Self-assembling multimeric integrin α5β1 ligands for cell attachment and spreading

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Introduction

The extracellular matrix (ECM) component involved in integrin-mediated cell adhesion is dependent on the integrin subtype, for example, osteoclasts bind collagen via integrins α1β1 and α2β1, whereas fibroblasts bind fibronectin (FN) via integrin α5β1 (Clark et al., 2000). The primary integrin-binding motif, arginine–glycine–aspartic acid (RGD), is found in several ECM proteins (Pierschbacher and Ruoslahti, 1984). However, although RGD binds integrin αvβ3 with the same affinity as its ECM ligand, vitronectin, this is not true for all integrins, especially integrin α5β1 which requires the ‘synergy site’ (PHSRN) on the 9th FIII domain (FIIF9) in addition to RGD on FIII10 (Mardon and Grant, 1994). This is often overlooked because many cell types will adhere to surfaces furnished with RGD (Massia and Hubbell, 1991), including cells expressing both integrins αvβ3 and α5β1 since RGD favours αvβ3 binding over α5β1 (Massia and Hubbell, 1991). This conceals the limitation that, for fibroblasts binding via integrin α5β1, cell spreading and signalling cascades are not fully invoked (Hotchin et al., 1999). Peptide amphiphiles harbouring PHSRN and RGD motifs have been reported but demonstrate very weak synergistic effects (Mardilovich and Kokkoli, 2004), corroborating direct evidence that the spatial relationship of the FIIF9–10 domain pair is critical to integrin α5β1 binding (Grant et al., 1997; Altroff et al., 2004).

With RGD being a poor mimic of the ECM, one option would be to coat surfaces with FN. This has been achieved with some control over FN organisation via control over its compact and extended conformation via adsorption to hydrophobic and hydrophilic substrates, respectively (Bergkvist et al., 2003; Baugh and Vogel, 2004). Although simple, this does not address the issues of adsorption induced denaturation and orientated or polyvalent ligand presentation to cells, as can be achieved by highly packed peptidic self-assembling monolayers (Storrie et al., 2007). Nor does the size and complexity of the FN molecule permit controlled cross-linking with polymeric supports on account of steric hindrance and multiple functional sites. Furthermore, the tendency of FN to form fibrils (Litvinovich et al., 1998) may restrict control over a well-defined structure on functionalised surfaces.

Since integrin-mediated cell adhesion depends not only on receptor occupancy but also on receptor clustering (Irvine et al., 2002), it has been shown that the cellular response depends not only on the average surface concentrations of ligand but also on ligand clustering or polyvalency (Maheshwari et al., 2000; Koo et al., 2002). In these studies, cell migration and actin polymerisation were found to be greater on surfaces presenting ‘combs’ of, on average, 3.6–5.4 RGD peptides than on surfaces with combs of 1.7 RGD peptides. These findings now raise questions over previous results for minimal RGD densities required to support cell attachment and spreading because these were based on random ligand distributions (Massia and Hubbell, 1991). Therefore, concerns over ligand density, orientation and polyvalency cannot be ignored if biomimetic supports are to realise their full potential. Unfortunately, the methods required to synthesise peptide combs are incompatible with their application to proteins whose function is dependent upon maintenance of an intact conformation. However, fusion of a protein ligand with a coiled-coil domain has previously been employed in the oligomerisation of proteins and peptides (Wilkins et al., 1996; Watson et al., 2006). Importantly, mutation of key amino acids in the heptad repeat of the GCN4 leucine zipper helix can be used to direct self-assembly of parallel coiled coils of defined cluster sizes (Harbury et al., 1993).

Thus, it should be possible to generate multimers of FIIF9–10 for orientated conjugation to surfaces with control over the cluster size and cluster density. These surfaces will be useful in characterising cell responses to various, well-defined integrin α5β1 ligand displays. To this aim, we describe the design and expression of soluble protein chimeras that comprise a mutant...
of the minimal integrin α5β1 binding domain of FN (FIII9–10; van der Walle et al., 2002), an IgG-derived hinge in order to minimise steric hindrance and mutant leucine zipper-derived helices to foster self-assembly to dimers, trimers and tetramers. In this work, we present the characterisation of these novel chimeras, their specific conjugation to surfaces, integrin in this work, we present the characterisation of these novel chimeras, their specific conjugation to surfaces, integrin

Material and methods

Materials

Unless otherwise stated, general chemical reagents were sourced from Sigma (Dorset, UK), from Fisher Scientific (Leicestershire, UK) or Melford Laboratories (Ipswich, UK), at analytical grade or equivalent quality. Restriction enzymes were from New England Biolabs (Hertfordshire, UK) and integrin α5β1 (cat. no. CC1055) was from Chemicon (UK).

