Structure of an Integrin-Ligand Complex Deduced from Solution X-ray Scattering and Site-Directed Mutagenesis

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Running title: Integrin-fibronectin complex
Summary

The structural basis of the interaction of integrin heterodimers with their physiological ligands is poorly understood. We have used solution X-ray scattering to visualize the head region of integrin α5β1 in an inactive (Ca$^{2+}$-occupied) state, and in complex with a fragment of fibronectin containing the RGD and synergy recognition sequences. Shape reconstructions of the data have been interpreted in terms of appropriate molecular models. The scattering data suggest that the head region undergoes no gross conformational changes upon ligand binding, but do lend support to a proposed outward movement of the hybrid domain in the β subunit. Fibronectin is observed to bind across the top of the head region, which contains an α subunit β-propeller and a β subunit vWF type A domain. The model of the complex indicates that the synergy region binds on the side of the β-propeller domain. In support of this suggestion, mutagenesis of a prominent loop region on the side of the propeller identifies two residues (Tyr$^{208}$ and Ile$^{210}$) involved in recognition of the synergy region. Our data provide the first view of a complex between an integrin and a macromolecular ligand in solution, at a nominal resolution of ~10 Å.
Introduction

Integrins are heterodimeric adhesion receptors with critical roles in development, cell migration and survival (1, 2). Ligand binding to integrins is tightly regulated in vivo and leads to the modulation of cytoskeletal assembly and spatial compartmentalization of signalling events. Affinity regulation of integrins is achieved largely through conformational changes in the extracellular (ligand-binding) domains (2, 3). The interaction of integrin α5β1 with the extracellular matrix glycoprotein fibronectin has served as a prototype for many of these studies. Fibronectin contains the archetypal integrin recognition sequence Arg-Gly-Asp (RGD1) in its tenth type III repeat (Fn10); however a second region of the protein strongly enhances its interaction with integrins such as α5β1 and αIIbβ3 (4-5). This synergy region is located in the adjacent ninth type III repeat (Fn9) and contains a key pentapeptide sequence Pro-His-Ser-Arg-Asn (PHSRN) (6).

Compared to the detailed understanding of the sites on fibronectin that bind integrin, our knowledge of the other side of the interface is limited. Early studies using mAbs directed against α5β1 suggested that the RGD binding site was mainly on the β subunit but also at or near an interface between the α and β subunits: in contrast, the synergy region appeared to be recognised mainly or exclusively by the α subunit (7). Importantly, the epitope of mAb P1D6 that solely blocked synergy site recognition was localised to the third N-terminal repeat of the α5 subunit (7-9). Using a gain-of-function approach, specificity for synergy region binding was also found to reside in this portion of the subunit (9).

Recent X-ray crystal structures of the extracellular domains of αVβ3 have shed new light on mechanisms of integrin-ligand binding. The ligand-binding “head” region of the integrin
contains a seven-bladed β-propeller module in the α subunit (corresponding to the seven N-terminal sequence repeats) and a vWF type A domain in the β subunit (βA domain, also referred to as I-like domain), which is inserted into an immunoglobulin-like ‘hybrid’ domain (10). The top face of the βA domain contains a metal-ion dependent adhesion site (MIDAS) and is closely interacting with the upper face of the α subunit β-propeller. A high-affinity cyclic RGD-containing peptide was found to bind in a groove between the propeller and βA domain with one of the carboxylate oxygens of the Asp side chain coordinating directly to a Mn²⁺ ion in the MIDAS site (11). The Arg side chain bound entirely to loops on the β-propeller. Only small conformational changes (restricted to the βA domain and the β-propeller) were observed between the ligand-free and ligand-occupied states (10, 11), leading to considerable controversy about the molecular basis of activation (12-15).

A number of issues concerning the structural basis of integrin-ligand binding are currently unresolved. (i) It is not known if the binding of integrins to their native macromolecular ligands occurs by the same or by different mechanisms as that observed for RGD peptide mimics. (ii) The location of synergy binding sites on the integrin have not been pinpointed. (iii) It is unclear if major conformational changes occur in the α and β head regions upon ligand binding (16), such as the subunit separation observed by low resolution rotary shadowing EM (17-18), or the coupling of ligand binding to a dramatic outward swing of the hybrid domain as observed by negative staining EM (13, 19).

