Biofunctionalization of a generic collagenous triple helix with the α2β1 integrin binding site allows molecular force measurements

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1. Introduction

The interactions of cells with collagens are of vital importance for tissue homeostasis. They transmit not only essential environmental cues but also mechanical forces. The signals from the extracellular matrix (ECM) make cells react in a tissue-specific manner, e.g. platelets are strongly activated by collagens, resulting in platelet aggregation and hemostasis (Farndale et al., 2004). Mechanical forces on the interstitial collagen network allow cell adhesion and contraction of the collagen fibrils, thus playing a role in fibrosis (Eckes and Krieg, 2004). Moreover, the tensile forces counteract the swelling pressure of proteoglycans, which are intermingled with the collagen network, thus regulating interstitial fluid pressure, which in turn determines transcapillary fluid exchange and edema formation (Reed and Rubin, 2010). Different collagen receptors mediate attachment of cells to collagens, among them the integrins α1β1, α2β1, α10β1, and α11β1 (Eble, 2005), the two discoidin domain receptors (DDR)-1 and 2, glycoprotein (GP) VI, and the leukocyte-associated Ig-like receptor-1 (LAIR-1) (Herr and Farndale, 2009; Leitinger and Hohenester, 2007).

Collagens, which are among the most abundant proteins in metazoans, form a large family. Structurally, they share the typical triple helix which is formed by the tight assembly of three

Abbreviations: AFM, atomic force microscopy; CD, circular dichroism; ECM, extracellular matrix; PBS, phosphate-buffered saline; TBS, tris-buffered saline; SEM, scanning electron microscopy.

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peptide chains with a characteristic Gly-Pro-Pro (GPP) repetitive sequence (recently reviewed in Gordon et al., 2010; Myllyharju and Kivirikko, 2004; Brodsky et al., 2008). Another hallmark of the collagens is the high content of hydroxylated prolyl residues, especially 4-hydroxy-prolyl, which are posttranslationally generated by prolyl-4-hydroxylases (Myllyharju, 2008). To fulfill their vital function as the force-bearing scaffold in connective tissues, collagens not only associate with themselves to form supramolecular structures, such as fibrils or network (Myllyharju and Kivirikko, 2004), but also interact with other ECM proteins in an organ- and tissue-specific manner. The list of collagen-binding ECM proteins is extensive. Examples are von Willebrand factor (vWF) and laminins, which form networks of their own (Durbeej, 2010; Sadler, 1998), and smaller ECM proteins, such as BM40/SPARC and nidogens/entactins (Breitkreutz et al., 2009; Clark and Sage, 2008). Additionally, the glycosaminoglycan chains of proteoglycans may interact with collagens (Ricard-Blum et al., 2006).

An essential prerequisite for the interaction of collagens with ECM molecules and with the cellular collagen receptors is the triple-helical structure (Herr and Farndale, 2009; Brodsky et al., 2008). It forms the molecular framework, in which certain amino acid side chains need to be presented for recognition by their interaction partners (Emsley et al., 2004; Golbik et al., 2000; Saccà et al., 2002). In contrast, prolyl hydroxylation of collagen is required for some, but not all of these interactions (Perret et al., 2003; Jarvis et al., 2008). Both triple-helical conformation and prolyl-hydroxylation have been the major challenges in producing chemical surrogates of collagenous interaction sites (Farndale et al., 2008; Saccà et al., 2002; Koide, 2007). Since the successful chemical synthesis of the collagenous α2β1 integrin recognition site (Knight et al., 1998), Farndale et al. (2007, 2008) have paved the way for the identification of several collagenous interaction sites within ECM proteins or collagen receptors, using a collagen peptide Toolkit. Thus, they unravelled the trimeric GFOGER sequence with O being 4-hydroxy-prolyl as recognition site for some collagen-binding integrins (Knight et al., 2000; Zhang et al., 2003). The molecular mechanisms of integrin binding and A-domain activation were unravelled using these collagenous peptides (Emsley et al., 2004). Additionally, the synthetic collagen triple-peptides were instrumental in elucidating the recognition sites for DDR2 (Konitsiotis et al., 2008), for GPVI which strongly depends on prolyl hydroxylation and oligomeric presentation (Jarvis et al., 2008; Smethurst et al., 2007), and for the ECM proteins vWF and SPARC/BM40 (Lisman et al., 2006; Hohenester et al., 2008).

To contribute to the yet poorly analyzed integrin-mediated force transmission between force-exerting cells and the force-bearing collagen network of the ECM, we recombinantly produced a triple-helical mini-collagen harboring the α2β1 integrin-binding relevant GFPGER sequence in a host collagen triple helix. The unhydroxylated mini-collagen was fully active for α2β1 integrin binding both at the molecular and cellular level. Moreover, in contrast to the insoluble and self-aggregating full-length collagens, the recombinant mini-collagen was an ideal tool to analyze the force transmission of individual α2β1 integrin molecules by atomic force microscopy (AFM).

2. Materials and methods

2.1. Materials and cells

The generation and production of the heterodimeric α2fos-β1jun and α1fos-β1jun ectodimers, called soluble α2β1 and α1β1 integrin, respectively, as well as the isolation of collagen I from fetal calf skin, were described previously (Eble et al., 2001, 2006). The antibodies, hVinc against vinculin (Sigma, Deisenhofen, Germany) and JA221 against the human integrin α2 subunit (a gift of D. Tuckwell, University of Manchester) were labelled with digoxigenin-3-O-methylcarbonyl-ε-amino caproic acid-N-hydroxysuccinimide (NHS) ester (Roche Diagnostics, Penzberg, Germany) and sulfo-NHS-LC-biotin (Pierce, Thermo Scientific, Rockford, IL, USA), respectively, in a 400–500 M excess for 2 h and dialyzed extensively. HT1080 fibrosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal calf serum (FCS; Invitrogen, Karlsruhe, Germany) and penicillin/streptomycin (PAA Laboratories, Cölbe, Germany). Cells were detached with 0.5 mM EDTA in PBS without trypsin and washed with DMEM. For adhesion and inhibition experiments, HT1080 were suspended in DMEM, supplemented with 25 mM HEPES, pH 7.4, 50 ng/ml insulin-like growth factor-1 (IGF-1) and 5 ng/ml lysophosphatidic acid (LPA). Primary dermal fibroblasts from wildtype and α2β1 integrin-deficient mice (Holtkötter et al., 2002) were cultured like HT1080 cells and used up to passage 6.

2.2. Construction of expression plasmids and production of recombinant mini-collagen

Codons for the motif GFPGER were inserted into the center of a $(GPP)_{10}$-coding sequence by two-step PCR and a synthetic $(GPP)_{10}$-foldon gene within the bacterial expression vector pHisTrx2 as template (Frank et al., 2001). The 27 amino acid-long foldon domain of the T4 phage protein fibritin was used to constrain the short GPP-repetitive peptides into a collagenous triple-helical quaternary structure (Frank et al., 2001). The amplicons coding for $(GPP)_5$GFPGER$(GPP)_5$-foldon (FC3) and $(GPP)_{10}$-foldon (FC0) were inserted into the NdeI-XhoI site of the expression plasmid pET15b (Novagen, EMD, Darmstadt, Germany). The recombinant proteins with an N-terminal thrombin-cleavable His_6-tag were expressed in BL21(DE3) (Novagen) and purified by immobilized-metal ion affinity chromatography (Qiagen, Hilden, Germany) with an imidazole step gradient. The proteins were dialyzed overnight against 20 mM ammonium acetate, lyophilized and dissolved in either PBS or TBS. Proteins were quantified with BCA assay (Pierce) and analyzed by gel electrophoresis according to Schägger and von Jagow (1987). Mini-collagens were stabilized by treatment with 1% glutaraldehyde (Sigma) for 30 min on ice. Subsequently, unreacted glutaraldehyde was inactivated by addition of tris.