Construction of FIII9–10-hinge-coiled-coil expression vectors

The construction of FIII9–10 has been described elsewhere (van der Walle et al., 2002). Peptide sequences of the hinge-coiled coils for the di-, tri- and tetramer are presented in Fig. 1A; the corresponding cDNAs used a codon bias for expression in Escherichia coli and were synthesised and cloned into pBluescript II KS (Stratagene, UK) by Epoch Biolabs, Inc. (USA). The pBluescript-hinge-coiled-coil constructs were digested with Psrl and HindIII and the resulting cassettes ligated into similarly digested pBluescript harbouring FIII9–10 cDNA between the Kpn1 and HindIII sites (i.e. cloned downstream of the FIII9–10 cDNA); the existing TAA (stop) codon for FIII9–10 was then mutated to TCA (serine) using the Quikchange™ protocol (Stratagene, UK). The pBluescript construct harbouring the FIII9–10-hinge-coiled-coil cDNA was digested with NheI and Psrl and the cassette ligated into similarly digested pRSETa (Invitrogen, UK), such that the protein chimeras were expressed with a hexahistidine tag at their N-termini. As a monomeric ligand control, FIII9–10 cloned into pRSETa (van der Walle et al., 2002) was mutated by extending the C-terminus with a GGC tripeptide (termed FIII9–10-GGC). All construct sequences were confirmed by the University of Dundee Sequencing Service (UK).

Expression and purification of the protein chimeras

Proteins were expressed in E. coli BL21 (DE3) LysS. Cells were grown in a Bioflow 110 fermenter (New Brunswick, UK) either in LB medium or modified M9 medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C and induced at 1.5 OD600nm (mid-exponential phase) with 0.1 mM IPTG for 3–4 h. Cells were harvested by centrifugation (6000 g, 10 min, 4°C) and frozen. Thawed cells were lysed by sonication in 250 mM NaCl, 10 mM imidazole and 50 mM NaHPO4, pH 8.0. Insoluble material was removed by centrifugation (18 000 g, 40 min, 4°C). The resulting soluble fraction was applied to a Ni-NTA column (His-Select, Sigma), washed with 300 mM NaCl, 50 mM imidazole and 50 mM NaH2PO4, pH 8.0, and eluted in the same buffer but containing 400 mM imidazole. Protein was precipitated with 45% w/v (NH4)2SO4 and redissolved in 10 mM NaH2PO4, 300 mM NaCl (pH either 7.0 or 7.8). Protein purity and Mr (relative molecular mass) were assessed by Coomassie staining of sodium dodecyl sulphate (SDS)–polyacrylamide gels and protein concentration was determined from A280 values with a calculated extinction coefficient (ε) of 22 900 M−1 cm−1 for the polypeptide chain of the chimeras and 21 620 M−1 cm−1 for FIII9–10-GGC.

Size-exclusion chromatography

Gel filtration was performed using a Superdex 200 HR 10/30 column (GE Healthcare, UK) run in 10 mM NaH2PO4, 50–500 mM NaCl, 1 mM dithiothreitol, pH 7.8, at a flow rate of 0.5 ml/min. The column was calibrated with the globular proteins myoglobin (17 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97 kDa), as previously described (Watson et al., 2006), obtaining an R2 value of 0.99 for the calibration curve. A 50 μl aliquot of protein at a concentration of ~2.5 mg/ml was injected.

Fig. 1. (A) IgG hinge-coiled-coil amino acid sequences of the chimeras. The N- to C-terminal order of the chimeric constructs follows N-FIII9–10-hinge-coiled-coil C. The FIII9–10 sequence is described in van der Walle et al. (2002). Cysteines substituted for serines in the wild-type IgG hinge are underlined. (B) Schematic diagram of the monomeric unit of the chimeras. (C) Cartoon of the trimeric chimera.
**Mass spectrometry**

Characterisation was undertaken at the University of Glasgow Functional Genomics Facility by a trypsin digest of the corresponding protein band excised from a Coomassie-stained gel and MALDI-TOF analysis. Resulting peptide masses were compared with databases using MASCOT.