Here we have addressed these questions by determining the solution conformation of α5β1 in an inactive state, and in complex with a fragment of fibronectin, by solution X-ray scattering. Site-directed mutagenesis was used to elucidate further the location of the synergy sequence
interaction site on the α5 subunit. Our data provide new insights into integrin activation and ligand binding.
EXPERIMENTAL PROCEDURES

Materials

A recombinant fragment of fibronectin containing type III repeats 6-10 (Fn6-10), and a mutant fragment with 16 amino acid residues in Fn9 (including the PHSRN synergy sequence) replaced with the corresponding inactive sequence from Fn8 [Fn6-10(SPSDN)] (20) were produced and purified as previously described (7). Fibronectin-fragment Sepharose was prepared by coupling Fn6-10 to cyanogen-bromide activated Sepharose (Sigma, Poole, UK) (2mg protein to 1ml of beads) according to the manufacturer’s instructions. His-tagged TEV protease expression vector was a gift from Gunter Stier (EMBL, Heidelberg, Germany). The enzyme was purified from cell lysates of transformed E.coli using Ni-NTA agarose (Qiagen). MAbs 16 and 13 were gifts from Dr. K. Yamada (National Institute of Dental Research, NIH, Bethesda, MD). MAbs SAM-2 and 4B4 were purchased from Beckman Coulter (High Wycombe, UK), and Monosan (Uden, The Netherlands), respectively. Mouse anti-human α5 mAbs SAM-1 and JBS5 were from Serotec (Oxford, UK). Mouse anti-human α5 mAb P1D6 was a gift from Dr. E. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA). Mouse anti-human mAb TS2/16 was a gift from F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). MAbs 12G10 and SNAKA52 were produced as described (8). All mAbs were used as purified IgG.

Sample preparation

Truncated α5 and β1 constructs [α5(1-613) and β1(1-455)] fused to the hinge region and C_12 and C_13 domains of human IgG_1 chain were made as previously described (21). A double-gene vector was constructed by linearizing the α5 vector using NotI and ligating it with a NotI-NotI fragment of the β1 vector which included the β1-Fc construct. NS0 cells (mouse...
myeloma, ECACC) were maintained in Iscove’s medium containing 10% fetal calf serum. Cells (10^7 in 1ml PBS) were transfected with 40 µg of PvuI-linearised double-gene vector by electroporation, and stably transfected cells were selected using glutamine-free medium. These cells were then cloned by limiting dilution and integrin expressing clones were identified by Fc capture ELISA (21). The highest expressing clone was selected for culture in an airlift fermenter (Celltech Chiroscience). Culture supernatant was concentrated 10-fold and integrin was purified using Protein A Sepharose (Amersham) and dialysed into 150 mM NaCl, 25 mM Tris-Cl, pH 7.4 (buffer A). The yield was approximately 15 mg/ litre of supernatant. Partially purified integrin was applied to a fibronectin fragment Sepharose affinity column in the presence of 2 mM MnCl₂ and eluted with buffer A containing 5mM EDTA. Fc domains were cleaved off using TEV protease. The cleaved Fc domains were removed using Protein A Sepharose (this procedure also removed any remaining uncleaved or partially cleaved integrin). Purified cleaved integrin was concentrated to approx. 4 mg/ml using a Centricon 80 centrifugal concentrator (Millipore) and dialysed into buffer A. Protein concentrations were measured using the BCA assay (Perbio, Chester, UK). Analysis by gel filtration on a Superose 12 column (Amersham Biosciences) and dynamic light scattering demonstrated that the integrin was >98% monomeric under these conditions. To produce the Ca^{2+}-occupied α5β1, CaCl₂ (100 mM) was added to a final concentration of 5 mM.

To produce the α5β1-FN complex, purified truncated integrin in buffer A was mixed in a 1:1 molar ratio with the Fn6-10 fragment in the same buffer, and MnCl₂ (100 mM) was added to a final concentration of 1 mM. Analysis by gel filtration on a TSK G2000 column (Anachem) indicated that essentially all the integrin formed a 1:1 complex with the fibronectin under these conditions.
Solution X-ray scattering experiments

