at ~280 nm thus denoting that most of the Tyr residues are solvent-exposed in the apoform and the fully Mg2+-loaded form (46). This observation markedly differs from what is observed with a typical EF-hand protein, such as calmodulin, for which Ca2+ binding affects both Tyr and Phe chromophores (47).
titration profiles at pH 6.0 are given in A in independent experiments see were carried out in duplicate. For the observed variations between two M and 6.0 and 8.3 (indicating that the basic organization of the isolated b affinity, respectively), of divalent cation-binding sites, CD spectra of the apoprotein and the fully Mg2+ loaded form of the protein hydrophobic core. In this case, there is a reduction in the molar ellipticity at 268 nm. The changes in molar ellipticity are presented as a function of the cation concentration for the Phe band at 268 nm. \( \theta \) and \( \theta_{\text{max}} \) correspond to the molar ellipticities of the apoform and of the cation-saturated form, respectively, and \( \theta \) to the molar ellipticity at a given cation concentration. The Mn2+ titration was carried out at pH 6.0 (cadoxylate buffer B), whereas the titration with Mg2+ was carried out at pH 8.3 (borate buffer A). In both cases, the initial protein concentration was 10 \( \mu \)M in all experiments, and the successive points along the titration profiles correspond to a progressive dilution of the solution not exceeding 2% (see “Experimental Procedures”). To establish the correspondence between the titrations at different pH values, the assays with Mg2+ was also carried out at both pH values, 6.0 and 8.3, yielding practically identical results. For the sake of comparison, some points of the titrations at pH 6.0 \( (\square) \) are given in A. B, normalized molar ellipticity changes upon binding of Ca2+ to the Mg2+-loaded \( \beta_1(121–329) \) protein; initial conditions: \( [\text{protein}] = 0.01 \text{mM} \) and \( [\text{Mg}^{2+}] = 0.6 \text{mM} \).

(10), were carried out under identical conditions, we conclude that both isolated proteins, \( \alpha_5(229–448) \) and \( \alpha_5(160–448) \), display identical \( K_d \) values for each class, I and II (high and low affinity, respectively), of divalent cation-binding sites, i.e. \(-30 \) and \(-120 \mu \)M, respectively, independently of the cation itself (Ca2+, Mg2+, and Mn2+).

\( \beta_1(121–329) \)—No difference is observed between the far-UV CD spectra of the apoprotein and the fully Mg2+-loaded form of \( \beta_1(121–329) \) (profiles 1 and 2, respectively; Fig. 1C), thus indicating that the basic organization of the isolated \( \beta_1 \) I-type domain is independent of cation binding. In contrast, a marked difference is apparent between both protein forms in the near-UV, at least in the 250–270 nm range (Fig. 1D), indicating that cation binding is associated with a conformational adaptation of the protein hydrophobic core. In this case, there is a reduction of the (absolute) intensity at 268 nm upon Mg2+ binding. If the spectrum in this region is dominated by the Phe chromophores, such a reduction in the molar ellipticity at 268 nm could be interpreted as due to the induction of an open form of \( \beta_1(121–329) \) upon divalent cation binding, with some of its Phe residues becoming more exposed to the external environment. It must be noted, however, that the CD spectrum of \( \beta_1(121–329) \), in the 255–270 nm range, corresponds to a composite profile with contributions from both Phe and S-S chromophores (see above) so that the observed variations between profile 1 (apoprotein) and profile 2 (fully Mg2+-loaded form) may include contributions from both chromophores without an exact delineation between these two types of contributions being readily feasible. As expected, profile 1 was restored (not shown) upon addition of EDTA in excess (50 mM) to the Mg2+-loaded form of \( \beta_1(121–329) \).

As shown in Fig. 3, we then carried out a titration of the apoprotein of \( \beta_1(121–329) \) with the three divalent cations, Ca2+, Mg2+, and Mn2+, used with \( \alpha_5(160–448) \). The ellipticity variations at 268 nm with \( \beta_1(121–329) \) resulted in a monophasic profile upon progressive addition of either Mn2+ or Mg2+ (Fig. 3A), thus indicating the occurrence of a single class of cation-binding sites. The absolute molar ellipticity variations observed with Mg2+ or Mn2+ at saturation are identical, suggesting that both cations induce a practically identical conformational variation upon binding to the protein. In contrast, even in the presence of a large excess of Ca2+ (up to 100 mM), the near-UV CD spectrum of the apoprotein of \( \beta_1(121–329) \), i.e. profile 1 in Fig. 1D, remained unchanged (CD data with Ca2+ not shown). This appears as a striking difference with what is observed with the \( \alpha_5 \) protein fragment for which no

![Figure 2](image1.png)  
**Fig. 2.** CD-monitored divalent cation binding to \( \alpha_5(160–448) \). Normalized molar ellipticity variations as a function of cation concentration for the dichroic Phe negative band centered at 268 nm upon addition of Ca2+ (A), Mg2+ (B), and Mn2+ (C) to the protein apoprotein. \( \theta \) corresponds to the molar ellipticity of the apoprotein (see profile 1 in Fig. 1B), \( \theta_{\text{max}} \) to the molar ellipticity of the cation-saturated form (profile 2 in Fig. 1B), and \( \theta \) to the molar ellipticity at a given cation concentration. Note (see also “Experimental Procedures”) that the titration by Mn2+ was carried out at pH 6.0 (cadoxylate buffer B), whereas the titration with Mg2+ was carried out at pH 8.3 (borate buffer A). The initial protein concentration was 10 \( \mu \)M in all experiments, and the successive points along the titration profiles correspond to a progressive dilution of the solution not exceeding 2% (see “Experimental Procedures”). To establish the correspondence between the titrations at different pH values, the assays with Mg2+ was also carried out at both pH values, 6.0 and 8.3, yielding practically identical results. For the sake of comparison, some points of the titrations at pH 6.0 \( (\square) \) are given in A. B, normalized molar ellipticity changes upon binding of Ca2+ to the Mg2+-loaded \( \beta_1(121–329) \) protein; initial conditions: \( [\text{protein}] = 0.01 \text{mM} \) and \( [\text{Mg}^{2+}] = 0.6 \text{mM} \).

![Figure 3](image2.png)  
**Fig. 3.** CD-monitored divalent cation binding to \( \beta_1(121–329) \). A, normalized molar ellipticity variations upon binding of Mn2+ \( (\square) \) and Mg2+ \( (\bullet) \) to the apoprotein. The changes in molar ellipticity are presented as a function of the cation concentration for the Phe band at 268 nm. \( \theta \) and \( \theta_{\text{max}} \) correspond to the molar ellipticities of the apoform and of the cation-saturated form, respectively, and \( \theta \) to the molar ellipticity at a given cation concentration. The Mn2+ titration was carried out at pH 6.0 (cadoxylate buffer B), whereas the titration with Mg2+ was carried out at pH 8.3 (borate buffer A). In both cases, the initial protein concentration was 10 \( \mu \)M, and the successive points along the titration profiles correspond to a progressive dilution of the solution not exceeding 2% (see “Experimental Procedures”). To establish the correspondence between the titrations at different pH values, the assays with Mg2+ was also carried out at both pH values, 6.0 and 8.3, yielding practically identical results. For the sake of comparison, some points of the titrations at pH 6.0 \( (\square) \) are given in A. B, normalized molar ellipticity changes upon binding of Ca2+ to the Mg2+-loaded \( \beta_1(121–329) \) protein; initial conditions: \( [\text{protein}] = 0.01 \text{mM} \) and \( [\text{Mg}^{2+}] = 0.6 \text{mM} \).
selectivity was observed for the binding of the different divalent cations (Fig. 2). We hypothesized that the ionic radius likely plays a major role in the effects observed upon cation binding to \( \beta_1\) (121–329). Indeed, a large cation, \( \text{Ba}^{2+} \) (1.34 Å ionic radius), induced no effect on the near-UV CD spectrum of the \( \beta_1\)-protein, whereas a small cation, \( \text{Zn}^{2+} \), induced a similar effect to the one observed with \( \text{Mg}^{2+} \) or \( \text{Mn}^{2+} \) with ionic radii below 0.8 Å (CD data with \( \text{Ba}^{2+} \) and \( \text{Zn}^{2+} \) not shown). So far the stoichiometry of cation binding by \( \beta_1\) (121–329) has not been determined. This originates from the fact that \( \text{Ca}^{2+} \) binds to the recombinant \( \beta_1\) protein with low affinity (see below); the quin2 assay, described above in the case of \( \alpha_\text{C} \) (160–448), could thus not be used. It is accepted that the integrin \( \beta \) subunits display a single cation-binding site resembling the MIDAS site found in the \( \alpha \) subunit I-domains (13, 14). Assuming that there is a single cation-binding site in \( \beta_1\) (121–329), \( K_\text{D} \) values of \( <5 \) and \( >85 \mu \text{M} \) were inferred from the titration profiles of Fig. 3A for \( \text{Mn}^{2+} \) and \( \text{Mg}^{2+} \), respectively. These values are closely related to the apparent \( K_\text{D} \) values reported for the \( \beta_1\) MIDAS-type site in \( \alpha_\beta \), i.e. 1–10 and 80–100 \( \mu \text{M} \) for \( \text{Mn}^{2+} \) and \( \text{Mg}^{2+} \), respectively (14).