**Circular dichroism**

Circular dichroism (CD) spectra were recorded at 20°C using a Jasco J810 spectropolarimeter. Spectra were recorded in the far- and near-ultraviolet (UV) region (185–260 and 250–320 nm, respectively). Spectra represent an average of eight scans, run at 50 nm/min⁻¹ with a 0.5 s time constant and 1 nm bandwidth. Protein samples were studied using concentrations between 0.8 and 1.0 mg/ml with a 0.2 mm pathlength cell for far-UV spectra and with a 2 or 5 mm pathlength cell for near-UV spectra. Proteins were extensively dialysed against 10 mM NaH₂PO₄, 500 mM NaF, pH 7.8, prior to analysis. Estimates of the percentage secondary structures from the CD spectra were obtained using the online CD secondary structure analysis server Dichroweb, with the Variable Selection Method and reference set 3 (Lobley et al., 2002; Whitmore and Wallace, 2004). CD spectra for proteins in 6 M guanidine hydrochloride (GdnHCl) were acquired using a Chirascan spectropolarimeter (Applied Photophysics, UK) in the near-UV region. Spectra represent an average of eight scans, with an acquisition time of 3 s per (1 nm) point and 1 nm bandwidth. Protein samples were studied using concentrations between 1.7 and 2.3 mg/ml with a 0.1 mm pathlength cell to minimise absorbance from the 6 M GdnHCl solution.

**Equilibrium chemical denaturation**

Equilibrium unfolding experiments were performed on recombinant FIII⁹-10-chimeras incubated in 0 to ~6 M GdnHCl in 10 mM NaH₂PO₄, 300 mM NaCl, pH 7.8. Protein samples were diluted in GdnHCl (0.1–0.15 mg/ml) and allowed to equilibrate for 2 h at 25°C before measuring fluorescence emitted at 350 nm, using an excitation wavelength of 278 nm on a Varian Cary Eclipse spectrophotometer, at 25°C. The data were fitted for a two-state unfolding mechanism as described previously (Greene and Pace, 1974) for the calculation of the Gibbs free energy (∆G) between the folded and unfolded states, extrapolated to 0 M GdnHCl to obtain the conformational stability of the protein, ∆G_H₂O.

**Particle size measurements**

The hydrodynamic diameters of the protein chimeras were determined by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, UK), for the proteins dialysed into 10 mM NaH₂PO₄, 300 mM NaCl, pH 7.0, and cleared from insoluble material by centrifugation prior to measurement. Values are given as the mean of three measurements.

**Surface plasmon resonance (BIACore™)**

Proteins were biotinylated via the sulfhydryl group of the C-terminal cysteine with PE₉-O⁻maleimid activated biotin (product no. 21901, Pierce, UK) according to the manufacturer’s recommendations. In brief, protein solutions were incubated with reagent and 5–10 mM Tris[2-carboxyethyl]phosphine (TCEP) for 2 h at room temperature. Excess non-reacted maleimide-PE₉-biotin was removed by extensive dialysis. Biotin incorporation was estimated using the HABA (4'-hydroxyazobenzene-2-carboxylic acid) method (product no. 28005, Pierce, UK). For biosensor analysis, a fully automated BIACore 3000 instrument was used as described previously (Beattie et al., 2008). Biotinylated FN-chimera (150 µg/ml in 10 mM NaH₂PO₄, 300 mM NaCl, pH 7.0) were directly captured on streptavidin-conjugated Sensor Chip SA (BIACore Ltd, UK) at a density of 1000–4000 RUs (1–4 ng/mm²). This surface was stable and integrin-binding capacity was constant during repeated binding/regeneration cycles. Integrin α5β1 (0–10 mM in 25 mM Tris–HCl, 150 mM NaCl, 1 mM Mn²⁺, 0.01% Tween 20, pH 7.4) was injected at a flow rate of 30 μl/min for 5 min. Dissociation of integrin α5β1 occurred over a 15 min period in analyte-free buffer. Regeneration of the FN surface was achieved by 2 × 30 s pulses of 50 mM NaOH, 20 mM EDTA, 2 M GdnHCl. BIACore running buffer was as described above for analyte dilution with the exception that Mn²⁺ was replaced with 3 mM EDTA. In control flow cells, biotinylated bovine serum albumin (BSA) (Sigma product no. A-8549) was immobilised at an equivalent level to biotinylated FN constructs. Binding of integrin α5β1 to BSA control surfaces was automatically subtracted.