Measurements were performed at station 2.1 (22) of the Synchrotron Radiation Source (SRS), Daresbury Laboratory, U.K. Solutions for the scattering experiments were prepared as described above. The studies were performed in a standard cell at sample concentrations of 0.5 to 4 mg/ml, according to established procedures (23, 24) and all samples were measured at 4°C. Scattering data were collected in time frames of 60s in order to be able to check for radiation damage, protein aggregation or deposition on the cell mica windows. Offline examination revealed that the samples were unaffected by the above shortcomings and allowed for a total data collection time of 90min and 100min for trα5β1-Ca2+ and trα5β1/Fn6-10 complex, respectively. The latter included averaging several runs with fresh samples and merging data from different concentrations and two experimental set-ups. Two sample-to-detector distances (1.25 m and 6.25 m) were used and the corresponding profiles merged so as to cover the momentum transfer interval \(0.006 \text{ Å}^{-1} \leq q \leq 0.62 \text{ Å}^{-1}\). The modulus of the momentum transfer is defined as \(q = \frac{4\pi \sin\Theta}{\lambda}\), where \(2\Theta\) is the scattering angle and \(\lambda\) is the wavelength (1.54 Å). The q-range was calibrated using silver behenate powder and wet rat tail collagen (based on diffraction spacings of 58.38 Å and 670 Å, respectively). The maximum scattering angle corresponds to a Bragg resolution of 10 Å. The radius of gyration, the forward scattering intensity, and the intra-particle distance distribution function \(p(r)\) were evaluated with the indirect Fourier transform program GNOM (25).

Ab initio modeling and generation of molecular envelopes

Particle shapes were restored from the experimental scattering profiles using the *ab initio* procedure based on the simulated annealing algorithm to a set of clustered spheres representing amino acid residues (26) (GASBOR version 17 or 18). This yields a 3D-distribution of scattering centers that reproduces the one dimensional profile obtained.
from the scattering data. To aid in the interpretation of the reconstructed shape in terms of defined protein structures, we generated ‘equivalent electron density maps’. These maps were obtained by repositioning the 3D-distribution of scatterers in the centre of a suitable box, and calculating the map by standard crystallographic Fourier synthesis using CNS (50). A 20Å resolution cut-off was imposed on these maps. A large number of simulations (>12) were performed for the different states of trα5β1, these simulations generated very similar but not identical shapes. A representative electron density envelope was selected from these on the basis that it showed the consistent shape features observed in the other reconstructions and also was readily interpretable from a structural point of view. It should be noted that these shape reconstructions only give an approximate fit to the experimental scattering data and thus can be used solely as a rough guide for fitting of structural models to the scattering data (see below).

**Homology modeling of trα5β1-Fc and analysis of structural models**

Since high-resolution structural information is available for integrin and fibronectin domains, homology models have been built, based on the crystal structures of integrin αVβ3 and fibronectin (PDB codes 1JV2, 1L5G and 1FNF), in order to exploit fully the observed scattering features in structural terms. The homology model of trα5β1 was built as previously described (15, 27), with the addition of the PSI domain. The human αV and α5 subunits are highly homologous, sharing 51% identity (69% similarity) in the construct used (residues 1-613). The human β3 and β1 are also highly homologous: 44% identity, 63% similarity in the construct used (residues 1-455). Hence, the structural modeling of the truncated α5β1 is likely to be reliable. The PSI domain (which was untraced in the αVβ3 crystal structure) was modeled on an EGF repeat (an α,β fold of similar size). PSI modeling was carried out in order to find a spacefilling shape for placement into scattering envelopes, rather than a true
structural identity. Integrin EGF repeat 3 (from 1JV2) was used as a template in XLOOK (28). An extended N-terminus was repositioned by hand to create a compact fold, and to give a better reconstruction of a small domain. The position of the PSI domain relative to the neighboring hybrid domain was manually adjusted to optimise the fit to the scattering data for the Ca\(^{2+}\)-occupied state. N-linked carbohydrates were modeled on those in the ICAM-1 structure (29) and were attached at Asn residues that fitted the consensus Asn-X-Ser/Thr sequence. Our model of Fn repeats 6-10 in the complex was guided by reconstruction of scattering data for the Fn6-10 fragment in isolation (not shown); flexibility at domain interfaces (30) was also allowed. The Fn6 domain (not present in the 1FNF structure) was modeled on Fn7. Superimposition of shape and structural models was performed with the program SUPCOMB (31). Models were built into the electron density maps using QUANTA (Accelrys Inc.). CRYSOL (32) was used for the simulation of scattering curves from the structural models; this analysis gives a discrepancy factor $\chi$ as a measure of how well the structural model fits the experimental data. The $\chi$ value is dependent on the structural model only, and is independent of the envelope reconstruction. A movement of the hybrid domain was modeled by performing an outward swing of this domain about a pivot point of Asp\(^{120}\) in $\beta$1 subunit. Head domain separation was modeled by moving apart the $\beta$-propeller and $\beta$A domain in increments of 2 Å.