Two possible explanations can be envisaged for the apparent lack of any conformational effect (based on near-UV CD evidence) when \( \beta_1\) (121–329) is brought in the presence of \( \text{Ca}^{2+} \): (i) either \( \beta_1\) (121–329) does not bind \( \text{Ca}^{2+} \) or (ii) bound \( \text{Ca}^{2+} \) is unable to induce any conformational adaptation of the protein. In order to assess if one or both possibilities applied to \( \beta_1\) (121–329), we carried out a CD-monitored \( \text{Ca}^{2+} \)-titration of the fully \( \text{Mn}^{2+} \)-loaded protein. As shown in Fig. 3B, a full reversal of the \( \text{Mg}^{2+} \)-induced CD effects was observed for \( \text{Ca}^{2+} \)-concentrations above 50 mM as judged by the molar ellipticity variations at 268 nm. In fact, above this \( \text{Ca}^{2+} \)-concentration, the entire near-UV CD spectrum of \( \beta_1\) (121–329) becomes superimposable (not shown) to that of the apoprotein (profile 1 in Fig. 1D), thus suggesting that \( \text{Mg}^{2+} \) is released from the \( \beta_1\) protein when \( \text{Ca}^{2+} \) binds to it. This situation is reminiscent of what is observed with the homologous \( \alpha \) subunit I-domains where \( \text{Ca}^{2+} \) displaces radioactive \( ^{85}\text{Mn}^{2+} \) from the \( \alpha_\text{C} \) recombinant I-domain when added in excess (48, 49). Our \( \text{Ca}^{2+}/\text{Mg}^{2+} \) competition experiment in Fig. 3B suggests that \( \text{Ca}^{2+} \) competes with \( \text{Mg}^{2+} \) for binding to \( \beta_1\) (121–329). Based on the competition profile of Fig. 3B, it appears that \( \text{Ca}^{2+} \) differs from \( \text{Mg}^{2+} \) by an affinity less than 2 orders of magnitude, but \( \text{Ca}^{2+} \) induces no significant conformational change, if any, in \( \beta_1\) (121–329).

We then used the PRE NMR approach to understand further some of the mechanistic aspects of the interaction of divalent cations with \( \beta_1\) (121–329). This method, as briefly presented under “Experimental Procedures,” is based on the fact that paramagnetically induced relaxation of water protons is markedly enhanced if the water molecules belong to the coordination sphere of a protein-bound paramagnetic cation in comparison to the relaxation observed with the free paramagnetic cation (38). In the case of \( \beta_1\) (121–329), \( \text{Mn}^{2+} \) was selected as a convenient paramagnetic probe due to its elevated electronic magnetic moment and to the fact that it interacts with the protein with a relatively high affinity (see above). As shown in Fig. 4, the progressive addition of \( \text{Mn}^{2+} \) to the \( \beta_1\) (121–329) apoform is accompanied by an enhancement of the water relaxation rates (1/\( T_1 \)). An enhancement factor, \( \epsilon = 2.1 \) (for a definition of this dimensionless factor see “Experimental Procedures”), was observed at the latest point of the titration at 1 mM \( \text{Mn}^{2+} \). Based on \( K_\text{D} = 5 \mu \text{M} \), as given above, an intrinsic enhancement factor \( \epsilon \) (defined under “Experimental Procedures”) of about 12.5 can be estimated (assuming a stoichiometric ratio of 1). Such an estimation assumes fast exchange conditions on the NMR time scale for water exchange, a condition that appears to apply to the present situation (38). This \( \epsilon \) value accounts for a relatively large hydration of the \( \beta_1\)-bound \( \text{Mn}^{2+} \) ion with at least 3 water molecules. This conclusion is in agreement with the structural organization of the MIDAS site in the I-domains of \( \alpha_\text{L} \) and \( \alpha_\text{M} \), where the coordination of \( \text{Mn}^{2+} \) involves 3 water oxygen atoms as part of the regular octahedral coordination of the central cation (50, 51). Our PRE results in Fig. 4 therefore suggest a strong structural resemblance between the divalent cation-binding site of \( \beta_1\) (121–329) and the MIDAS site of the integrin \( \alpha \) I-domains, in agreement with a previous proposal (13, 14).

We then analyzed the effects of adding \( \text{Ca}^{2+} \) to the \( \text{Mn}^{2+} \)-loaded \( \beta_1\) (121–329) protein by PRE. Addition of \( \text{Ca}^{2+} \) in excess (to a final concentration of 100 mM; see Fig. 3B), using the last point of the \( \text{Mn}^{2+} \) titration in Fig. 4, resulted in a relaxation rate (1/\( T_1 \)) identical to that of free \( \text{Mn}^{2+} \). This clearly establishes that \( \text{Ca}^{2+} \) competes with \( \text{Mn}^{2+} \) for binding to \( \beta_1\) (121–329). The reversal of the \( \text{Mg}^{2+} \)-induced conformational effect observed on the CD spectrum of \( \beta_1\) (121–329) when an excess of \( \text{Ca}^{2+} \) is added to the fully \( \text{Mg}^{2+} \)-loaded protein (Fig. 3B) can thus be interpreted as due to the release of \( \text{Mg}^{2+} \) upon \( \text{Ca}^{2+} \) binding to the protein. Whether both divalent cations, \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \), bind to the same site of \( \beta_1\) (121–329), or not, remains an open question (see “Discussion”).

The trichloroacetic acid-precipitated apofoms of \( \alpha_\text{C} \)-C (160–448) and \( \beta_1\) (121–329) used in this work (see under “Experimental Procedures”) need some consideration in terms of their structural integrity. The fact that both apofoms are characterized by far-UV CD spectra identical to those of the corresponding cation-loaded native forms (see Fig. 1) and that conformational reversibility is observed, based on near-UV CD evidence, for cation binding and removal, establishes that the trichloroacetic acid-precipitated proteins retain the basic folding features of the native proteins. However, it would be necessary to...
investigate if cation removal through trichloroacetic acid precipitation is really identical to cation removal through competition using a cation-chelating agent.\(^3\) EDTA readily generates the apoforms of both proteins, \(\alpha_c\)-(160–448) and \(\beta_1\)-(121–329). However, it is known that the apoforms of \(\text{Ca}^{2+}/\text{Mg}^{2+}\)-binding proteins bind EDTA in a selective manner, and this binding of a metal-chelating molecule biases the interaction studies conducted with metal cations using EDTA-generated protein apoforms (52).