**Cell attachment and spreading assays**

Baby hamster kidney (BHK) cells were maintained in G-MEM (21710-025, Invitrogen) containing 10% FCS and 5% tryptose phosphate broth solution (T8159, Sigma) at 37°C in 5% CO₂ incubator. Cell spreading and attachment assay were carried out according to methods described previously by Mardon (Mardon and Grant, 1994), with some modifications. In each experiment, the surface of each 96 well flat-bottomed plates (Nunclon) was coated with 100 ng/ml of NeutrAvidin (31000, Pierce) solution (25 μg/ml in PBS) overnight at room temperature. Uncoated plastic was blocked with SuperBlock® blocking buffer (37515, Pierce) for 2 h at room temperature. The surface of replicate wells was then coated with doubling dilutions of 6 μM of chimeras (dimer, trimer and tetramer) and FIII⁹-10-GGC in buffer for 1 h at room temperature. A total of 10⁴ BHK cells were inoculated into each well in G-MEM, and incubated for 1 h at 37°C in 5% CO₂ incubator. Adherent cells were washed gently with PBS and fixed with 4% formaldehyde; 4% gluteraldehyde in PBS. Cells were viewed by the use of phase contrast microscopy. In each experiment, at least 200 cells in four fields were counted in each duplicate well, and scored for either rounded or spread morphology. Cells that appeared elongated and non-refractile, with the nucleus and nucleoli clearly visible, were scored as spread; refractile cells in which the nucleus was not visible were scored as round. The number of spread cells was expressed as a percentage of the total number of cells counted. The adherent cells were then assessed by staining with 0.1% crystal violet for 30 min, solubilised dye with methanol and measured absorption at 540 nm. The number of adherent cells was expressed as a percentage normalised by the largest reading value in the plate.

The cell functional data obtained are expressed as the mean ± standard error (SD) for three replicates. Statistical analysis of the effects of increasing concentrations of...
immobilised ligand (chimeras and FIII9'–10-GGC) on cell attachment and spreading was performed using Friedman’s non-parametric test. A significance level of \( P < 0.05 \) was used to denote significance in all cases. All analyses were performed using GraphPad Prism version 4.1, GraphPad Software, CA, USA.

Results

Design and expression of the protein chimeras

The protein chimeras consist of: (i) the integrin-binding ligand FIII9'–10, (ii) an IgG hinge (spacer) to prevent steric clashes between integrin receptors, (iii) five complete heptad repeats based on the GCN4 leucine zipper wherein isoleucine and leucine are variously placed in positions ‘a’ and ‘d’ of each heptad repeat; self-assembly via the coiled coil thus generating the FIII9'–10-dimer, -trimer and -tetramer (Fig. 1). These multimers were considered to approximate the size of the RGD comb clusters (Koo et al., 2002). For controlled attachment of the protein chimeras to a substrate or surface, a unique C-terminal cysteine was introduced to facilitate selective biotinylation via a thioether bond, followed by binding to avidin-coated surfaces, or potentially direct covalent binding via the free thiol to gold surfaces. Thus, the chimeras are 273 residues in length, with 183 residues derived from FIII9'–10, 40 residues from the leucine zipper, 30 residues from the IgG hinge and the remainder from the affinity tag.

Proteins were obtained at medium yields following expression in *E. coli*, with purification proceeding to homogeneity. Yields for the FIII9'–10-dimer, -trimer and -tetramer were \(~40, 20\) and \(10\) mg/l, respectively. Varying the concentration of inducer (IPTG) between \(0.1\) and \(1\) mM did not result in any difference in protein expression. There was no significant difference in the quantity of expressed protein whether cells were grown in LB or modified M9 salt medium; however, the protein pattern of the cell-free extract was cleaner in the case of the mineral salt medium.