2.3. CD spectroscopy of recombinant mini-collagen

CD spectroscopy was performed with an Aviv model 400 spectrometer (Aviv Biomedical, Lakewood, NJ, USA) and a 1 mm quartz cell. Acquired spectra were normalized for concentration and path length to calculate the molar ellipticity after subtraction of the buffer contribution. For melting curves the CD signal was measured at 225 nm with a heating rate of 0.2 °C/min.

2.4. Binding-ELISA

Mini-collagens, FC3 without and with prior glutaraldehyde stabilization, and FC0, dissolved in TBS, pH 7.4, 2 mM $MgCl_2$ (TBS/Mg-buffer), were immobilised at 12.5 μg/ml to a microtiter plate at 4 °C overnight. Binding assays with soluble integrins were carried out as previously described (Eble et al., 2001, 2006).

2.5. Cell adhesion assays

Mini-collagens, FC3 and FC0 at 12.5 μg/ml and collagen I at 10 μg/ml were adsorbed to a Maxisorp microtiter plate (Nunc, Wiesbaden, Germany) at 4 °C overnight. After washing and blocking with 0.1% BSA in PBS, pH 7.4, cells were plated at a density

of 4×10^5 ml^{-1} for 1 h. For inhibition assays, different concentrations of glutaraldehyde-treated FC3 mini-collagen (FC3GA) were added to the cells either during pre-incubation, or concomitantly to seeding, or after cells have already adhered. Adherent cells were quantified by crystal violet staining (Aumailley et al., 1989) and examined microscopically. Cell spreading was quantified using ImageJ 1.42j (http://rsb.info.nih.gov/ij). The spreading index was determined from micrographs as the ratio of cell-covered area to cell number.

2.6. Confocal laser scanning microscopy

Chamber slides (Nunc) were coated with mini-collagens, FC3 and FC0, at 20 μg/ml, and with collagen I at 10 μg/ml overnight at 4 °C. After washing and blocking with 0.1% BSA in PBS, pH 7.4, cells were plated at 5×10^4 ml^{-1} in serum-free medium for 2 h in the incubator. Bound cells were fixed with 1% formaldehyde in PBS, pH 7.4, 2 mM $MgCl_2$ for 8 min, washed and permeabilized with blocking buffer (2.5% methanol, 0.5% saponin, 2% horse serum in PBS, pH 7.4). To detect α2β1 integrin and vinculin, biotinylated JA221 and digoxigenated hVinc antibodies, respectively, were applied in blocking buffer. Excess antibodies were washed off the specimens, and bound antibodies were detected with fluorescein-conjugated anti-digoxigenin antibodies (Roche Diagnostics) and Alexa555-labelled streptavidin (Molecular Probes, Eugene, OR, USA), respectively. After mounting the specimens with Fluoromount G (Molecular Probes), images were acquired with a confocal laser scanning microscope (Nikon PCM 2000). Five pictures were averaged to minimize background signal.

2.7. Scanning electron microscopy (SEM)

Silicon wafers were coated with 12.5 μg/ml FC3 or FC0 or with 10 μg/ml collagen I over night at 4 °C. After washing and blocking the wafers with 0.1% BSA in PBS, pH 7.4, cells were plated at 1×10^5 ml^{-1} in serum-free medium for 2 h in the incubator. After another washing step with PBS, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, adherent cells were fixed with 1% glutaraldehyde in the same buffer for 30 min. Specimens were prepared for SEM and analyzed as described previously (Rosenow et al., 2008).

2.8. Atomic force microscopy

Permanox plastic slides (Nunc) were coated overnight at 4 °C with 10 μg/ml recombinant α2β1 integrin in TBS, pH 7.4, 2 mM $MgCl_2$, 1 mM $MnCl_2$. Cantilevers (Veeco Instruments GmbH, Mannheim, Germany), with a spring constant of 17.3 pN/nm, were washed three times with chloroform, and their tips were amino-functionalized with 9 M ethanolamine in water-free dimethylsulfoxide (DMSO) at RT overnight. After three washing steps with DMSO and ethanol, the amino-functionalized cantilevers were allowed to react for 2 h at RT with the linker, aldehyde-PEG-NHS (obtained from H. Gruber, Institute for Biophysics, University of Linz, Linz, Austria) in a 6.6 g/l solution of chloroform supplemented with 2%(v/v) triethylamine. After removing unbound linker with chloroform, the second functional group of the linker covalently reacted with mini-collagen in PBS at 12.5 μg/ml for 2 h at 4 °C. Excess mini-collagen molecules were washed off with PBS thrice, and unreacted cross-linker was inactivated with a 0.5 M hydroxylamine in PBS. Finally, the mini-collagens on the cantilever tip were stabilized with 2.5% glutaraldehyde solution in PBS for 30 min.

Force spectroscopy was performed with a NanoWizard BioAFM (JPK instruments AG, Berlin, Germany). The same cantilever was used to measure the rupture forces of cantilever-fixed mini-collagen to the plastic-immobilized α2β1 integrin first in TBS, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM $MnCl_2$, then in TBS, pH 7.4 with 10 mM EDTA, and again with TBS, pH 7.4, 2 mM $MgCl_2$ and 1 mM $MnCl_2$. The last step confirmed that the α2β1 integrin has retained its function after having been incubated with the EDTA-containing buffer. The mini-collagen-decorated cantilever was pressed to the substratum-bound α2β1 integrin for 1 s and subsequently retracted with a velocity of 500 nm/s. Retractions were repeated about 1000 times. From every force–distance curve, the rupture force for the ultimate separation of cantilever from substratum was determined to avoid that several mini-collagens are pulled in parallel. Thus, single integrin–ligand interactions were analyzed (see boxed inset with dashed line in Fig. 9A).

3. Results

3.1. Production and characterization of a recombinant mini-collagen

Most collagens have a tendency to assemble into insoluble, highly ordered aggregates, which expose multiple recognition sites for cell adhesion molecules, such as integrins. Interaction with other collagen-binding matrix proteins further increases the number of potential cell-adhesive sites complicating the investigation of defined cell–matrix-contacts. To analyze the interaction of individual α2β1 integrin receptors with their cognate collagen recognition sites at both cellular and molecular level, we generated a recombinant mini-collagen, called FC3, which bears the non-hydroxylated α2β1 integrin recognition site GFPGER (Knight et al., 2000) in the middle of a collagenous $(GPP)_{10}$ repeat sequence (Fig. 1). The collagenous peptide sequence was fused at its C-terminus to a 27 amino acid long foldon trimerization motif, which serves as a nucleation center and brings three chains together into a collagenous triple helix (Frank et al., 2001). Both the parental structure lacking the integrin recognition site, termed FC0, and the modified mini-collagen FC3 were produced in a bacterial expression system. The N-terminal His-tag allowed the purification by Ni-chelating affinity chromatography. Although the three collagen chains were not held together by an interchain disulfide bridge, the foldon-driven trimerization of both FC3 and FC0 was already apparent in SDS–PAGE (Fig. 2). Being robust against SDS in the sample buffer, the three collagen chains migrated as trimer (left lanes in Fig. 2), unless they had been heated for 10 min at 95 °C (right lanes in Fig. 2). Even in the presence of SDS, a minor fraction of the three mini-collagen chains associated into trimers upon cooling the sample, with a higher trimerization yield of the FC0 compared to the FC3 mini-collagen. Although the individual chains of both FC0 and FC3 migrated at an apparent molecular mass of about 10 kDa, the trimers ran much slower at an apparent molecular mass of about 50 kDa, indicative of the stiff and extended shape that is typical of triple-helical collagen molecules.