**Ligand-binding Properties of \(\alpha_c\)-(160–448) and of \(\beta_1\)-(121–329)**

The immobilization assay depicted in Fig. 5A was used to assess the ligand binding capacities of both recombinant proteins, \(\alpha_c\)-(160–448) and \(\beta_1\)-(121–329). The ligand used was a fibronectin recombinant fragment, 3Fn7–10 (23), that contains the RGD adhesion motif located in the type III module 3Fn10, as well as the synergetic regions located in 3Fn9 (53). As shown in Fig. 5B, His-tagged \(\alpha_c\)-(160–448) binds 3Fn7–10 in the presence of either \(\text{Mg}^{2+}\) or \(\text{Ca}^{2+}\). The stoichiometric ratio of the complex was determined through covalent cross-linking (see "Experimental Procedures") using the untagged proteins, as previously reported (10). At saturating concentrations of \(\text{Mg}^{2+}\), a major peak appears by gel filtration (data not shown) with an estimated mass of about 70 kDa, in agreement with the formation of the 1:1 complex \(\alpha_c\)-(160–448)/3Fn7–10 (calculated mass 72.5 kDa). Recombinant \(\beta_1\)-(121–329) also binds 3Fn7–10 in the presence of \(\text{Mg}^{2+}\) in excess (Fig. 5C) to form a 1:1 complex (stoichiometric ratio inferred from cross-linking and size-exclusion chromatography using the untagged \(\beta_1\)-protein; data not shown). As shown in Fig. 5C, the \(\beta_1\)-(121–329)/3Fn7–10 binary complex is formed in the presence of \(\text{Mg}^{2+}\) (or \(\text{Mn}^{2+}\), not shown), whereas no interaction occurs in the presence of \(\text{Ca}^{2+}\), as judged by the immobilization assay.

Both \(\alpha_c\)-3Fn7–10 and \(\beta_1\)-3Fn7–10 binary complexes are dissociated in the presence of the cyclic RGD-containing peptide, G(Pen)*ELRGDWC* (where Pen indicates l-phenylalani-nyl[\(\beta\)-d-methyl-l-cysteiny] (see Fig. 5, B and C, lanes 1, respectively). This peptide is known to compete efficiently with fibronectin for binding to native \(\alpha_c\beta_1\) (54). These competition experiments with the cyclic RGD-containing peptide suggest that the RGD motif in Fn participates in ligand recognition by both isolated integrin components, \(\alpha_c\)-(160–448) and \(\beta_1\)-(121–329) (see also "Discussion"). We also investigated the competition between two simple compounds, \(n\)-butyrate and guanidinium chloride (mimicking the carboxylate and the guanidinium groups of RGD, respectively), and the 3Fn7–10 ligand for binding to \(\alpha_c\)-(160–448) or \(\beta_1\)-(121–329). As shown in Fig. 5B, the \(\alpha_c\)-(160–448)/3Fn7–10 binary complex is readily dissociated in the presence of 50 mM \(n\)-butyrate, whereas no dissociation is observed with GdnHCl at the same concentration. In contrast, the \(\beta_1\)-(121–329)/3Fn7–10 binary complex is dissociated in the presence of either 50 mM GdnHCl or 50 mM \(n\)-butyrate (Fig. 5C). These results will be discussed below in light of the model recently proposed (10) for RGD binding to an \(\alpha\beta\) heterodimeric integrin.

We subsequently investigated whether the synergistic motifs found in module 3Fn9 (53) are necessary for ligand recognition by one or both integrin components, \(\alpha_c\)-(160–448) and \(\beta_1\)-(121–329). As previously reported (10), the recombinant fragment 3Fn10–11 (24) was selected for this purpose since it includes the RGD-containing 10th type III module but lacks the 9th type III module. As shown in Fig. 6, no complex was observed between the immobilized His-tagged \(\alpha_c\)-(160–448) protein and 3Fn10–11, even in the presence of \(\text{Ca}^{2+}\) or \(\text{Mg}^{2+}\) in excess (Fig. 6, lane 5) but not in the presence of \(\text{Ca}^{2+}\) (not

---

\(^{3}\) J.-L. Bâneres, F. Roquet, A. Martin, and J. Parelo, unpublished data.
Cation and Ligand Binding by a Minimal αβ₁ Integrin

Functional Role of Cation Binding to β₅(121–329)

It has been recently shown that ligand binding to the α₂ I-domain results in metal ion displacement to generate a metal-free I-domain-ligand complex (57). A similar mechanism was initially proposed for the β subunit I-type domains (18). By using the isolated β₁(121–329) protein, we investigated by PRE the binding status of Mn²⁺ in the presence of the RGD peptide, G(Pen)⁵ELRGDGWC⁵. The choice of a small RGD ligand, instead of a fibronectin fragment, such as 3Fn7–10, was guided by the fact that a small ligand will not introduce any significant change in the local correlation times of the water molecules surrounding Mn²⁺ upon complex formation (see under “Experimental Procedures”), whereas this might not be the case with a high molecular weight ligand. A solution of Mn²⁺-loaded β₁(121–329) was titrated with the G(Pen)⁵ELRGDGWC⁵ peptide, and water relaxation rates were measured as a function of peptide concentration (Fig. 7). We used the last point in the Mn²⁺ titration of Fig. 4 to initiate a PRE-monitored peptide titration. As apparent in Fig. 7, the initial enhancement factor ε = −2.1 progressively returns in a monophasic manner to the value ε = 1 characteristic of free Mn²⁺. In contrast, no variation of the water relaxation rate was observed upon addition of the control peptide GAC⁵VRLNSLAC⁵GA (54), at least in the range of concentrations used. The PRE results observed with the cyclic RGD-containing peptide appear as direct proof that binding of this RGD ligand to the Mn²⁺-saturated β₁ protein is characterized by a complete release of Mn²⁺ to form a metal-free ligand-integrin complex. It is likely that this is also the case of Mg²⁺ upon binding of an RGD ligand, such as 3Fn7–10, to β₁(121–329), although we presently provide no direct evidence in this respect (see “Discussion”).

αβ Heterodimer Formation

We used the two-step immobilization assay described under “Experimental Procedures” to determine if the α₅ and β₁ recombinant fragments form a stable αβ heterodimer (or mini-integrin). HisTag-α₅(160–448) and untagged β₁(121–329) were incubated at a 1:1 molar ratio in the presence of 1 mM of G(Pen)⁵ELRGDGWC⁵; lane 3, SDS-PAGE analysis of the Ni-NTA agarose-bound αβ heterodimer; HisTag-α₅(160–448) and β₁(121–329) were used as markers (lanes 1 and 2, respectively). B, SDS-PAGE analysis of the immobilization between the heterodimer HisTag-α₅(160–448)β₁(121–329) and 3Fn7–10 (Fn). The immobilization assay was carried as described under “Experimental Procedures.” Lane 4, SDS-PAGE analysis of the immobilized proteins in the absence of any competing compound; lane 5, immobilized proteins in the presence of 1 mM of G(Pen)⁵ELRGDGWC⁵; lane 6, immobilized proteins in the presence of 50 mM sodium n-butyrate (COO⁻); lane 7, immobilized proteins in the presence of 50 mM GlnHCl; lanes 1–3 correspond to 3Fn7–10, HisTag-α₅(160–448), and β₁(121–329), respectively, used as markers (34.3 and 23.5 kDa, respectively). A and B, the symbol * denotes the protein directly bound to the Ni-NTA support.