Characterisation of chimeras

Mass spectrometry by peptide mass fingerprinting identified the FN and a GCN4 leucine zipper components. All proteins were expressed in and purified from *E. coli* to near homogeneity, with the purity of the proteins being \(>95\%\) (Fig. 2). CD spectroscopy in the far-UV region was employed to probe the secondary structure of the chimeras. Since the chimeras need at least \(150\) mM NaCl to remain stable, the proteins were dialysed against NaF to minimise absorption due to the chloride ions. The CD spectra for the FIII9'–10-dimer, -trimer and -tetramer were similar and exhibited minima at \(208\) and \(222\) nm and a maximum at \(192\) nm indicating contributions from \(\alpha\)-helical structure (Fig. 3A). The far-UV CD spectrum for the isolated FIII9'–10 domain pair shows no \(\alpha\)-helical contribution (Pereira et al., 2008) and has an unusual positive band at \(226\) nm representing aromatic contributions in the far-UV (Khan et al., 1989). Therefore, the appearance of characteristic \(\alpha\)-helical bands in the CD spectra for the chimeras is significant since this would be consistent with helical secondary structure adopted by the coiled coil (the IgG hinge being disordered in the crystal structure, Hoffman et al., 2000). Thus, the CD spectra were qualitatively consistent with the approximate predictions and to consolidate this interpretation, the fractional secondary structure components were estimated using the Dichroweb online server. The percentage \(\beta\)-sheet structure for the isolated FIII9'–10 domain pair has

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**Fig. 2.** Fifteen per cent of SDS–polyacrylamide gel of purified chimeras. Lane 1: molecular mass markers (values in kDa); lane 2: dimer; lane 3: trimer; lane 4: tetramer.

**Fig. 3.** (A) Far- and (B) near-UV CD spectra of the dimer (solid line), trimer (dashed line) and tetramer (dotted line) in \(10\) mM NaHPO4, \(500\) mM NaF, pH 7.8. (C) Near-UV CD spectra of the dimer (solid line), trimer (dashed line) and tetramer (dotted line) in \(6\) M GdnHCl, \(10\) mM NaHPO4, \(300\) mM NaCl, pH 7.8.
been estimated to be 39% (Pereira et al., 2008), compared with 45% calculated from NMR data acquired for murine FIII9–10 (Protein Data Bank reference 2mfn) (Copie et al., 1998). The chimera β-sheet values obtained in this study were estimated to be 37–40%. The NRMSD (normalised root mean square deviation) values for the analyses were all <0.07 which is within the acceptable range for this ‘goodness of fit’ parameter (Whitmore and Wallace, 2004) (Table I). The calculated percentages for α-helix in the spectra were less than that would be predicted by the fraction of polypeptide relating to the leucine zipper segment. It is likely that this was due to the contribution from the positive FIII9–10 band at 226 nm (i.e. of the opposite sign in the same region in the far-UV spectrum). The overlap of the FIII9–10 aromatic and coiled-coil α-helix contributions in the spectra make it difficult to extract further information relating to helical interactions consistent with a coiled coil (a [θ]222: [θ]205 ratio > 1 would be evidence for this; Canaves et al., 1998). The near-UV CD spectra for the chimeras were also similar in shape, showing pronounced aromatic contributions >260 nm (Fig. 3B).

In order to investigate whether the introduction of the coiled coil and hinge resulted in any change of conformational stability of FIII9–10, equilibrium denaturation experiments were performed. For all three chimeras, a 2-step unfolding curve was observed (Fig. 4), which is in good agreement with previous equilibrium denaturation studies of FIII9–10 and FIII9–10 demonstrating that the initial step represents the unfolding of FIII9 and the second step the unfolding of FIII10 (previously proven by NMR studies together with equilibrium and kinetic unfolding experiments) (Spitzfaden et al., 1997; Altroff et al., 2001). Given that CD spectra in the near-UV region provide information on the local tertiary structure, comparison of the near-UV CD spectra for the proteins in 6 M GdnHCl with the corresponding spectra for the natively folded proteins showed that the FN domains were in a fully denatured state in 6 M GdnHCl (Fig. 3C).

Table I. Secondary structure estimates from CD spectra (acquired in 500 mM NaF)

<table>
<thead>
<tr>
<th>FIII9–10 construct</th>
<th>% helix</th>
<th>% β-sheet</th>
<th>% unordered</th>
<th>NRMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer</td>
<td>6</td>
<td>40</td>
<td>53</td>
<td>0.063</td>
</tr>
<tr>
<td>Trimer</td>
<td>7</td>
<td>37</td>
<td>55</td>
<td>0.050</td>
</tr>
<tr>
<td>Tetramer</td>
<td>6</td>
<td>40</td>
<td>55</td>
<td>0.068</td>
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<tr>
<td>Calculated*</td>
<td>15</td>
<td>35</td>
<td>49</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*Relative contributions from theoretical calculation based on crystal structures (O’Shea et al., 1991; Leahy et al., 1996; Hoffman et al., 2000).