Bacteria cannot posttranslationally modify the prolyl residues of collagens to stabilize the collagenous triple helix. Since the triple-helicity of collagens is a prerequisite for α2β1 integrin binding, the correct folding of the three recombinant mini-collagen chains into a triple-helical structure needed to be confirmed. Circular dichroism (CD) studies showed almost identical spectra for FC3 and FC0 with minimal and maximal molar ellipticities at 195 nm and 225 nm, respectively, which are typical of collagenous triple helices (Fig. 3A). Thermal stability of the mini-collagen triple helices was determined by monitoring the molar ellipticity signal at 225 nm with increasing temperature (Fig. 3B). The biphasic melting curve indicated that FC3 contained two entities with distinct thermal stabilities. The high-melting moiety of FC3 showed a transition temperature of 65 °C, which is similar to the melting temperature of the monophasic transition curve of FC0 at 67 °C. Therefore, the high melting entity of FC3 likely corresponds to the foldon domain with

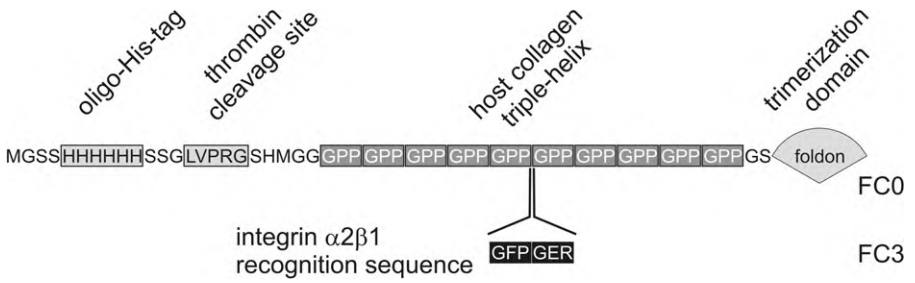

Fig. 1. Schematic presentation of the foldon-collagen (FC) constructs, the mini-collagens FC0 and FC3. The mini-collagen FC0 consists of three peptide chains, each 82 amino acid long. Each chain contains an oligo-His-tag, a thrombin cleavage site, 10 GPP repeats, and the foldon trimerization domain. Brought in close vicinity by the foldon domain, the three repetitive GPP-sequences spontaneously form a triple helix. In mini-collagen FC3, additional six amino acids comprising the α2β1 integrin binding sequence are inserted in the middle of the collagen host triple-helix, thereby arranged into a collagenous triple-helical conformation.

the adjacent part of the collagen host triple helix. Consequently, the foldon-distal moiety of the collagen triple helix, which is N-terminal to the α2β1 integrin recognition sequence, may account for the unfolding signal around 48 °C. Importantly, the majority of the FC3 molecules persisted in the fully triple-helical collagenous conformation at temperatures used in molecular binding assays and cell culture experiments.

3.2. Biological functionality of the mini-collagen FC3

The triple-helical framework of collagens is an indispensable prerequisite for most of their biological activities, such as α2β1 integrin binding and hence cellular interaction. Like wild-type collagen I, the recombinant mini-collagen FC3 was directly bound by both α1β1 and α2β1 integrin in a dose-dependent and saturable manner with K_d values of 19.3 ± 7.4 nM and 1.4 ± 0.1 nM, respectively (Fig. 4). The binding depended on divalent cations, a characteristic feature of integrin binding to ECM ligands. As opposed to FC3, FC0 failed to bind to either integrin, proving that the insertion of the six amino acids GFPGER into the molecular framework of the collagen triple helix biofunctionalized the parental

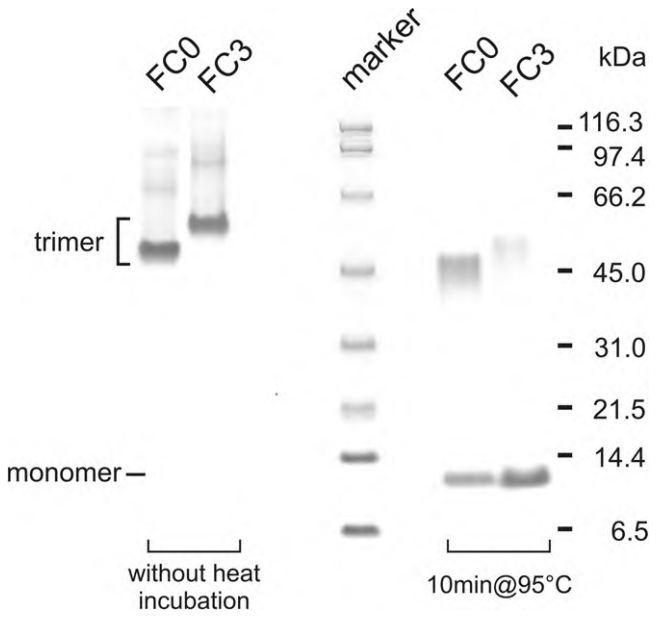

Fig. 2. Tricine-gel electrophoresis of the mini-collagens FC0 and FC3. Two microgram of the mini-collagens were incubated in SDS-containing sample buffer either without (left two lanes) or with (right two lanes) prior heating to 95 °C for 10 min. After cooling, the samples were applied to an 8% polyacrylamide gel with a tricine running buffer according to Schägger and von Jagow (1987). The gel was stained with Coomassie dye. The molecular masses of marker proteins are indicated on the right side of the gel.

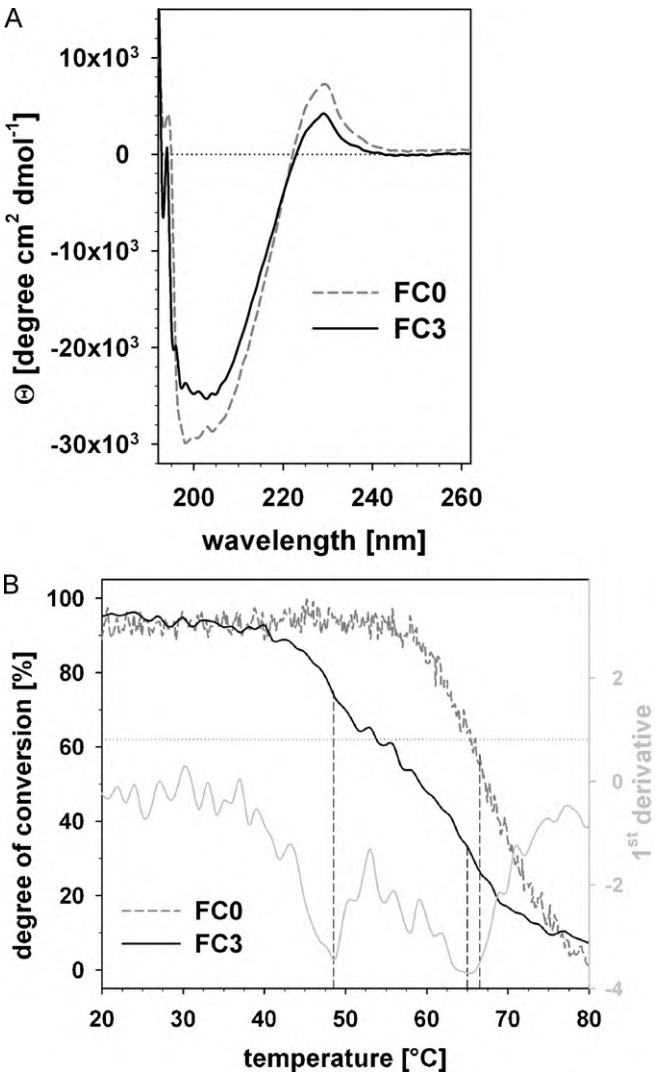

Fig. 3. Triple-helicity of the mini-collagens (A) and thermal stability of their triple helix (B). (A) CD spectra of FC0 (dashed line) and FC3 (solid line) at a chain concentration of 9 μM were recorded in PBS, pH7.4 at 25 °C. The molar ellipticity minimum and maximum at around 195 nm and 225 nm, respectively, are typical for a collagenous triple helix conformation. (B) The melting profiles of both mini-collagens, FC3 (solid line) and FC0 (dashed line) were determined at 225 nm at a chain concentration of 15 μM. Spectra and melting profiles were acquired at least four times. Data points represent the average of five buffer-corrected measurements of a representative acquisition. The horizontal reference line separates the two denaturation phases of FC3. The vertical lines denote the turning point of the FC0 melting curve and the two melting points of FC3, as deduced by the 1st derivative of its conversion curve (gray solid line, right axis). The 1st derivative had been smoothed by averaging the values in a temperature range of 5.0 ± 0.1 °C.