Interactions between the αβ Heterodimer and Fibronectin

We analyzed the interaction between the minimized αβ heterodimeric complex and 3Fn7–10 using the two-step immobilization assay. As shown in Fig. 8B, a stable ternary complex

![Fig. 7. Cation displacement upon β₁(121–329) ligand complex formation. A 0.1 mM solution of Mn²⁺-loaded β₁(121–329) in cacodylate buffer B (last point of the metal titration in Fig. 4 at [Mn²⁺] = 1 mM) was titrated at increasing concentrations of the peptide G(Pen)⁵ELRGDGWC⁵ (●) or the control peptide GAC⁵VRLNSLAC⁵GA (□). The symbol * denotes cyclization. The dimensionless enhancement factor ε (see definition under “Experimental Procedures”) is presented as a function of the peptide concentration.](http://www.jbc.org/content/18/22/12012/suppl/DC1/fig7)

![Fig. 8. Heterodimer α₅(160–448) β₁(121–329) and interaction with 3Fn7–10. A, analysis of the interaction between HisTag-α₅(160–448) (immobilized protein, α-IN) and β₁(121–329) (β-IN) using the immobilization assay described under “Experimental Procedures.” The assays were carried out in borate buffer A containing 1 mM Mg²⁺: lane 3, SDS-PAGE analysis of the Ni-NTA agarose-bound αβ heterodimer; HisTag-α₅(160–448) and β₁(121–329) were used as markers (lanes 1 and 2, respectively). B, SDS-PAGE analysis of the immobilization between the heterodimer HisTag-α₅(160–448)β₁(121–329) and 3Fn7–10 (Fn). The immobilization assay was carried as described under “Experimental Procedures.” Lane 4, SDS-PAGE analysis of the immobilized proteins in the absence of any competing compound; lane 5, immobilized proteins in the presence of 1 mM of G(Pen)⁵ELRGDGWC⁵; lane 6, immobilized proteins in the presence of 50 mM sodium n-butyrate (COO⁻); lane 7, immobilized proteins in the presence of 50 mM GlnHCl; lanes 1–3 correspond to 3Fn7–10, HisTag-α₅(160–448), and β₁(121–329), respectively, used as markers (34.3 and 23.5 kDa, respectively). A and B, the symbol * denotes the protein directly bound to the Ni-NTA support.)
Mg2+ integrin in the cross-linking experiment in the presence of graphic profile (Fig. 9B) 90 kDa). The presence of a small amount of free heterodimer to form a ternary 1:1:1 complex (calculated mass 1, rabbit phosphorylase B (97.0 kDa); 3, soybean trypsin inhibitor (21.5 kDa).)

Fig. 9. Stoichiometry of the binary αβ and ternary αβ/Fn complexes. A, an equimolecular mixture of α5(160–448) (30.5 kDa) and β3(121–329) (23.5 kDa) was cross-linked, as described under "Experimental Procedures," in the absence (c) or in the presence (c) of 50 mM EDTA and analyzed through size-exclusion chromatography. B, an equimolecular mixture of α5(160–448), β3(121–329), and 3Fn7–10 (39.9 kDa) was cross-linked in the absence (c) or in the presence (c) of 1 mM G(Pen)*ELRGDGWC*. Each fraction corresponds to 1 ml. The position of the molecular weight markers used is indicated by an arrow; 1, rabbit phosphorylase B (97.0 kDa); 2, bovine serum albumin (66.0 kDa); 3, hen egg white ovalbumin (45.0 kDa); 4, bovine carboxy anhydrrase (31.0 kDa); 5, soybean trypsin inhibitor (21.5 kDa).

α5(160–448)β3(121–329)3Fn7–10 is formed in the presence of Mg2+ (lane 4). As expected, this ternary complex was not formed in the presence of the cyclic RGD-containing peptide (see Fig. 8B, lane 5, and Fig. 9B), thus establishing the RGD dependence of the interaction between the 3Fn7–10 ligand and the minimized α5β3 integrin. We also investigated the competition between n-butyrate, as well as guanidinium chloride, and the RGD-containing 3Fn7–10 ligand for binding to the α5(160–448)β3(121–329) heterodimer. As shown in Fig. 8B (lanes 6 and 7), the ternary complex was totally dissociated in the presence of 50 mM n-butyrate, whereas no dissociation was observed with GdnHCl at the same concentration.

The stoichiometry of the ternary complex was determined through covalent cross-linking. As shown in Fig. 9B, the cross-linked ternary complex displays an estimated mass of ~85 kDa, indicating that one 3Fn7–10 ligand molecule is bound per heterodimer to form a ternary 1:1:1 complex (calculated mass 90 kDa). The presence of a small amount of free α5β3 mini-integrin in the cross-linking experiment in the presence of Mg2+ in excess (see Fig. 9B) likely denotes an imbalance in the initial input ratios rather than a dissociation of the complex, since no free Fn ligand was concomitantly observed in the final reaction mixture. In the presence of the RGD peptide, G(Pen)*ELRGDGWC*, the ternary complex is not formed, and no cross-linked ternary complex was thus visualized. Both molecular species, the initial binary complex α5(160–448)β3(121–329) and free 3Fn7–10, are primarily visualized on the chromatographic profile (Fig. 9B). We nevertheless note a minor amount of ternary complex in this profile, and this is certainly due to the relative affinities of both RGD-containing ligands, 3Fn7–10 and G(Pen)*ELRGDGWC*, for the α5β3 mini-integrin.

Conformational Adaptation of the Integrin and Ligand Components in the Complexes

We summarize in Fig. 10 our CD observations (near-UV region) with regard to the conformational changes induced upon formation of the different binary complexes and the ternary ligand-receptor complex described above. In all cases, comparisons (difference spectra) were made using the Mg2+-load forms of both isolated integrin fragments (there is no divalent cation dependence on the conformation of the fibronectin ligand, as initially reported; see Ref. 10). For the sake of clarity, we present in Fig. 11A an illustration of the different conformational states adopted by both α and β components that make up the minimized α5β3 integrin, as well as by the Fn ligand, as a complement to the CD results presented in Fig. 10. It thus appears that (i) the binary complex α5(160–448)3Fn7–10 involves changes at the level of both Phe and Trp bands without affecting the Tyr bands (Fig. 10A). Similar effects were already reported (10) in the case of the related binary complex α5(229–448)W406L3Fn7–10 (from the spectrum of the α5(160–448)β3(121–329) complex 4 recorded under saturating Mg2+ concentrations. D, difference spectrum obtained after subtracting the sum of the individual CD spectra of the Mg2+-loaded forms of α5(160–448) (β3(121–329) (4) and 3Fn7–10 (5), from the spectrum of the α5(160–448)β3(121–329) complex 6 recorded under saturating Mg2+ concentrations. The numbers in bold correspond to the molecular assemblies defined in Fig. 11. The contribution of the different aromatic chromophores Phe, Tyr, and Trp is indicated in A.)
are closely related to those previously observed with the α5-(229–448) fragment (10), then the adhesion motif and its αβ heterodimeric integrin receptor. The interaction between the ligand and the α subunit (α-IN) involves a direct coordination of a divalent metal cation (located in the first EF-hand type domain as a thioredoxin-fused recombinant protein in an E. coli host strain compatible with disulfide bonding (59). As reported under “Results,” the production of the isolated β1 I-type domain as a thioroadoxin-fused recombinant protein in an E. coli host strain compatible with disulfide bonding, in conjunction with the co-expression of bacterial chaperones, is characterized by relatively good yields of a soluble, natively folded, recombinant protein including an intramolecular disulfide bridge. The CD analysis of β1-(121–329) indicates that this isolated β1 I-type domain corresponds to a highly organized structure, including up to 65% of α-helical and β-strand residues in total (see Table I). This experimentally derived secondary structure organization can be compared with the ones inferred from theoretical models used for predicting the folding of the integrin β subunit I-type domains. A first model has been proposed (13, 59) in which the β I-type domain displays a tertiary fold of the integrin β subunit I-type domains. A first model has been proposed (13, 59) in which the β I-type domain displays a tertiary fold closely resembling that determined for the I-domains of αM, α1, and α5 by x-ray crystallography (12, 60, 61). The tertiary fold in the αM I-domain includes 38% of helical residues and 27% of β-stranded residues (60). These values appear very close to those experimentally measured in this work by CD with the isolated β1 I-type domain (see Table I). However, Lin et al. (14) have recently concluded that the fold of the β subunit I-type domains is expected to differ significantly from the one observed with the α I-domains. According to their predictions, the secondary structure organization would correspond to much a lower content of α-helix and β-strand residues (~21 and 11%, respectively), in contrast with our experimental results with β1-(121–329) (see Table I).
Cation and Ligand Binding by a Minimal α₂β₁ Integrin

Residues in the α subunit I-domain crystal structures (α₁, PDB entries ILFA and IZON; α₂, PDB entries I1O6 and I1JM; α₃, PDB entry 1AOX) that are involved in divalent cation coordination in the MIDAS site are given in bold. The putative cation-coordinating residues in the β subunit I-type domains (13, 63, 64) are also given in bold. The underlined DDL sequence in the β subunit I-type domains corresponds to a putative RGD-binding motif.(15).