Characteristic of chimera self-assembly

Particle size distributions relating to the hydrodynamic diameters of the chimeras at different protein concentrations (1 and 4 mg/ml) were in the range of 10–14 nm (Table III). This is in agreement with a hydrodynamic diameter for FIII9–10 of 6.2 nm (Pereira et al., 2008) (corresponding to the domain pair dimensions in the crystal structure, Leahy et al., 1996) in addition to the predicted coiled-coil dimension of 5 nm (using the crystal structure, O’Shea et al., 1991). For DLS measurement by volume, the particle
fractions shown in Table III accounted for 97–100% of the population and this suggests that the protein solutions were homogenous with no evidence for the formation of larger aggregates. Estimation of mean hydrodynamic diameter at different protein concentrations (1 and 4 mg/ml) showed a small concentration dependence, with slightly smaller sizes observed in more dilute solution. This may be a result of a shift in equilibrium of monomer/oligomer towards the monomer with decreasing concentration, although the concentration of 1 mg/ml (30 μM) lies well above the known dissociation constant (K_d) of 0.5–10 μM for coiled-coil proteins (Thomas et al., 1997), such that the majority of the chimera molecules would be expected to be present as the oligomer. It is not yet known if fusion of FIII9–10 affects the leucine zipper coiled-coil K_d.

Subsequently, self-assembly of the chimeras was analysed by size-exclusion chromatography. Fig. 5 shows the typical elution profiles for the three chimeras in phosphate buffer containing 150 mM NaCl. In 150 mM NaCl, three main peaks were detected with elution volumes (V_e) in the order of the relative sizes of the proteins: 11.1, 11.8 and 12.6 ml corresponding to the traces for the FIII9–10-tetramer, -trimer and -dimer, respectively. It is clear that the apparent molecular weights estimated from the calibrated gel column do not match the theoretical molecular weights (Table IV); this is likely to be due to calibration with globular proteins whose tertiary structures contrasts with those of the chimeras. The relationship between apparent M_r and V_e relies on a correspondence between the hydrodynamic properties of the protein standards and the test protein, Cabre et al., 1989.) Nevertheless, calibration and M_r estimation were required to analyse the elution traces for evidence of the monomeric form of the chimeras, which, based on the calibration curve and theoretical M_r of 29.8 kDa, would elute after 15.6 ml. Since no elution peak was near this value (also taking into account uncertainty over the calibration), this suggests that the concentration of the monomeric form, if present at all, was below the detection limit. Absence of the monomer is in agreement with the DLS measurements and the known K_d of coiled coils, estimating a concentration of ~0.8 mg/ml (24 μM) for the proteins on the column, assuming a 1:3 dilution for an injection concentration of ~2.5 mg/ml.

Interestingly, the FIII9–10-dimer chimera, which was designed to form dimers, yielded two main elution peaks with one of these peaks having an identical V_e to the elution peak of FIII9–10-trimer (Fig. 5). This suggests that FIII9–10-dimer is actually a mixture of a dimer and what appears to be a trimer. Spiking a FIII9–10-dimer solution with FIII9–10-trimer confirmed that the peak eluting at a V_e of 11.8 ml was indeed identical to the peak obtained with FIII9–10-trimer. Altering the salt concentration (50, 150, 500 mM NaCl) in the buffer resulted in a change of the relative AUCs (area under the curve) for these two peaks: at 500 mM NaCl, a greater proportion of the FIII9–10-dimer sample eluted with a V_e of 11.8 ml (corresponding to the apparent trimer), and at 50 mM NaCl, the elution trace was indicative of protein precipitation on the column (data not shown). It is not clear yet which part of the molecule is responsible for this behaviour, although Coussen et al. (2002) reported a tendency of multimeric FIII7–10 to precipitate in low salt buffer and Burkhard et al. (2000) reported coiled coils with isoleucine and leucine at the a and d positions of the heptad repeat which switched between dimeric and trimeric forms on increasing salt concentration above 150 mM.