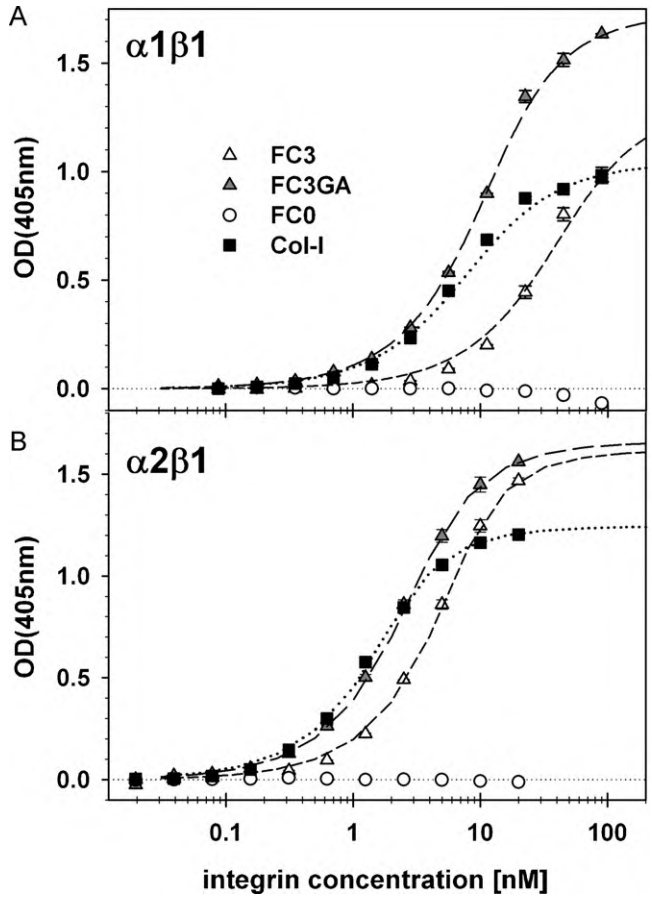

Fig. 4. Both α1β1 and α2β1 integrin bind to FC3 but not to FC0. Binding of soluble α1β1 (A) and α2β1 (B) integrin ectodomains to immobilized FC3, either without (open triangles) or with (filled triangles, FC3GA) prior glutaraldehyde treatment, and to FC0 (open circles), and to wild-type collagen-I (filled squares), was measured in the presence of either 1 mM Mn^{2+} ions or 10 mM EDTA. The β1 integrin-activating antibody 9EG7 was added to α2β1 integrin, but was not required for full activation of α1β1 integrin. Bound integrin was quantified in an ELISA, in which the conversion of the alkaline phosphatase substrate *p*-nitrophenyl-phosphate was measured at 405 nm. The values measured in the presence of 10 mM EDTA were subtracted from values measured in the presence of Mn^{2+} and 9EG7 to obtain the integrin-specific, divalent cation-dependent interaction signal OD405 nm. The data were approximated by regression curves using the following affinity constants which were calculated with a linearization algorithm of optical titration curves according to Heyn and Weischet (1975): K_d-values for α1β1 and α2β1 integrin for the titrations of FC3 (short dashed line): 19.3 ± 7.4 nM and 1.4 ± 0.1 nM, respectively, of FC3GA (long dash line): 4.8 ± 0.1 nM and 1.1 ± 0.03 nM, respectively, and of collagen-I (dotted line): 5.2 ± 0.3 nM and 0.62 ± 0.01 nM, respectively. The titrations were performed in at least three independent experiments with duplicates.

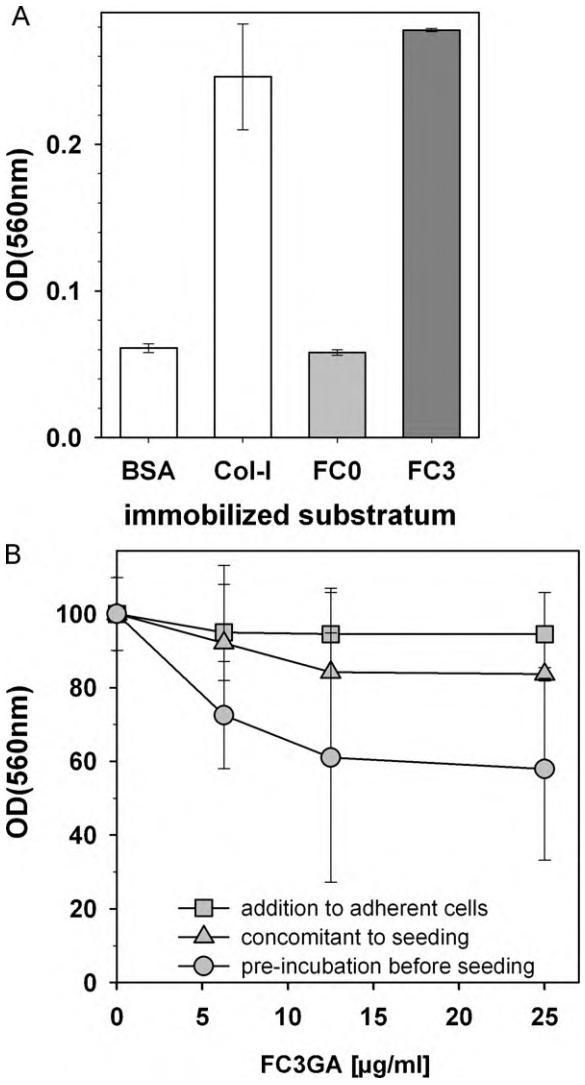

Fig. 5. Adhesion of HT1080 fibrosarcoma cells to mini-collagens (A) and the inhibitory potential of soluble mini-collagens on cell adhesion (B). (A) HT1080 cells were allowed to adhere to wells coated with BSA, collagen-I, and the mini-collagens, FC0 and FC3, for 20 min. Immobilized FC3, but not FC0 supported cell adhesion similarly to collagen-I. (B) HT1080 cells were plated on collagen-I coated wells. Subsequently, FC3 molecules with glutaraldehyde-stabilized triple helix (FC3GA) were added to already adherent cells, or to suspended cells during their adhesion, or to suspended cells, which had been pre-treated with FC3GA for 20 min. After 20 min, non-adherent cells were washed off. Adherent cells were quantified with crystal violet staining in both (A) and (B). Data are means ± S.D. of triplicate measurements of a representative experiment performed three times.

FC0 host triple helix for integrin binding. As the insertion of the α2β1 integrin recognition site seemed to destabilize the host triple helix, we tested whether chemical cross-linking of the three FC3 chains with glutaraldehyde would increase the integrin binding signals. Indeed, cross-linked FC3 (termed FC3GA) showed higher binding signals than the non-modified FC3 construct (K_d values of FC3GA: 4.8 ± 0.1 nM and 1.1 ± 0.03 nM for α1β1 and α2β1 integrin, respectively). The binding signals and the approximated affinity constants almost reached those obtained with native collagen I with K_d values of 5.2 ± 0.3 nM and 0.62 ± 0.01 nM for α1β1 and α2β1 integrin, respectively (Fig. 4). Obviously, treatment of FC3 with glutaraldehyde increased its triple-helical stability without compromising α2β1 integrin binding. Integrin α2β1 bound better to both collagen-I and glutaraldehyde-stabilized FC3 than α1β1 integrin (Fig. 4A vs. B). The preference of integrin binding was even more pronounced for non-cross-linked FC3, which was recognized by α2β1 integrin with much higher affinity (K_d = 1.4 ± 0.1 nM) than by α1β1 integrin (K_d = 19.3 ± 7.4 nM). Therefore, albeit not an exclusive α2β1 ligand, non-cross-linked FC3 showed a preference for α2β1 integrin over α1β1 integrin.