Analysis of $\alpha$5 subunit mutants

Mutations in the $\alpha$5 subunit were carried out using oligonucleotide-directed PCR mutagenesis, as described (21). Oligonucleotides were purchased from MWG Biotech (Southampton, UK). The presence of the mutations (and the lack of any other changes to the wild-type sequence) was verified by DNA sequencing. Chinese hamster ovary cells L761h variant (21) were maintained in Dulbecco’s modified Eagle’s medium supplemented with
10\% fetal calf serum, 2 mM glutamine and 1\% non-essential amino acids (growth medium). 75-cm\(^2\) flasks of sub-confluent CHOL761h cells were transfected with 5 \(\mu\)g of wild-type or mutant \(\alpha_5(1-613)-\text{Fc}\) and 5 \(\mu\)g of wild-type \(\beta_1\) (1-455)-\text{Fc} DNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. After 4 days, culture supernatants were harvested by centrifugation at 1000x \(g\) for 5 min. Wild-type or mutant heterodimers were purified using Protein A-Sepharose essentially as described before (27). Sandwich ELISA assays for epitope expression and ligand binding assays were carried out as before (7, 21, 27). Although wild-type or mutant heterodimers showed little ligand binding after indirect capture using polyclonal goat-anti-human Fc (15), the heterodimers were fully active after direct coating onto 96-well plates (the conditions used for these assays). In each assay involving a comparison between different heterodimers the binding of mAb TS2/16 (used at 5 \(\mu\)g/ml) was used to normalize for any differences between the amounts of the different heterodimers bound to the wells (15).
RESULTS AND DISCUSSION

Currently only very low resolution structural information of integrin-ligand complexes is available (e.g. by rotary shadowing, ref. 33). In principle, negative staining electron microscopy offers potentially higher resolution (19); however, adsorption of macromolecules to the carbon film may involve flattening and distortion, and the high concentration of heavy metal ions used for staining could induce structural changes. Solution X-ray scattering avoids these potential pitfalls and has proven to be a powerful technique for obtaining information on shape and domain organization of biomolecules (although the fine details of the molecular architecture cannot be determined) (34). This is the case even in ab initio studies in the absence of any high resolution structural information. Nevertheless, where crystallographic data are available, as is the case for integrins, the interpretation of a scattering profile can be performed in a highly structurally relevant manner. This approach is analogous to fitting atomic subunits into low-resolution electron microscopy densities of large biomolecular assemblies (35). The scattering technique, however, is less time-consuming and not restricted to large, symmetrical structures (> 200kD). For our studies we used a previously described recombinant truncated version of α5β1 (trα5β1, ref. 21), which is a glycoprotein of molecular mass ~160 kDa. A homology model of trα5β1 was built as described in the Methods section.

Solution structure of inactive α5β1

To determine the conformation of the integrin in a low affinity state, X-ray scattering measurements were made in the presence of 5mM Ca^{2+}, a cation that only very weakly supports ligand binding by α5β1 (36). The geometrical parameters obtained from the scattering data (Table I) and ab initio shape reconstructions indicated that the integrin head region adopts a compact conformation under these conditions, a representative envelope
reconstruction is shown in Fig. 1A, B. This compact shape is consistent with the domain organization observed in the αVβ3 crystal structure (10), and the homology model of trα5β1 (with the identical quaternary organization of domains as seen in the αVβ3 structure) corresponded well with these shapes (Fig. 1A, B). Because of its dependence on geometric shape, scattering data can be extremely sensitive to domain orientations and hence to conformational changes. This feature allows a robust quantitative analysis of different domain arrangements to be performed. The theoretical scattering profile of the homology model of trα5β1 was in very good agreement with the experimental data (discrepancy factor χ=1.37, Fig. 1C). Deviations of the theoretical scattering profile from the experimental data could be due to a number of reasons, including the mobility of the many carbohydrate groups, domain flexibility, and uncertainties in the modeling of the PSI domain (see Experimental Section). Any significant domain movements incorporated into the structural model (e.g. of the hybrid domain) led to a much poorer fit to the experimental data (Fig. 1D). In summary, these results suggest that Ca2+-occupied α5β1 in solution adopts a conformation very similar to that seen in the αVβ3 structure (10).