Integrin-binding activity of the chimeras and cell attachment-spreadng

Fig. 6 shows the binding of integrin α5β1 to immobilised di-, tri- and tetrameric chimera surfaces as determined by real-time surface plasmon resonance (SPR)-based biosensor technology. Binding to each of these surfaces was low but was clearly identified above background levels of binding. Although the binding activity did not increase in line with higher states of FIII9–10 oligomerisation, this was to be expected since solubilised and purified integrin α5β1 clearly cannot undergo cell-mediated clustering responses. Attachment and spreading of BHK fibroblasts to surfaces coated with the immobilised chimeras and FIII9–10-GGC increased with increasing

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### Table III. DLS measurement of protein hydrodynamic diameter

<table>
<thead>
<tr>
<th></th>
<th>FIII9–10-dimer</th>
<th>FIII9–10-trimer</th>
<th>FIII9–10-tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 mg/ml</td>
<td>1 mg/ml</td>
<td>4 mg/ml</td>
</tr>
<tr>
<td>Mean, by volume, ± SD (nm)</td>
<td>10.85 ± 0.18</td>
<td>10.22 ± 0.13</td>
<td>13.05 ± 0.31</td>
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### Table IV. Size-exclusion chromatography

<table>
<thead>
<tr>
<th>FIII9–10 construct</th>
<th>Elution volume (ml)</th>
<th>Apparent molecular mass* (kDa)</th>
<th>Calculated molecular mass* (kDa)</th>
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</thead>
<tbody>
<tr>
<td>Dimer</td>
<td>12.6</td>
<td>94.0</td>
<td>59.5</td>
</tr>
<tr>
<td></td>
<td>11.8</td>
<td>127.7</td>
<td>82.9</td>
</tr>
<tr>
<td>Trimer</td>
<td>11.3</td>
<td>127.7</td>
<td>82.9</td>
</tr>
<tr>
<td>Tetrmer</td>
<td>11.1</td>
<td>168.0</td>
<td>119.0</td>
</tr>
<tr>
<td>FIII9–10-GGC</td>
<td>15.6^a</td>
<td>n.a.</td>
<td>29.7</td>
</tr>
</tbody>
</table>

*Based on amino acid sequence; ^Based on column calibration; ^Two elution peaks; ^Theoretical estimate.

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Fig. 5. Gel filtration chromatography of the chimeras on a Superdex 200 column equilibrated in 10 mM NaHPO4, 150 mM NaCl, 1 mM DTT, pH 7.8. Traces correspond to the dimer (solid line), trimer (dashed line) and tetramer (dotted line).
multimerisation (Fig. 7). However, statistical analysis showed the difference in the means for cell attachment not to be significant ($P = 0.052$). In contrast, for the same surfaces, the observed increase in cell spreading with the increase in chimera multimerisation was shown to be statistically significant ($P = 0.017$), i.e. cell spreading was greater on surfaces coated with immobilised chimera in the order: tetramer > trimer > dimer > FIII9–10-GGC.

**Discussion**

Soluble polyvalent cell-adhesive ligands binding integrin α5β1 were engineered as chimeras expressed in *E. coli* and purified to high homogeneity. Extending the N- and C-termini of the coiled coil with FIII9–10 and IgG hinge fragments did not appear to have perturbed the propensity of the coiled-coil domains to oligomerise, and conversely, the coiled-coil motifs did not affect the folding of FIII9–10 which maintained integrin α5β1 binding activity. Therefore, each building block in the protein chimeras performed in principle the role it was intended for. In addition, the chimeras show good stability, an important bonus for their practical applications in biomaterials. It was necessary to determine experimentally the oligomerisation state of each chimera, since this will have direct consequences for integrin clustering and cell adhesion response. Although the coiled-coil sequences used here have been previously reported to form exclusively dimers, trimers or tetramers (Harbury *et al.*, 1993), gel-filtration revealed that the dimeric construct (FIII9–10-dimer) forms a mixture of dimers and trimers under the conditions used, with molar ratio of trimer:dimer increasing with increasing ionic strength of the buffer. Conformational plasticity of coiled-coil structures has been reported previously (Erb *et al.*, 1997; Thomas *et al.*, 1997; Burkhardt *et al.*, 2000); however, not for the leucine zipper variations used in this study. The hallmark of coiled coils is the distinctive packing of amino acid side chains in the hydrophobic core of the helix bundles, called ‘knobs-into-holes’ packing, which presents the major stabilising force (Harbury *et al.*, 1993). However, ionic interactions between the side chains of neighbouring helices affect stability, orientation and stoichiometry of coiled coils (Burkhardt *et al.*, 2002). Accordingly, inter- and intra-helical salt bridges between polar residues in positions $g$ and $e$ of the heptad repeat are particularly important, with higher salt concentration favouring the higher oligomerisation state (Burkhardt *et al.*, 2000, 2002). This involves higher ionic strengths reducing the contribution of ionic interactions to coiled-coil stability, due to increased shielding of charged side chains, while increasing the hydrophobic interaction. Therefore, the dimer–trimer transition for FIII9–10-dimer was possibly due to improved shielding of the hydrophobic side chains from the very polar environment in a trimeric coiled coil compared with a dimeric coiled coil, although the packing of the side chains would favour the dimeric structure. Further characterisation of the monomer–multimer behaviour of the chimeras, including estimation of the dissociation constant, could be investigated by analytical ultracentrifugation.