Next, we tested the biological activity of the recombinant mini-collagen FC3 at the cellular level. To this end, HT1080 cells, which are characterized by high α2β1 integrin expression, were allowed to adhere to immobilized FC3. Similar binding was detected to FC3 and collagen-I, while cells did not adhere to FC0 (Fig. 5A). The adhesion signal to FC0 approached background levels like to BSA-blocked wells. Since adhesion of HT1080 cells to FC3 was as high as to collagen-I, we asked whether adhesion of HT1080 cells to collagen-I would be inhibitable by soluble mini-collagen FC3 (Fig. 5B). To warrant triple-helicity of FC3 at 37 °C in solution, we used glutaraldehyde-cross-linked FC3 (FC3GA) molecules. Interestingly, cells which had adhered to collagen-I could not be detached with FC3GA, whereas FC3GA reduced attachment of suspended

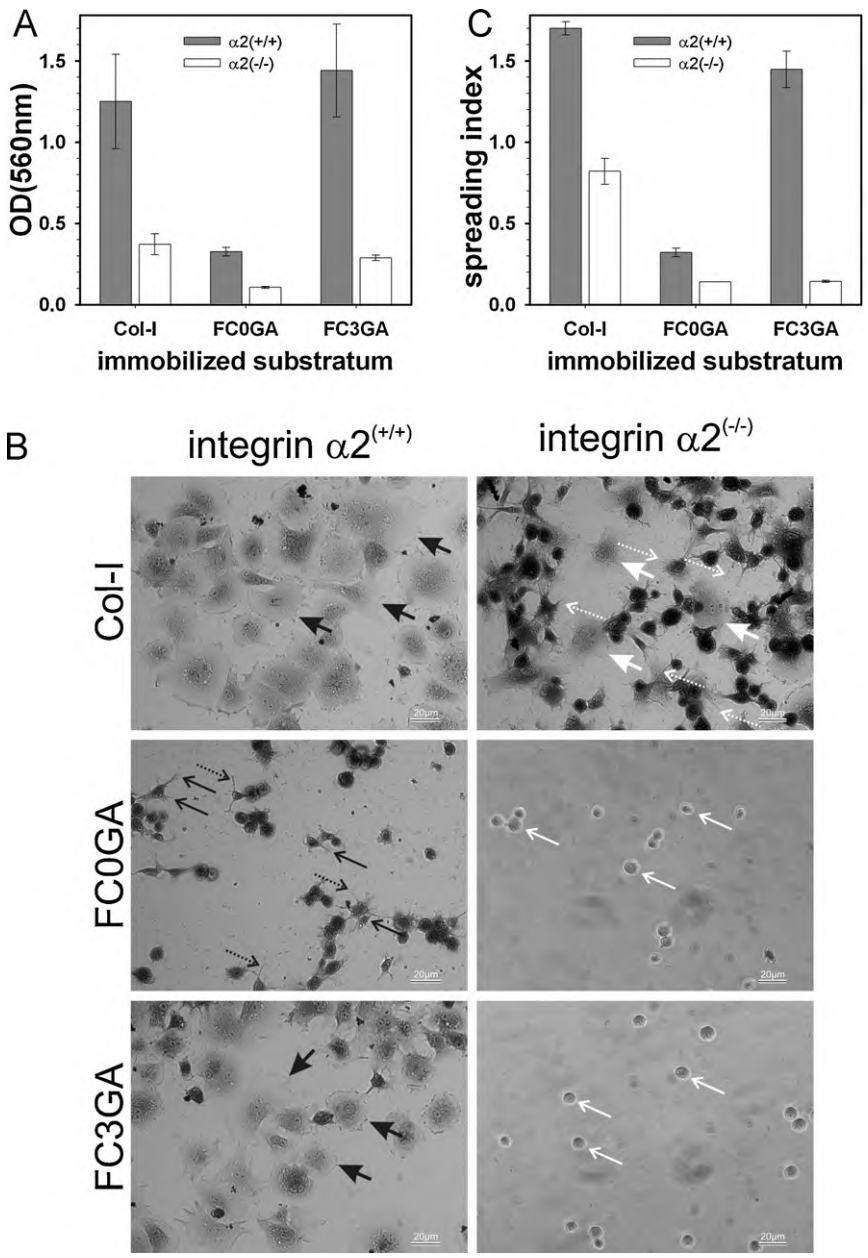

Fig. 6. Skin fibroblasts from wild-type and integrin α2-deficient mice show differences in adhesion (A), morphology (B) and spreading index (C) on collagen-I, and the mini-collagens FC0 and FC3. Primary skin fibroblasts from wild-type (integrin α2(+/+)) (filled bars) and integrin α2-deficient (integrin α2(−/−)) (open bars) animals were plated on collagen-I and the glutaraldehyde (GA)-treated mini-collagens, FC0GA and FC3GA. Cell attachment to the different substrates was quantified with crystal violet staining (A). Subsequently, morphologies of the cells attached to these different substrata in selected fields were visualized by light microscopy at a magnification of 20× (left column: wild-type cells, right column: integrin α2-deficient cells; arrows are explained in the text) (B). The morphological differences were quantified as spreading index, i.e. cell-covered area per cell number, for both wild-type (filled bars) and integrin α2-deficient (open bars) cells on collagen-I and the GA-treated mini-collagens FC0 and FC3 (C). Data are means ± S.D. of triplicate measurements of a representative experiments carried out three times.

cells to collagen-I when it was administered during adhesion or 20 min prior to plating (Fig. 5B). This effect was consistently observed at different concentrations of the mini-collagen.

To test for the receptor specificity of cell attachment to FC3, we compared the adhesion of primary dermal fibroblasts, which had been isolated from the skin of integrin α2-expressing and -deficient mice (Fig. 6A). Quantification of adherent cells showed that integrin α2β1 expressing cells attached with a similar signal to both collagen-I and FC3GA, whereas the adhesion signal to FC0GA approached background values. Compared to wild-type skin-fibroblasts, integrin α2-deficient cells showed similar attachment signals to the three different substrates, however at a lower signal level (Fig. 6A). Morphologically, the differences were more striking (Fig. 6B). α2β1 integrin expressing fibroblasts (Fig. 6B, left column and Fig. 6C) spread intensively with fully extended lamellipodia on both collagen-I and the mini-collagen FC3GA (bold black arrows in Fig. 6B), but on FC0GA they showed only minimal cell protrusions with scarce and tiny lamellipodia (thin black arrows) and filamentous protrusions (dotted black arrows). Integrin α2(−/−) fibroblasts showed a certain degree of cell spreading on collagen-I with lamellipodia (bold white arrows) and long filopodia-like protrusions (dotted white arrows). In contrast, only poor adhesion and virtually no flattening of the cells was observed on either FC3GA or FC0GA (thin white arrows in Fig. 6B, right column and Fig. 6C). This latter observation strongly suggested that in integrin α2-deficient fibroblasts other collagen-binding integrins, such as