**Solution structure of ligand-occupied α5β1**

To determine the conformation of the integrin in a high affinity ligand-occupied state, X-ray scattering measurements were made in the presence of the activating cation Mn2+, and the complex was formed by adding an equimolar concentration of a fibronectin fragment containing type III repeats 6-10 (Fn6-10). This fragment contains both the synergy and RGD recognition sequences in repeats 9 and 10, respectively. *Ab initio* shape reconstructions and structural modeling of the scattering data indicated that the integrin adopts a grossly similar overall conformation to that of the low affinity state, with the fibronectin fragment binding across the top of the integrin head (Fig. 2A, B). Modeling of the fibronectin repeats in the
complex was guided by scattering analyses of the isolated Fn6-10 fragment. These analyses showed that the repeats form a conformationally mobile structure, rather than a rigid and elongated chain (data not shown). The position of the repeats in the complex was consistent with binding of the RGD-containing loop of Fn10 at the interface between the propeller and the βA domain, as observed in the RGD-occupied αVβ3 crystal structure (11). No interaction of fibronectin with the cation-binding sites on the lower face of the propeller (37) was apparent. The model of the complex suggests that, although the position of Fn10 may be fixed, the location of the other repeats, particularly Fn6-Fn8, is variable. As a starting model for the integrin we used the same conformation as for the Ca$^{2+}$-occupied structure. The theoretical scattering pattern of the complex with the integrin in this conformation gave a good agreement with the experimental data ($\chi=1.60$, Fig. 2C), suggesting that no dramatic shape changes take place upon ligand binding. To investigate if major domain movements within the integrin head could be accommodated in our model of the complex, we simulated the effect of either a separation of the α and β subunit head domains (16) or an opening of the head region involving a swing out of the hybrid domain (13, 19). The results showed that while a separation of the β-propeller and βA domain led to a much poorer fit to the experimental data (Fig. 2D), a limited movement (~45° outward swing) of the hybrid domain gave a significant improvement in the $\chi$ value ($\chi=1.44$, Fig. 2E), and accurately fitted the experimental $D_{\text{max}}$ and $R_g$ values (Table I). The improvement in the fit to the experimental data was mainly due to an improvement in the low to medium angle range (Fig. 2F), a region that defines domain orientations (34). We have recently provided biochemical evidence that activation of the head region does involve a swing of the hybrid domain away from the α subunit, and that this motion appears to be an essential feature of signal transduction (15). Nevertheless, while the scattering data allow for some movement of this domain in solution, it may be more limited in extent than that suggested by some negatively-stained EM images.
The probable existence of hybrid domain movement in the active integrin supports the proposal that the Ca\(^{2+}\)-occupied \(\alpha V\beta 3\) structure (10) is in a low affinity conformation (12, 13, 15).

*Site-directed mutagenesis of the \(\alpha 5\) subunit \(\beta\)-propeller domain reveals a site of interaction with the synergy region of fibronectin*

In the model of the complex, Fn9 is located close to the side of the \(\beta\)-propeller domain suggesting that the synergy sequence could interact with this region of the \(\alpha\) subunit. Our previous studies of the interaction of fibronectin with \(\alpha 5\beta 1\) suggested that the synergy sequence interacts with blades 2 and 3 of the \(\beta\)-propeller (7-9). Significantly, the epitope of mAb P1D6 that specifically blocks synergy site recognition localizes to blade 3 (38, 9). On the side of the \(\beta\)-propeller in blade 3 there is a prominent loop, corresponding to residues Tyr\(^{204}\) - Gln\(^{214}\) of \(\alpha 5\), which includes part of the P1D6 epitope (Leu\(^{212}\)). We performed alanine-scanning mutagenesis of residues within this loop and tested whether these mutations had any effect on the binding of the integrin to Fn6-10. Mutations that affect only the binding of the synergy sequence would be expected to reduce the affinity of the interaction by a maximum of \(~40\)-fold, since the Fn6-10(SPSDN) fragment, in which the synergy region is inactivated (20) bound to wild-type tr\(\alpha 5\beta 1\) with \(~40\)-fold lower affinity than the native Fn6-10 fragment (Table II). The results (Table II) showed that the affinity of the interaction was decreased \(~5\)-fold by the mutation Y208A and \(~200\)-fold by the mutation I210A. Mutation of the other residues in this loop had no effect on ligand binding. To test whether the mutations affected binding of the RGD sequence or of the synergy sequence to \(\alpha 5\beta 1\), we compared the binding of these mutant integrins to the Fn6-10(SPSDN) fragment. Mutations that solely affect recognition of the synergy region should show no change in the affinity of Fn6-10(SPSDN) binding. The data (Table II) showed that the Y208A mutation had no effect on the affinity of
Fn6-10(PSDNS) binding, hence mutation specifically affected synergy sequence recognition. The I210A mutation had a small effect on the interaction with Fn6-10(PSDNS) (~5-fold lower affinity). Hence, although this latter mutation mainly affects synergy sequence binding, it also has some effect on RGD recognition. In contrast, a control mutation F187A, which is predicted to solely affect the interaction with of the RGD sequence (11), strongly perturbed binding to both the Fn6-10 and the Fn6-10(PSDNS) fragments (data not shown).