Based on the CD evidence reported in this work, β₁-(121–329) undergoes a conformational adaptation upon Mg²⁺ (or Mn²⁺) binding (Fig. 3A). The observed variations essentially affect the 250–280 nm range of the CD spectrum, whereas the intensity of the Trp- and Tyr-associated bands in the 280–300 nm range is only slightly altered (see Fig. 1D). The reduction in the absolute CD signal intensity at 268 nm can be interpreted as an enhanced exposure to the external medium of some of the Phe residues upon Mg²⁺ (or Mn²⁺) binding. Such a conformational adaptation upon divalent cation binding is reminiscent of what has been previously observed with the integrin α subunit I-domains. Based on crystallographic evidence (51, 62), two different conformational states have been defined for the human α₁ I-domain as follows: (i) a “closed” conformation in which all Phe residues are buried in the protein structure, and (ii) an “open” form that is characterized by the exposure of some of the Phe residues to the solvent. The transition from 2 to 4, in Fig. 11A, illustrates such a cation-induced conformational rearrangement in the case of the isolated β₁ I-type domain.

A remarkable observation is that Ca²⁺ is unable to induce any conformational adaptation of the isolated β₁-(121–329) protein in contrast to Mg²⁺ or Mn²⁺. However, Ca²⁺ can expel a β₁-bound Mn²⁺ ion from its binding site when it is present in excess, based on the PRE results presented in Fig. 4. The differences observed between both cations, Ca²⁺ and Mn²⁺, could be linked to the difference in their ionic radii. As indicated by site-directed mutagenesis (13, 14, 63, 64), the putative cation-binding sites in the I-type domains of the β₁, β₂, and β₃ subunits present a strong sequence homology with the MIDAS sites in the α I-domains with regard to the number and the chemical nature of the metal-coordinating residues (see Table II). Our PRE measurements with the isolated β₁ I-type domain also lead to the conclusion that the divalent cation-binding site in this integrin domain adopts a MIDAS-type topology. This conclusion is based on the observation of a relatively large hydration of the Mn²⁺ ion when bound to the β₁ I-type domain (3 water molecules are likely to coordinate the central cation; see “Results”), in agreement with the known hydration of Mn²⁺ in the crystal structures of the isolated I-domains of α₁ and α₃ (50, 51). Coordination in proteins of “small” cations, such as Mg²⁺ or Mn²⁺, versus “large” cations, such as Ca²⁺, differs by the number of coordinating oxygen atoms. Hexa-coordination is generally the rule for Mg²⁺ and Mn²⁺, whereas hepta-coordination is largely observed for Ca²⁺ (65–68). It could be that hepta-coordination is not adapted to the intrinsic features of the MIDAS site. No crystal structure of a Ca²⁺-substituted MIDAS site has been reported so far. Two mutually excluding possibilities can be envisaged to explain the effects of Ca²⁺ on cation binding by β₁-(121–329) as well as on the conformation of this isolated β₁ I-type domain as follows: (i) upon replacement of the small cation Mg²⁺ (or Mn²⁺) by Ca²⁺ within the β₁ MIDAS-type site, a rearrangement of the coordination sphere is induced to accommodate an additional coordinating atom (conversion from hexa- to hepta-coordination), whereas the protein conformation is reverted to an apoform-like conformation; (ii) Ca²⁺ binds to a distinct cation-binding site, with reduced affinity, and this additional site could share one of its cation-coordinating residues with the adjacent MIDAS-type site (hypothesis of overlapping Mg²⁺- and Ca²⁺-binding sites). At the structural level, the latter possibility is reminiscent of what is observed with the typical EF-hand parvalbumin, in which one of the high affinity divalent cation-binding sites displays a satellite (low affinity) site with both sites sharing a common coordinating residue (66). If such an overlap between sites occurs in β₁, it would be expected that, upon binding, Ca²⁺ will significantly alter the affinity of β₁-(121–329) for Mg²⁺ (or Mn²⁺) so that the initially bound cation will be released from β₁. Such a mechanism requires validation by further structural studies with the isolated β₁-(121–329) protein reported here. Both isolated α₁-(160–448) and β₁-(121–329) fragments, taken separately, bind 3Fn7–10 in a divalent cation-dependent manner. However, whereas all three divalent cations, Ca²⁺, Mg²⁺, and Mn²⁺ (see Fig. 2), promote the same ligand-binding (or Mn²⁺)-binding sites). At the structural level, the latter possibility is reminiscent of what is observed with the typical EF-hand parvalbumin, in which one of the high affinity divalent cation-binding sites displays a satellite (low affinity) site with both sites sharing a common coordinating residue (66). If such an overlap between sites occurs in β₁, it would be expected that, upon binding, Ca²⁺ will significantly alter the affinity of β₁-(121–329) for Mg²⁺ (or Mn²⁺) so that the initially bound cation will be released from β₁. Such a mechanism requires validation by further structural studies with the isolated β₁-(121–329) protein reported here. Both isolated α₁-(160–448) and β₁-(121–329) fragments, taken separately, bind 3Fn7–10 in a divalent cation-dependent manner.
GdnHCl and this is not the case of the binary complex α5 Fn-5 is a strong indication that the interactions between the RGD ligand and the α or β subunit essentially differ by their chemical nature, in agreement with a model previously proposed (10). In this model, the RGD motif interacts with the integrin ligand-binding sites by anchoring its aspartyl carboxylate and its arginyln guanidinium on the α subunit and on the β subunit, respectively (Fig. 11C). As previously noted (10), other carboxylic acids with a lesser number of carbon atoms were not effective in dissociating the α5 Fn binary complex, and this could translate that n-butyrate interacts in a specific manner with the RGD-binding site within the α5 EF-hand domain. The fact that GdnHCl is effective in dissociating the β1 Fn binary complex and is ineffective in the case of the α5 Fn binary complex (Table III) also suggests that the effects of such a simple guanidinium compound are rather specific. The observation that n-butyrate also dissociates the β1 Fn complex (Table III) can be rationalized if one assumes that the chemical group in the β subunit that directly interacts with the RGD motif is an integrin carboxylate group (Fig. 11C), possibly originating from one Asp residue from the highly conserved DDL motif that has been identified as an integrin RGD-binding site (15).