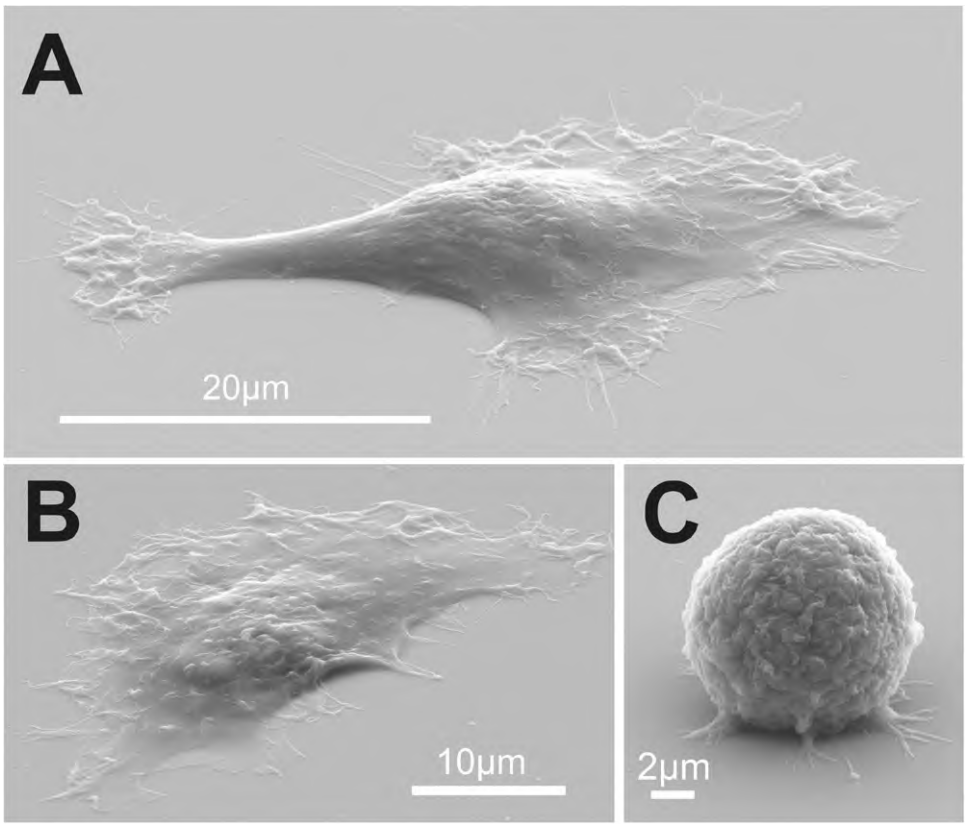

Fig. 7. Mini-collagen FC3, but not FC0, induces changes in cell morphology similar to collagen-I. Collagen-I (A), the mini-collagen FC3 (B) and FC0 (C) were immobilized as substrates for HT1080 cell adhesion. After adhesion for 2 h, cells were fixed and visualized by SEM.

α1β1 or α11β1 (Zhang et al., 2006), only compensate for the loss of α2β1 integrin with respect to adhesion, but apparently fail to compensate and exert forces to the substratum as α2β1 integrin does, and hence fail to induce or sustain cell spreading.

To further analyze the morphological changes of cells on FC3, we visualized adherent HT1080 by SEM (Fig. 7). On collagen-I, HT1080 cells showed the typical appearance of migrating cells with a leading front (towards the right in Fig. 7A) and a trailing end at opposite poles. Filopodia were seen as membrane spikes, whereas lamellipodia formation was accompanied by intense cell spreading. On FC3 (Fig. 7B), cells adopted a similarly flattened, yet less polarized appearance. Pronounced lamellipodia formation showed an integrin-induced cytoskeletal rearrangement. Therefore, cells attached firmly to both collagen-I and FC3. In contrast, the few HT1080 cells, which had remained attached to the FC0-coated surface, entirely lacked lamellipodia, failed to rearrange their cytoskeleton, did not spread and hence showed an almost spherical shape (Fig. 7C). Consequently, the absence of any cellular interaction with FC0 led to the removal of almost all cells from the surface upon washing.

To assess integrin-mediated signaling from the matrix into the cell, we analyzed the recruitment of α2β1 integrins into focal adhesions as one of the early steps in integrin signaling. Again, HT1080 cells spread well on collagen-I and FC3, and formed vinculin- and integrin α2β1-positive focal adhesions, but remained spherical on FC0 (Fig. 8). Interestingly, there are some slight differences in the number and distribution of focal adhesions on collagen-I and FC3. On FC3, cells tend to form slightly less focal adhesions, which are preferentially concentrated at the front of several pronounced lamellipodia. However, a leading edge pointing into the direction of movement is hardly established. Moreover, although cells move with the same velocity on both FC3 and collagen-I, they show a reduced persistence in the migratory direction and rather crawl back and forth. In contrast, on collagen-I, cells tend to from more focal adhesions, which are distributed along a clearly recognizable leading edge. Furthermore cells kept their direction of migration more persistently on collagen-I.

The recruitment of α2β1 integrins into focal adhesions demonstrated that, first, the triple-helical α2β1 integrin recognition motif within the short FC3 molecule is fully competent to elicit integrin-mediated signals that trigger cytoskeletal rearrangements and, second, that this mini-collagen is a potent α2β1 integrin agonist with an efficiency that is similar to authentic collagen-I.

3.3. Assessment of intermolecular forces between α2β1 integrin and FC3

Cells exert forces via their integrins to subjacent matrix proteins, leading to morphological changes of cells and to remodeling of the ECM. The integrin–matrix interactions have to withstand these cellular forces to avoid detachment of the cells and tissue disintegration. We employed FC3 as a tool to measure molecular forces between α2β1 integrin and its collagenous recognition site by using atomic force microscopy (AFM). To this end, immobilized α2β1 integrin was probed with a cantilever which had been covalently functionalized with glutaraldehyde-stabilized FC3. Force spectra were recorded in the presence of either Mg^{2+} and Mn^{2+} ions or EDTA (Figs. 9 and 10). Two representative force–distance curves are shown in Fig. 9. Intense interactions occurred in the presence of divalent cations (Fig. 9A), whereas EDTA strongly reduced the number of binding interactions (Fig. 9B). In more than 1000 recordings, we obtained more than 920 analyzable spectra for each condition. The histogram of the rupture forces measured in the presence of Mg^{2+} and Mn^{2+} ions (Fig. 10, black bars) could be approximated with a double Gaussian normal distribution, representing two populations. The low force peak at 77 ± 23 pN is similar