To examine whether the effect of the mutations on ligand binding was direct or indirect, sandwich ELISA experiments were performed to test if the mutations altered the conformation of the head region (Table III); the mutation L209A (which did not affect the affinity of ligand binding) was used as a control. The results showed that the expression of all \(\alpha\) and \(\beta\) subunit epitopes, with the exception of P1D6, was unchanged by the Y208A mutation. The I210A mutation resulted in strongly decreased expression of both the P1D6 and mAb16 epitopes, although all other epitopes in this region of the \(\beta\)-propeller were unaffected. Hence, neither the Y208A nor the I210A mutation caused any gross structural changes. An effect of these mutations on the P1D6 epitope is not unexpected since Try\(^{208}\) and Ile\(^{210}\) lie very close to Leu\(^{212}\). It is also possible that Ile\(^{210}\) could form part of the mAb16 epitope because the P1D6 and mAb16 epitopes are closely overlapping (38). Therefore, although it is likely that these mutations directly affect the interaction of the integrin with the synergy region, we cannot rule out the possibility that the effect of the mutations is partly indirect, i.e. due to a local structural perturbation. Nonetheless, since the mutations had little or no effect on RGD recognition, the data strongly support the notion that this region of the \(\alpha\) subunit participates in synergy sequence binding. The RGD and synergy sites lie on the same face of the fibronectin molecule separated by ~ 35 Å (30, 39), suggesting that they could form an extended integrin-binding surface. Tyr\(^{208}\) and Ile\(^{210}\) are positioned ~35 Å away from the
MIDAS cation of the β1 subunit (the key site of RGD binding), and are therefore ideally placed to interact with the synergy sequence (Fig. 3). In contrast to the RGD sequence, which lies at the apex of an exposed loop, the synergy region is a flatter, more extensive surface (39). This surface may be complementary to the relatively flat surface of the side of the α subunit β-propeller. Our model of the complex is also compatible with the observation that regions of Fn in addition to the RGD loop are in close proximity to α5β1 and are capable of contributing to the integrin-ligand interface (40).

Recently, it has been questioned whether the synergy region has a direct role in ligand binding because mutations in this site in the context of fibronectin fragments containing only Fn9 and Fn10 affect structural stability (41) (although this is not the case for synergy site mutations within a larger fragment, ref. 42, as used here). However, our data showing selective loss of binding to the Fn6-10 fragment by the Y208A and I210A mutations provide firm support for a direct interaction between the synergy sequence and the integrin. Since the synergy region also contributes to the strength of the α5β1-Fn bond (43-44), evidence for a direction interaction is compelling. Tyr208 and Ile210 are well conserved between α5 subunits from different species, but the residues at these positions differ between the fibronectin-binding integrin α subunits (α3, α5, αV, and αIIb). These differences may contribute to the differential requirements for the synergy region between the different integrins (9, 45, 46).

In summary, our data reveal new details of interaction of integrins with their macromolecular ligands in solution. These results are likely to be widely applicable. For example, there is evidence for an interaction of αIIbβ3 with the synergy region (5), and for a binding site for fibrinogen on the side of the αIIbβ3 β-propeller (47). Our findings should prove valuable in
the development of anti-integrin therapies for the treatment of inflammatory, vascular and metastatic disease (48).
Footnotes

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1Abbreviations: RGD, Arg-Gly-Asp; βA domain, β subunit vWF A-domain; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; trα5β1-Fc, recombinant soluble integrin heterodimer containing C-terminally truncated α5 and β1 subunits (α5 residues1-613 and β1 residues 1-455) fused to the Fc region of human IgGγ1; CHO, Chinese hamster ovary; S.D., standard deviation; Fn6-10, recombinant fragment of fibronectin containing type III repeats 6-10 (Fn6 through Fn10); Fn6-10(SPSDN) recombinant fragment of fibronectin containing Fn6 through Fn10 in which the synergy region in Fn9 is replaced by an inactive sequence from Fn8 (PHRSN replaced by SPSDN).