There is apparently a paradox between the proposed dual binding of the RGD motif by each subunit of the integrin αβ1, as illustrated in Fig. 11C, and the results obtained for the interaction between 3Fn10–11 (containing the RGD motif but devoid of the synergistic regions in 3Fn9) and the isolated α5 and β subunits (Fig. 6). If we conclude, in the absence of any stable α5(160–448)/3Fn10–11 binary complex (as judged by our immobilization assay), that there is no direct contact between the ligand adhesion RGD motif and the integrin α5 subunit, then it is not easy to rationalize the observed competition between the cyclic RGD peptide, G(Pen)*ELRGDGWC*, and 3Fn7–10 for binding to isolated α5(160–448) (see Fig. 5B, lane 5). It was previously concluded (55, 56) that the α5 subunit is essentially involved in interactions with the synergistic regions found in the 9th type III module of Fn. A possibility accounting for our observation that the cyclic peptide GrPen)*ELRGDGWC* readily dissociates the binary complex between 3Fn7–10 and α5(160–448) would then be that RGD, in the small cyclic peptide, also directly competes with the Fn synergistic regions for binding to α5. This is a possibility that cannot be ruled out presently. If RGD was to interact solely with α5 through an intermolecular metal-coordination bond, as shown in Fig. 11C, then this contact would certainly not be sufficient, in energetic terms, to ensure the stability of the binary complex. α5(160–448)/3Fn10–11, in the absence of other stabilizing contacts (synergistic contacts). However, a direct contact between the Asp carboxylate from RGD and the metal cation bound to one of the EF-hands in α5 could be highly relevant to an accurate positioning of the RGD-containing ligand within the binding pocket of the integrin receptor.

If most of the binding energy between α5(160–448) and 3Fn7–10 originates from other contacts besides this assumed specific RGD contact with α5, what then is the exact role of n-butyrate in dissociating this binary complex? The synergistic pentapeptide motif PHSRN 5 in 3Fn9 (53) displays several structural elements in common with the RGD motif, i.e., an Arg residue as well oxygenated polar side chains (from Ser and Asn). Based on mutation studies, Aota et al. (53) concluded that the arginine residue of PHSRN is important for cell-adhesive function. If this is so, Arg in PHSRN might then interact with a negatively charged residue (carboxylate) from α5, and this interaction would be weakened by the presence of n-butyrate concomitantly with the weakening of the contact between RGD-Asp and the integrin EF-hand. The inefficiency of GdnHCl to dissociate the α5 Fn binary complex (Fig. 5B, lane 7) could result from the fact that Arg, in the synergistic PHSRN sequence, is only one among the different stabilizing intermolecular contacts in the complex. However, such conclusions still remain speculative, and it appears necessary to evaluate the contributions of the different intermolecular contacts to the global binding energy on a quantitative basis. Although energetically weak, some of the aforementioned interactions could strongly influence the specificity of ligand recognition by the integrin receptor.

By using Tb3+ as a luminescence probe, Dickeson et al. (57) have recently shown with the isolated I-domain of α5 that the presence of a metal cation is required for initial binding of a collagen-derived peptide, and subsequently ligand binding results in cation displacement from the integrin domain thus generating a metal-free ligand-receptor complex. This mechanism, in which a cation promotes a ligand–competent conformation and the cation is subsequently released upon formation of the ligand-receptor complex also applies apparently to the case of the β I-type domains, as initially suggested by D’Souza et al. (18). Our results with the isolated I-type domain of β1 are in agreement with this “cation displacement” mechanism, as indicated by the PRE experiment reported in Fig. 7. Such a cation release from the β I-type domains upon formation of the ligand-integrin complex remains basically unexplained in mechanistic terms. It must be noted that the β I-type domains are systematically characterized by the occurrence of a totally conserved DLD pentapeptide (see Table II). Pasqualini et al. (15) showed that the C*WDDLLWC* cyclic peptide, including the conserved β DLD tripeptide sequence (Table II), is ligand–competent and recognizes RGD-containing fibronecrtin fragments, thus suggesting that the DLD sequence could correspond to an essential RGD-binding element in the integrin β subunits. An interesting feature of the DLD motif is that it systematically includes the highly hydrophobic residue Leu that is adjacent to the Asp-Asp dipeptide (see Table II). Such a hydrophobic environment could help stabilize a salt bridge between the guanidinium (from the RGD arginyl residue) and a carboxylate (from DLD in the β subunit) by increasing the pKα of the interacting carboxylic group (70).

If the integrin DLD sequence plays such a central role in ligand recognition, an attractive mechanism accounting for the release of Mn2+ from β1-(121–329), as induced by RGD ligand binding (as well as by Ca2+ binding), would be that the DLD motif in the β I-type domains acts by itself as a metal-coordinating element. As previously noted by D’Souza et al. (18), the second aspartyl residue in DLD occupies the relative position 9 with regard to Asp-1 in the MIDAS-like DXXSXS sequence (see Table II). This conserved “Asp-9” could act, in the integrin β subunit.

4 PRE/NMR experiments (J.-L. Banéres and J. Parello, unpublished information) indicate that the binding of an RGD ligand to the Mn2+-loaded protein α5-(229–448) results in displacement of the coordinating water from the EF-1 cation-binding site, in agreement with the “missing-ligand” mechanism initially proposed by Edwards et al. (77).

5 Site-directed mutation studies at the level of the four EF hands of the α5 divalent cation-binding domain α5-(229–448) indicate that the N-terminal EF-1 hand is responsible by itself for ligand recognition (J.-L. Banéres, M. Green, D. Mesnier, H. LeCalvez, and J. Parello, unpublished information).

---

6 Mutation of either Asp residue in the DLD motif abrogates ligand recognition by β1-(121–329): D. Chazalette, J. L. Banéres, and J. Parello, manuscript in preparation. This suggests a central role of a carboxylate group in the interaction between the β1 subunit and the guanidinium group from the RGD motif in agreement with our competition experiments using n-butyrate.
subunits, as a specific metal-coordinating residue, as is the case of the aspartyl residue usually found at the relative position 9 in the typical EF-hand loops (7). One possibility would be that Asp-9 in the integrin β subunits switches from a metal-coordinating state (contributing to Mg$^{2+}$ or Mn$^{2+}$ coordination) to a ligand-binding state, thus resulting in the loss of the initially bound Mg$^{2+}$ or Mn$^{2+}$ ion after binding of the RGD ligand. This hypothetical proposal assumes that the divalent cation-binding site in the β-I-type domains departs somewhat from the typical MIDAS coordination in the sense that Asp-9 in the β subunits could contribute to the hexa-coordination of Mg$^{2+}$ (or Mn$^{2+}$). There is evidence for the occurrence of a long range metal-coordinating residue in the integrin β-I-type domains, i.e. Asp-217 in β3 (13) and Asp-232 in β2 (63), as in the MIDAS sites (see Table II). However, there is no evidence for the occurrence of another long range metal coordination as observed with Thr in the MIDAS sites of the α I-domains (Table II). As reported by Tozer et al. (13), substitution of Thr-197 in β2 (a candidate for the missing MIDAS metal-coordinating Thr residue) did not affect ligand binding function by integrin αMβ2, thus suggesting that no metal-coordinating Thr exists in the MIDAS-type site of this β subunit. It might be that the totally conserved Asp-9 in the integrin β subunit sequences acts as a direct metal-coordinating residue in place of the missing MIDAS Thr residue. Such an hypothesis needs to be tested through site-directed mutagenesis at the level of the DDL sequence.3 Similarly, Ca$^{2+}$ could interact with the DDL motif thus rendering Asp-9 unavailable for Mg$^{2+}$ (or Mn$^{2+}$) binding and therefore accounting for the release of these small divalent cations, in the presence of Ca$^{2+}$ in excess. If Asp-9 effectively acts as an additional Mg$^{2+}$ (Mn$^{2+}$)-coordinating residue in β2 (in the absence of any RGD ligand), then the number of protein residues involved in metal coordination would amount to five (see Table II). This is in apparent contradiction with our PRE results that indicate that Mn$^{2+}$ bound to β2-(121–329) remains hydrated by 3 water molecules at least. However, the MIDAS site itself in the α I-domains that involves five protein metal-coordinating residues (see Table II) still retains 3 water molecules directly coordinating Mn$^{2+}$ (first sphere), as shown by x-ray crystallography (50, 51). Two among the five metal-coordinating residues of α1 (i.e. Asp-137 and Thr-206; see Table II) do not directly coordinate the central cation and are relayed by one water molecule.