The chimeras presented in this paper were designed for engineering of surfaces displaying FIII9–10 clusters. Rather than permitting random ligand orientations at the surface via non-specific adsorption or non-specific covalent binding of FN multimers as previously reported (Coussen *et al.*, 2002; van der Walle *et al.*, 2002), the chimeras were anchored in a defined orientation via a single cysteine at the C-terminus facilitating specific binding with ‘leg-down’ orientation. In this manner, the multimeric coiled-coil leg should free the FIII9–10-hinge ‘arms’ such that multiple integrin receptors at a cell surface can be bound. Here, targeted biotinylation via thioether bond was used to anchor the chimera to a streptavidin (BIAcore) chip surface. Since integrin clustering by localisation to the focal adhesions subsequent to FN ligand occupancy is a cellular response (Irvine *et al.*, 2002; LaFlamme *et al.*, 1992), the binding data generated using the immobilised chimera and solubilised integrin simply represented the avidity of the FIII9–10-integrin interaction. However, the SPR studies did demonstrate that these surfaces

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**Fig. 6.** Binding of integrin αβ1 analyte to immobilised dimer, trimer and tetramer, examined at 0, 0.31, 0.62, 1.25, 2.5, 5 and 10 nM. Analyte was injected in duplicate and in random order across chip surfaces. Association time was 5 min, followed by 15 min dissociation in analyte-free buffer. A zero analyte control was also included. Biotinylated-BSA was immobilised at an equivalent level in the control flow cell and served as a control for specificity of chimera-integrin αβ1 binding.

**Fig. 7.** BHK fibroblast attachment (A) and spreading (B) to biotinylated chimeras at coating concentrations of 3 μM. Results are expressed as the percentage of maximum cell attachment and the percentage of cells spread. Error bars represent the standard errors of three independent experiments.
could be repeatedly washed while retaining excellent operational stability, evincing proof of concept for the design strategy for chimera immobilisation. Although the distance between the FIII9–10 ligands in the chimeras could not be calculated a priori, our previous data for RGD multimers and data for FIII7–10 multimers with modular titin (spacer) arms (Coussen et al., 2002; Watson et al., 2006) suggested that the IgG-hinge would accommodate multiply bound integrins, assuming a head diameter of around 100 Å (Adair et al., 2005). This assumption was evinced by the cell adhesion data which demonstrated that cell spreading increased as the polyvalency of the chimeras increased. This would only have been possible had the chimeras been able to engage integrin clusters. It is therefore possible that since FN exists as dimers in fibrillar arrangements, this is to promote cell spreading. It would be interesting to further test these chimeras for evidence of cell adhesion reinforcement, as observed for clustered RGD displays (Koo et al., 2002).

In summary, we have engineered ‘second generation’ cell-adhesive ligands that harbour key structures of FN ECM for cell-binding via integrin α5β1 and improve cell spreading activity. The chimeras described here are far simpler than the previously described FIII7–10 multimers but retain integrin α5β1 binding activity, in contrast to RGD multimers. We anticipate that these chimeras could ultimately be useful for the creation of highly defined synthetic ECM analogs, in which ligand concentration and spatial distribution can be modulated upon a passive background.

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