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Figure Legends

FIG. 1. Modeling of the low affinity (Ca\(^{2+}\)-occupied) state of tr\(\alpha 5\)\(\beta 1\). (A) Envelope of a representative ab initio model is shown in grey ‘chicken-wire’ depiction. Homology model of tr\(\alpha 5\)\(\beta 1\) is depicted in PyMol\(^2\); \(\alpha 5\) subunit is in red, \(\beta\) subunit is in blue, Asn-linked glycans are in purple. It should be noted that the envelope serves as only an approximate guide for the conformation of the integrin, which was optimized to match the scattering data (see text). The hole in envelope between the \(\alpha\) and \(\beta\) subunits was not seen in all ab initio models. (B) Side-view of the model shown in (A) (after a 90° rotation about the z-axis in an anti-clockwise direction). (C) Solution scattering profile of Ca\(^{2+}\)-occupied tr\(\alpha 5\)\(\beta 1\). Experimental data points are shown in blue. Smooth curve represents the theoretical scattering profile of the homology model of tr\(\alpha 5\)\(\beta 1\) with no domain movements incorporated into the structure. Inset shows calculated distance distribution function \(p(r)\). \(I(q)\) and \(p(r)\) functions have been normalized so that \(I(0)\) and the area under \(p(r)\) are scaled to unity. (D) Analysis of effect of hybrid domain movement on the discrepancy factor \(\chi\). \(\chi\) value is plotted as a function of outward swing of the hybrid domain about a pivot point at the hybrid domain/\(\beta A\) junction. Analysis of models was in (C) and (D) was performed using CRYSOL (32).

FIG. 2. Modeling of the ligand-occupied state of tr\(\alpha 5\)\(\beta 1\). (A) Envelope of a representative ab initio model is shown in grey ‘chicken-wire’ depiction. This was used as an approximate guide for the shape of the tr\(\alpha 5\)\(\beta 1\)/Fn6-10 complex (depicted in PyMol\(^2\)). \(\alpha 5\) subunit is in red, \(\beta\) subunit is in blue, Asn-linked glycans are in purple, and the Fn6-10 fragment is in green. Since the scattering results provide a low-resolution, averaged view of the complex, the shape of the envelope in the region of the fibronectin repeats may circumscribe the range of positions occupied by these repeats relative to the integrin head domains. Many different
orientations of the fibronectin repeats were tested in order to optimise the fit to the experimental data. (B) Side-view of the model shown in (A) (after a 90° rotation about the z-axis in an anti-clockwise direction). (C) Solution scattering profile of ligand-occupied trα5β1. Experimental data points shown in red. Smooth curve represents the theoretical scattering profile of the structural model, in which the homology model of trα5β1 has no domain movements incorporated into the structure. Inset shows calculated distance distribution function \( p(r) \). \( I(q) \) and \( p(r) \) functions have been normalized so that \( I(0) \) and the area under \( p(r) \) are scaled to unity. (D) Analysis of effect of head domain separation on the discrepancy factor \( \chi \). \( \chi \) value is plotted as a function of a movement apart of the α5 and β1 subunits. (E) Analysis of effect of hybrid domain movement on the discrepancy factor \( \chi \). \( \chi \) value is plotted as a function of outward swing of the hybrid domain about a pivot point at the hybrid domain/βA junction. (F) Effect of hybrid domain movement on the fit to the experimental data. Smooth curve represents the theoretical scattering profile of the structural model, in which the homology model of trα5β1 has a 45° outward swing of the hybrid domain incorporated into the structure. Experimental data points shown in red. Analysis of models in (C), (D), (E) and (F) was performed using CRYSOL (32).

FIG. 3. Close-up view of putative fibronectin-binding site on the head region of α5β1.

Integrin and fibronectin are depicted as in Fig. 2, except that, for clarity, Asn-linked glycans are not shown. Side chains of Tyr208 and Ile210 in the α5 subunit are portrayed as orange sticks. Side chains of Arg1374 of the synergy region (in Fn9), and Arg1493 and Asp1495 of the RGD loop (in Fn10) are depicted as green sticks. MIDAS site cation is shown as a cyan sphere. Scale bar = 35 Å.
Tables

TABLE I

*Geometrical parameters (radius of gyration, $R_g$, and maximum particle size, $D_{max}$) of $\text{tr} \alpha5 \beta1$ in two different states and comparison with values deduced from the model that provided the best agreement with the experimental data.*

Values are given in Å.

| Structure           | $R_g$  | $D_{max}$ | $R_g$ model | $D_{max}$ model |
|---------------------|--------|-----------|-------------|-----------------|
| $\text{Tr} \alpha5 \beta1$-Ca$^{2+}$ | 44.3 ± 1.0 | 148 ± 10  | 41.1        | 144             |
| $\text{Tr} \alpha5 \beta1$/Fn6-10 complex | 51.8 ± 1.0 | 178 ± 10  | 50.9        | 184             |
TABLE II