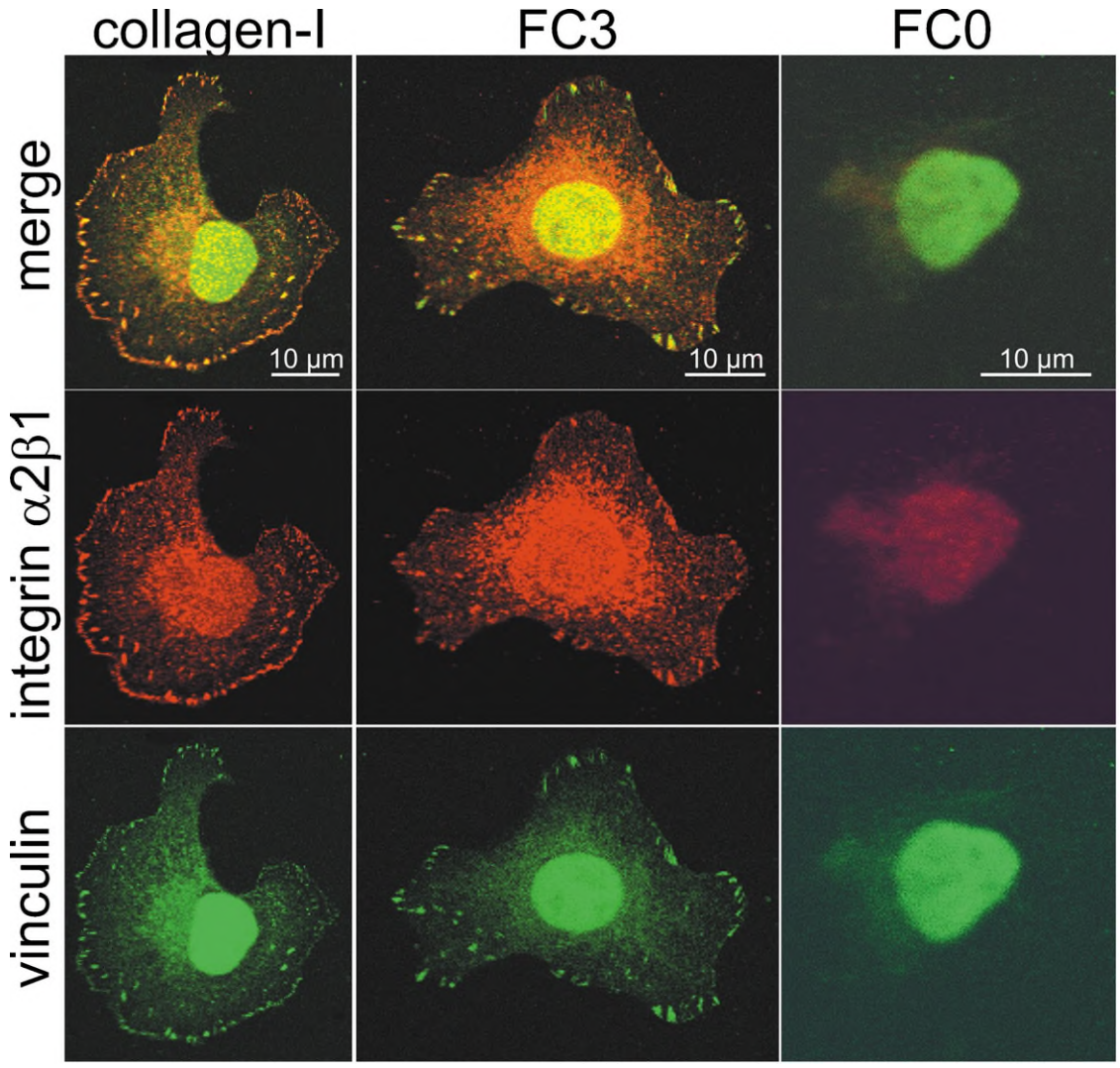

Fig. 8. FC3 agonistically induces the recruitment of α2β1 integrins into focal adhesions. HT1080 cells were allowed to spread for 2 h on chamber slides, which had been coated with collagen-I (10 μg/ml) or the mini-collagens, FC3 and FC0 (each 20 μg/ml). Cells were fixed, stained for α2β1 integrin and vinculin, a marker for focal adhesions, and analyzed by confocal laser scanning microscopy.

to the maximum of the single peak-force histogram at 59 ± 20 pN, measured in the presence of EDTA (Fig. 10, white bars), and is therefore considered as non-specific background. In the presence of divalent cations, we observed a population of integrin–ligand interactions rupturing at (159 ± 56) pN. This represents the rupture force for the specific, divalent cation-dependent interaction of α2β1 integrin with its recognition site within the mini-collagen FC3.

4. Discussion and conclusion

Chemically synthesized collagen molecules have mainly been used to analyze the structural requirements of triple helix stability and the mechanism of collagen binding to various cellular receptors and ECM proteins (reviewed in Brodsky et al., 2008; Farndale et al., 2008). Here, we describe the recombinant production of a biofunctionalized triple-helical mini-collagen FC3 to be used as an alternative to native collagen-I. Based on the excellent work of Engel and co-workers, we utilized the expression construct, in which three collagen chains consisting of ten GPP repeats are trimerized by a foldon domain (Frank et al., 2001). We inserted the α2β1 integrin recognition sequence, GFPGER (Knight et al., 2000), in the middle of the collagen host sequence, which forces the inserted hexapeptide sequences into a triple-helical conformation. The insertion sequence theoretically reduces the melting temperature of the collagen triple helix from 44 °C to 34 °C, as calculated by the Collagen Stability Calculator (http://compbio.cs.princeton-edu/csc/) (Persikov et al., 2005). Circular dichrosim measurements confirmed the triple-helical conformation of FC3. However, the insertion of the six amino acids likely separated the triple-helical FC3 construct into two entities with different thermal stabilities. While the second triplet GER of the insertion sequence contributed to the triple-helical stability similar to a GPP triplet sequence, the first triplet GFP with the phenylalanine in the X-position remarkably destabilized the triple helix (Persikov et al., 2005), in line with crystallographic studies (Emsley et al., 2004). The C-terminally adjacent foldon domain stabilized the five GPP-repeat long triple helix between the foldon domain and the insertion site. This foldon-proximal portion of the FC3 triple helix melted at a temperature of 65 °C, similar to the FC0 construct with its homogenous GPP-triple helix. The foldon-distal part of the host triple helix of FC3 showed a lower melting temperature of 48 °C. The different melting properties resulted in a biphasic melting profile of FC3 in contrast to the monophasic melting curve of FC0 with its uniform GPP-repeats. Connecting the three collagen chains by glutaraldehyde cross-linking stabilized the recombinant mini-collagen resulting

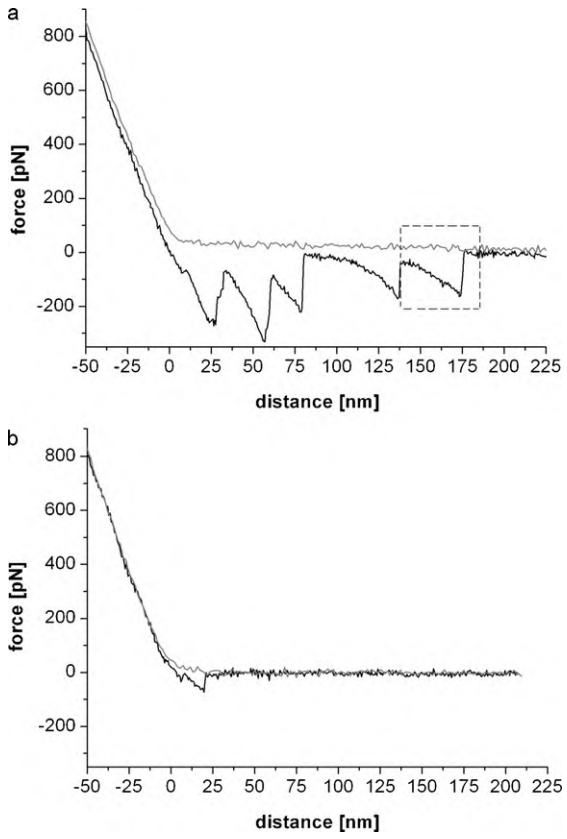

Fig. 9. Representative force–distance curves for the interaction between α2β1 integrin and mini-collagen FC3 in the presence (A) and absence (B) of divalent cations. The interaction of a FC3-coated cantilever and α2β1 integrin-coated substratum was recorded as force–distance curve in the presence of 2 mM Mg^{2+} and 1 mM Mn^{2+} ions (A) or 10 mM EDTA (B) for every retraction cycle. The gray curve is the trace curve when the cantilever approaches the substratum, while the retrace curve (black line) represents the pulling event. The ultimate rupture event of the retrace curve (indicated as dashed box in (A)) was used to determine the interaction between single molecules. For each condition, up to 1000 force–distance curves were acquired, and the measurements were performed three times.