Based on the CD evidence presented in this work, it appears that β2-(121–329) does not undergo any apparent conformational change upon formation of the β2-(121–329)-3Fn7–10 binary complex, although the formation of this complex is likely to be accompanied by the loss of the initially bound Mg$^{2+}$ ion, as illustrated in Fig. 11A. Strictly speaking, our results only established the loss of Mn$^{2+}$ from β2-(121–329) upon binding of an RGD peptide (Fig. 7). Extension to the case of Mg$^{2+}$ and macromolecular RGD ligands appears substantiated. However, there is still a somewhat puzzling situation with regard to the conformational features of β2-(121–329) in the different complexes investigated in this work. Indeed, if we assume that the aforesaid of β2-(121–329) is present in the binary complex 8 (labeled β5 in Fig. 11A), a non-null CD difference spectrum 8 – (4 + 5) would then be expected in contrast to what is observed experimentally (see Fig. 10B). We are thus led to the conclusion that, after loss of the metal cation from β2-(121–329), the resulting metal-free state (or β5) within the binary complex 8 (see Fig. 11A) corresponds to a conformation that is practically identical, based on the CD data, to that observed with the isolated Mg$^{2+}$-loaded form of β2-(121–329). One possibility would be that 3Fn7–10 stabilizes the metal-induced ligand-competent conformation of β2-(121–329) within the binary complex 8, even after release of the protein-bound Mg$^{2+}$ ion, as illustrated in Fig. 11A. The observation that the CD difference spectrum, 9 – (3 + 4 + 5) (Fig. 10D), is practically identical to the CD difference spectrum, 7 – (3 + 5) (Fig. 10A), also implies that the β-component in the ternary complex 9 adopts a similar conformation as the one observed with the Mg$^{2+}$-loaded form of β2-(121–329), although it is probable that Mg$^{2+}$ does not rebind to the β-component in the ternary complex 9 (see transition 6 to 9 in Fig. 11A). The ionic content of β2-(121–329) in this ternary complex still remains an open question. Strictly speaking, we did not establish in this work if any divalent cation is displaced from the β-component of the mini-integrin 6 upon interaction with the Fn RGD-containing ligand (conversion from 6 to 9 in Fig. 11A). However, evidence is available that release of Mn$^{2+}$ from the intact αMβ2 integrin occurs upon ligand binding (18), thus suggesting that the β1-component in our ternary complex 9 (Fig. 11A) is presumably devoid of any bound divalent cation.

The spectral changes induced at the level of the aromatic chromophores upon formation of the binary complex α2-Fn (complex 7 in Fig. 11A), as well as of the ternary complex α2β1-Fn (complex 9 in Fig. 11A), appear to be associated rather exclusively with conformational changes that occur within the hydrophobic regions of the Fn ligand molecule (see “Results”). Such a conclusion derives from the fact that both CD difference spectra, in Fig. 10, A and D, are quantitatively very close to the difference spectrum previously reported (10) with a mutant form of the isolated α2 EF-hand domain in which the single Trp-406 residue was substituted by Leu. One possible explanation accounting for the specific conformational deformability of the Fn ligand can be found in the fact that both type III modules of fibronectin, 3Fn9 and 3Fn10, are involved in intermolecular contacts with the integrin α subunit (see above). The Fn adhesive motifs, RGD (in 3Fn10) and PHSRN (in 3Fn9), are distant by 30–40 Å in the crystal structure of free 3Fn7–10 (23). An analysis of the shape of the minimal ligand-competent EF-hand domain, α2-(229–448), by small angle neutron scattering2 in solution indicates that this domain corresponds to a globular protein with a nearly spherical distribution of its mass and only includes intramolecular distances well below 30 Å. An adaptation of the Fn ligand, resulting in a reduction of the distance between both sites, RGD in 3Fn10 and PHSRN in 3Fn9, would then be required to lead to a productive complex with the integrin α subunit. This could occur through bending of the multi-module Fn filament at the level of the hinge region between both modules 3Fn9 and 3Fn10. This mechanism appears substantiated by a recent NMR analysis indicating that the isolated 3Fn9–10 fragment in solution displays a certain degree of flexibility (71). A bending of the 3Fn9–10 motif at the level of its hinge region could be responsible for changes within the cores of the 9th and/or 10th type III modules of Fn. These structural changes, although subtle, would thus be detected in the near-UV region of the CD spectra (Phe and Trp aromatic chromophores). Site-directed mutagenesis of the Trp residues in Fn7–10 (one tryptophan per type III module), in combination with CD and fluorescence studies, will certainly allows us to better delineate the role of the hydrophobic regions within each Fn type III module on the formation and stability of the integrin-fibronectin complex.2

The question that finally arises is to know whether all the

---

2 The shape factor of α2-(229–448) does not exceed 1.1 (the shape factor corresponds to the ratio between the experimental radius of gyration and the gyration radius calculated for a spherical distribution of the protein mass); small angle neutron scattering results to be published (J.-L. Banères, T. Calmettes, and J. Pareillo, unpublished information).
conformational states identified in this work (see Fig. 11), using our soluble minimized αβ mini-integrin (as well as its isolated α- and β-components), are relevant to the function of this integrin receptor under physiological conditions. It appears unlikely that the apoforms, 1 and 2 (Fig. 11), will play any physiological role if one takes into consideration the concentration range of divalent cations in the extracellular environment.

One of the remarkable features derived from this work is that the isolated β1-(121–329) protein is unable to recognize its Fn ligand in the presence of Ca2+. This suggests that the Ca2+ to Mg2+ ratio in vivo will be essential for switching the integrin β-component from a ligand-competent conformation (high affinity complex) to a ligand-in competent one (low affinity complex), as illustrated in Fig. 11B. In the extracellular medium where both divalent cations, Ca2+ and Mg2+, are present at similar concentrations (in the millimolar range), it can be anticipated, based on the measured affinity constants in vitro, that the β-integrin subunits will be essentially substituted by Mg2+ within their MIDAS-type sites, whereas the α-integrin subunits, with their EF-hand type sites, will correspond to mixed states of occupation by both cations, Ca2+ and Mg2+. Such hybrid α(Ca2+/Mg2+)/β(Mg2+) complexes (see 6 in Fig. 11B) are expected to be ligand-competent in vivo, as suggested by the results reported in this work. Ligand binding by α(Ca2+/Mg2+)/β(Mg2+) will generally involve the loss of the Mg2+ ion from the β subunit, whereas the EF-hand type sites of the α subunit will remain occupied by divalent cations (transition 6 to 9 in Fig. 11B). If an imbalance of the Ca2+ to Mg2+ ratio in vivo occurs with Ca2+ concentrations reaching values comparable to the concentrations used in our in vitro studies, the physiologically active form 6 might be converted into an inactive α(Ca2+/βCa2+) form (state 6’ in Fig. 11B) in both subunits α and β which are occupied by Ca2+. Liberation of Ca2+ from mineralized bone can lead to an increase of free Ca2+ concentration at the level of the osteoclast up to 40 mM (72). As established in this work, the Ca2+-loaded form of the β1-I-type domain is ligand-in competent (see Fig. 5C, lane 4). It is thus concluded that the Ca2+-substituted binary complex 6, if present in vivo, will only recognize the RGD-containing Fn ligand through its α-component to form a ternary complex 9 with a lower stability than the active ternary complex 9 (Fig. 11B). In this respect, we observed that 3Fn7–10 is still recognized, under in vitro conditions, by the minimized αβ mini-integrin in the presence of Ca2+ exclusively, based on our immobilization assay (SDS-PAGE data not shown). However, in the presence of Ca2+, the immobilized complex, 3Fn7–10αβ, is more susceptible to dissociation by a competing RGD-containing peptide than is the case in the presence of Mg2+. One possible explanation is that the binding of 3Fn7–10 to the mini-integrin receptor as a whole becomes significantly weaker in the presence of Ca2+ than in the presence of Mg2+. It is not clear whether Ca2+ is released from the β subunit upon formation of the low affinity complex 9’, by analogy with what is likely to occur upon formation of the high affinity complex 9, in the presence of Mg2+. Our results with the isolated α and β1 fragments thus provide a possible explanation for the observed decrease in the affinity of the native αβ mini-integrin for fibronectin in the presence of Ca2+ (73). The inefficiency of the β subunit to bind Fn in the presence of Ca2+ (Fig. 5C, lane 4) is likely to be responsible for such a decrease in the affinity of the native integrin for fibronectin. It thus appears that a shift in the relative concentrations of extracellular Ca2+ and Mg2+ that would favor the binding of Ca2+ by the integrin receptor, under in vitro conditions, could limit adhesion, as suggested by Hu et al. (74) in the case of integrin α5β3 in the bone. As illustrated in Fig. 11B, we anticipate that the conversion of 6 into 6’ upon substitution of Mg2+ by Ca2+ within the β I-domain type region is accompanied by a conformational change of this β domain which adopts an apoform-like structure based on our studies with our soluble αβ mini-integrin and its isolated α and β components (see Fig. 11A). Such a prediction needs to be validated experimentally using the difference CD spectroscopy approach used here. Knowledge of the exact conformational features that are associated with divalent cation and ligand interactions needs to await the determination of three-dimensional structures at atomic resolution. Our present study, however, with the soluble mini-integrin αβ, allowed us to delineate an extended repertoire of conformational events (see Fig. 11) that likely play a direct role in the regulation of EM-cell adhesion.