*Effect of α5 subunit β-propeller mutations on apparent affinity of Fn6-10 or Fn6-10(SPSDN) binding to trα5β1 in a solid phase assay.*

Values shown are mean ± S.D., and are expressed as fold-change (increase) in apparent $K_D$ (Ap. $K_D$) relative to the wild-type integrin. N=3, except where otherwise stated. Ap.$K_D$ of Fn6-10 binding to wild-type trα5β1 was ~5nM, Ap.$K_D$ of Fn6-10(SPSDN) binding to wild-type trα5β1 was ~200nM, in close agreement with previously published data (7). ND = not determined.

| Mutant | Fn6-10 binding: fold change in Ap.$K_D$ | Fn6-10(SPSDN) binding: fold change in Ap.$K_D$ |
|--------|----------------------------------------|-----------------------------------------------|
| Y204A  | 1.82 ± 0.37                            | ND                                            |
| Y205A  | 1.27 ± 0.28                            | ND                                            |
| P206A  | 1.12 ± 0.23                            | ND                                            |
| E207A  | 1.73 ± 0.60                            | ND                                            |
| Y208A  | 5.38 ± 0.79 (n=5)                      | 1.05 ± 0.14                                   |
| L209A  | 0.89 ± 0.10                            | 1.21 ± 0.13                                   |
| I210A  | 198 ± 62 (n=4)                         | 5.19 ± 1.69                                   |
| N211A  | 1.38 ± 0.1                             | ND                                            |
| L212A  | 1.51 ± 0.2                             | ND                                            |
| V213A  | 0.93 ± 0.14                            | ND                                            |
### TABLE III

**Summary of mAb binding to α5 subunit β-propeller mutants.**

Results are mean ± S.D., expressed as a percentage of binding to wild-type tro5β1.

| mAb     | Epitope<sup>d</sup> | Y208A   | L209A   | I210A   |
|---------|---------------------|---------|---------|---------|
| SNAKA52 | W1-3<sup>a</sup>   | 88 ± 7  | 103 ± 24| 123 ± 15|
| SAM-1   | W2/W3<sup>b</sup>  | 103 ± 4 | 118 ± 6 | 75 ± 5  |
| SAM-2   | W2/W3<sup>b</sup>  | 107 ± 10| 121 ± 5 | 80 ± 10 |
| JBS5    | W1-3<sup>a</sup>   | 93 ± 3  | 98 ± 2  | 94 ± 3  |
| 16      | W2/3<sup>b</sup>   | 93 ± 7  | 98 ± 2  | 0<sup>e</sup> |
| P1D6    | W3<sup>c</sup>     | 0<sup>e</sup> | 116 ± 1 | 14 ± 6  |
| 13      | βA                  | 81 ± 10 | 91 ± 10 | 89 ± 2  |
| 4B4     | βA                  | 95 ± 5  | 94 ± 5  | 97 ± 13 |
| TS2/16  | βA                  | 93 ± 2  | 99 ± 5  | 104 ± 5 |
| 12G10   | βA                  | 100 ± 3 | 97 ± 8  | 113 ± 6 |

<sup>a</sup> blades 1-3 of α5 subunit β-propeller, <sup>b</sup> blades 2-3 of α5 subunit β-propeller, <sup>c</sup> blades 3 of α5 subunit β-propeller.

<sup>d</sup>For references see Mould et al. (9), Humphries et al. (49).

<sup>e</sup>A value of zero indicates no detectable binding of the antibody above the level to supernatant from mock transfected cells.
FIG. 1

A

β-propeller

Thigh domain

Hybrid domain

B

βA domain

PSI domain
FIG. 1C

$q = 4\pi \sin \Theta / \lambda$ \[Å^{-1}\]

FIG. 1D

Discrepancy factor ($\chi$)

Angle of rotation (°)
FIG. 2C

![Graph showing intensity vs. q = 4\pi \sin \Theta / \lambda [Å⁻¹]].

FIG. 2D

![Graph showing discrepancy factor (χ) vs. head domain separation (Å)].
FIG. 2E

![Graph showing Discrepancy factor vs. Angle of rotation.](image)

FIG. 2F

![Graph showing Intensity vs. q = 4\pi \sin \Theta / \lambda.](image)
Structure of an integrin-ligand complex deduced from solution X-ray scattering and site-directed mutagenesis
Paul Mould, Emlyn J.H. Symonds, Patrick A. Buckley, Gunter J. Grossmann, Paul A. McEwan, Stephanie J. Barton, Janet A. Askari, Susan E. Craig, Jordi Bella and Martin J Humphries

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