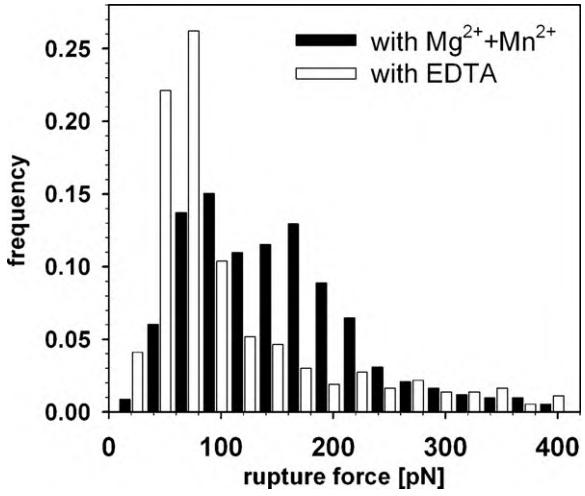

Fig. 10. Molecular forces between α2β1 integrin and the mini-collagen FC3 as determined by AFM. Recombinant α2β1 integrin was immobilized on a plastic slide as described in Section 2. FC3 was chemically fixed to the tip of an AFM cantilever and its triple helix stabilized by glutaraldehyde treatment. The cantilever tip was repetitively approached and removed from the α2β1 integrin-coated slide in the presence of 2 mM Mg^{2+} and 1 mM Mn^{2+}-ions (n = 922, black bars) or of 10 mM EDTA (n = 974, white bars) at a speed of 500 nm/s. Rupture forces of a representative experiment (n = 3) were determined from the cantilever deformation curves and binned into classes with a width of 25 pN.

in a very stable trimer which did not melt below 65 °C (data not shown).

Our mini-collagen FC3 was fully active in binding to the α2β1 integrin both at the molecular and cellular level. Remarkably, α2β1 integrin interacted with the chemically stabilized mini-collagen FC3 with a similar affinity to tissue-derived collagen-I. Moreover, when immobilized, it acted as an agonist of α2β1 integrin-dependent cell functions, such as cell spreading and lamellipodia formation, to nearly the same extent as the collagen-I molecule. Interestingly, the GFPGER-sequence presented in the non-hydroxylated triple-helical environment bound to α2β1 integrin with a remarkable specificity. Although the prolyl-hydroxylated GFOGER was reported as binding site not only for α2β1 integrin, but also for the other collagen-binding integrins, α1β1 and α11β1 (Knight et al., 2000; Zhang et al., 2003), the latter integrins failed to compensate functionally for the loss of α2β1 integrin, when integrin α2-deficient mouse skin fibroblasts were plated on non-hydroxylated FC3. Fibroblasts from α2-knockout mice adhered and spread on collagen-I, indicative of the presence of compensating collagen receptors, presumably α1β1 and α11β1 integrins (Zhang et al., 2006; Gullberg, 2009). Yet, these integrins were unable to transmit forces sufficiently strong to induce spreading of integrin α2-deficient fibroblasts on FC3. Obviously, non-hydroxylated FC3 represents a higher affinity substrate for α2β1 integrin than for the other collagen-binding integrins. This is in contrast to previous studies using prolyl-hydroxylated synthetic peptides (Knight et al., 2000; Zhang et al., 2003). Therefore, the different collagen-binding integrins seem to have differential requirements for prolyl hydroxylation in their ligands, which is in line with our previous observations (Perret et al., 2003). Thus, α2β1 integrin binds to its collagenous recognition sequence irrespective of prolyl-hydroxylation of the collagen triple helix, whereas α1β1 integrin needs a prolyl-hydroxylated collagen ligand. The fact, that α1β1 integrin-bearing RuGli cells largely failed to spread on FC3 (data not shown), further corroborated this hypothesis. However, prolyl hydroxylation may also affect the preferences for different integrins more indirectly by stabilizing the triple-helical conformation (Brodsky et al., 2008). Chemical cross-linking the three collagen chains, which stabilized the mini-collagen triple helix independently of prolyl hydroxylation, increased the affinity of FC3 for α1β1 integrin five-fold, whereas its affinity for α2β1 integrin remained almost unaltered. Therefore, non-hydroxylated, non cross-linked FC3 showed a much higher preference for α2β1 than for α1β1 integrin. Moreover, as mini-collagen FC3 does not bear any known recognition sites for other cellular receptors or matrix proteins, our studies on the recruitment of α2β1 integrin into focal adhesions indicated that the triple-helical integrin recognition site is fully sufficient for α2β1 integrin-triggered cell spreading. Additional co-signaling events, such as the syndecan–integrin α5β1 cooperation in fibronectin-induced cell spreading (Humphries et al., 2005), were not required. Nevertheless, it cannot be ruled out that collagen-I may affect other cell reactions, such as migration, differently than FC3. Partially aggregated into fibrils, collagen-I could bind to the cell culture plastic more efficiently than FC3. This could explain the directionally more persistent cell migration and formation of lamellipodia on collagen-I.

In contrast to native collagen-I, the mini-collagen FC3 does not form insoluble and supramolecular aggregates. This feature allowed us to analyze single molecule interactions between FC3 and α2β1 integrin by AFM. Previous studies on the interaction forces of cantilever-bound single cells with immobilized collagen-I had shown that the cellular interaction of α2β1 integrin with collagen-I is a multi-step process, which is influenced by several parameters (Taubenberger et al., 2007; Tulla et al., 2008). Initially, only a single α2β1 integrin establishes contact with collagen-I, while at a later time multiple integrin receptors are activated and recruited

into focal contacts, so that they cooperatively exert higher forces (Taubenberger et al., 2007; Tulla et al., 2008). Our studies allowed the detailed analysis at the molecular level of this initial cell–matrix contact, in which an isolated single α2β1 integrin binds to one mini-collagen molecule. Moreover, in our experimental setting, additional cellular effects, such as integrin activation (Tulla et al., 2008), cytoskeleton rearrangement (Grashoff et al., 2010) and plasmalemma tension, did not occur. At comparable loading rates, the rupture force to detach α2β1 integrin from its collagen ligand is higher than that of other integrins and their non-collagenous ligands (reviewed in Weisel et al., 2003), but similar to the forces of cell-anchored, non-activated α2β1 integrin (Tulla et al., 2008). This underlines the importance of α2β1 integrin-mediated force transmission between cells and their surrounding force-bearing collagen network. As these forces contribute to the interstitial fluid pressure, which plays an important role in transcapillary fluid flow (Reed and Rubin, 2010), the force-transmitting α2β1 integrin may be an important target to treat edema formation and to normalize tissue pressure in pathological conditions. Moreover, α2β1 integrin may be targeted to treat dysregulated force exertion of fibroblasts in fibrotic diseases (Eckes and Krieg, 2004).

In conclusion, the recombinant production of a mini-collagen FC3, which harbors a single α2β1 integrin binding site in a triple-helical collagenous conformation, provides an ideal tool to study α2β1 integrin-mediated cell contacts at both the molecular and cellular level. Unlike collagen-I, it is a small and soluble molecule, which does not form supramolecular and insoluble complexes. Moreover, it lacks contact sites for other cellular receptors or matrix molecules, endowing it with a specificity for collagen-binding integrins and a preference for α2β1 integrin. Due to the lack of hydroxylated prolyl-residues, it is unable to activate platelets via GP VI, which induces platelet activation and thrombosis along with α2β1 integrin (Farndale et al., 2004). However, when used as soluble agent, the mini-collagen FC3 was unable to effectively block α2β1 integrin-mediated cell adhesion, which plays a role in metastasis (Rosenow et al., 2008), thus limiting its applicability in tumor therapy (Schmidmaier and Baumann, 2008). By contrast, our studies imply its suitability to biofunctionalize surfaces of biomedical materials, which need to be tightly connected with the surrounding cells in a force-resistant manner. The α2β1 integrin-mediated linkage is able to withstand high mechanical forces. Moreover, our results on the force load for an individual α2β1 integrin sheds light on the less well understood biomechanical function of α2β1 integrin at the molecular level.

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