Recently, Cieriewski et al. (75) have reported the expression of human β3-(95–373) in E. coli and established that Ca2+ inhibits the binding of fibrinogen to the isolated β fragment, in contrast to intact αββ3. As noted by these authors (75), recombinant β3-(95–373) binds fibrinogen in the presence of Mg2+ and at low concentrations of Ca2+, whereas high concentrations of Ca2+ abolish the interaction. These observations are totally consistent with our results with recombinant β1-(121–329) establishing that Ca2+ inhibits ligand recognition through release of Mn2+ (and probably Mg2+) from the isolated β1 I-type domain (see “Results”). As discussed above, Ca2+ might interfere with the Mg2+/Mn2+-binding site on the β1 I-type domain by eliciting a common coordinating residue so that Ca2+, at high concentrations, will be able to destabilize the primary MIDAS-type site (with high affinity for Mg2+ or Mn2+) by occupying a vicinal site (with low affinity for Ca2+). The stoichiometry of Ca2+ binding to the β I-type domain is not known presently.

As a general comment, we note that both mini-integrins, α5-(160–448)β1-(121–329), described here, and α1m-(1–233)β3(111–318), previously reported by McKay et al. (16), essentially differ by their α components. Both αβ heterodimeric assemblies only display the α repeat III in common. Besides repeat III, our αβ1 mini-integrin displays a full EF-hand type domain (repeats IV through VII), whereas this Ca2+/Mg2+-binding domain is completely absent from the α1mβ3 mini-integrin. Both mini-integrins essentially include a similar β I-type domain taking into account the sequence homologies between both β1 and β3 domains (see Table II). Although both mini-integrins display RGD-dependent ligand recognition, it is not clear if they do have the same cation-binding requirements. The lack of the α1m EF-hand domain in the α1mβ3 mini-integrin, combined with the RGD-dependent recognition of fibrinogen by the minimized integrin receptor, would suggest that the Ca2+/Mg2+-binding domain of α1m is not required for ligand recognition. However, Gulino et al. (9) using the recombinant construct α1m-(171–464), i.e. repeats III to VII, showed that this isolated domain from α1m is ligand-competent and recognizes fibrinogen upon occupation of all four sites by divalent cations (Ca2+, Mg2+, and Mn2+). Our isolated α1m EF-hand domain, α1m-(160–448), that also encompasses repeats III to VII recognizes the RGD-containing Fn ligands in a divalent cation-dependent manner (Ref. 10 and this work). However, it has been shown that the isolated fragment α5-(229–448) that only encompasses repeats IV to VII is similarly ligand-competent in an RGD- and divalent cation-dependent manner (10). This result emphasizes the central role of the α5 EF-hand type sites as part of the integrin ligand recognition pocket. It is likely that in both integrins α5β3 and α1mβ3, the α EF-hand domains play a similar role with regard to their cation- and ligand-binding properties. In this respect, the α1mβ3 mini-integrin reported by McKay et al. (16) lacks such a contribution from the α1m subunit. However, the ligand competence of the mini-integrin
αβ₅β₃ can be explained based on the model of Fig. 11C, since part of the interactions involved in the stabilization of the ligand-integrin complex (interaction between the RGD guanidinium and the DLL site in the β subunit) is still possible between the αβ₅β₃ mini-integrin and the RGD ligand.

In closing, the soluble αβ₃ mini-integrin, α₅(160–448)β₃(121–329), with ~500 residues out of a total of ~1800 in the native αβ₅β₃ integrin, needs to be viewed as a molecular assembly mimicking most, if not all, of the essential interactions that are encountered between the extracellular part of the integrin receptor and its RGD-containing fibronectin ligand. We note, however, that both recombinant fragments α₅(160–448) and β₃(121–329) from human integrin αβ₅ lack the potential N-linked glycosylated moieties, and this could affect the binding properties of the fibronectin ligands in comparison to the glycosylated native receptor. Of particular interest is the fact that in α₅ three potential glycosylation sites (i.e. Asn-216, Asn-266, and Asn-275) are flanking the EF-1 domain in repeat IV. This EF-hand is essential for ligand recognition. Our minimized αβ₅ construct with two structurally well-defined domains, from both its α and β subunits, apparently includes all cation-binding sites that occur in the native αβ₅ heterodimeric integrin and certainly most of the structural elements that make up the fibronectin-binding pocket, as well as the essential elements that are involved in αβ₅ heterodimerization. This soluble recombinant αβ₅ integrin therefore appears to be well adapted for analyzing the molecular bases of integrin-ligand recognition at the structural and pharmacological levels.

Acknowledgments—We are grateful to H. P. Erickson (Duke University, Durham, NC) for providing us with the 3Fn⁷–10 recombinant fragment and for a critical reading of the manuscript. We thank E. Ruoslahti (Burnham Institute, La Jolla, CA) for providing us with the RGD-containing peptide and K. R. Ely for providing the 3Fn¹⁰–¹¹ recombinant fragments. We are grateful to Drs. J. H. Vidal for help in the PRE/NMR experiments.

REFERENCES
1. Hynes, R. O. (1992) Cell 69, 11–25
2. Smith, J. W. (1994) in: Integrins: Molecular and Biological Responses to Extracellular Matrix (Cheresh, D. A., and Mecham, R. P., eds) pp. 1–32, Academic Press, San Diego
3. Ruoslahti, E. (1996) Annu. Rev. Cell Dev. Biol. 12, 697–715
4. Corbi, A. L., Miller, L. J., O’Connor, K., Larson, R. S., and Springer, T. A. (1994) in: Integrin Structure and Function (Cheresh, D. A., and Mecham, R. P., eds) pp. 1–32, Academic Press, San Diego
5. Fasman, G., ed) pp. 69–108, Conformational Analysis of Biomolecules (Fasman, G., ed) pp. 69–108, Plenum Publishing Corp., New York
A Minimized Human Integrin $\alpha_\beta_1$ That Retains Ligand Recognition

Jean-Louis Banères, Françoise Roquet, Aimée Martin and Joseph Parello

J. Biol. Chem. 2000, 275:5888-5903.
doi: 10.1074/jbc.275.8.5888

Access the most updated version of this article at http://www.jbc.org/content/275/8/5888

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 71 references, 35 of which can be accessed free at http://www.jbc.org/content/275/8/5888.full.html#ref-list-1