Asparagine deamidation at the NGR sequence in the 5th type I repeat of fibronectin (FN-I5) generates isoDGR, an αvβ3 integrin-binding motif regulating endothelial cell adhesion and proliferation. By NMR and molecular dynamics studies, we analyzed the structure of CisoDGRC (isoDGR-2C), a cyclic β-peptide mimicking the FN-I5 site, and compared it with NGR, RGD, or DGR-containing cyclopeptides. Docking experiments show that isoDGR, exploiting an inverted orientation as compared with RGD, favorably interacts with the RGD-binding site of αvβ3, both recapitulating canonical RGD-αvβ3 contacts and establishing additional polar interactions. Conversely, NGR and DGR motifs lack the fundamental pharmacophoric requirements for high receptor affinity. Therefore, unlike NGR and DGR, isoDGR is a new natural recognition motif of the RGD-binding pocket of αvβ3. These findings contribute to explain the different functional properties of FN-I5 before and after deamidation, and provide support for the hypothesis that NGR → isoDGR transition can work as a molecular timer for activating latent integrin-binding sites in proteins, thus regulating protein function.

A number of cellular interactions with the extracellular matrix (ECM) are mediated by fibronectins, which are large adhesive glycoproteins (~450 kDa) involved in several key processes, including embryogenesis, angiogenesis, inflammation, hemostasis, thrombosis, and tissue repair (1, 2). Fibronectins are soluble elements of plasma and other body fluids, as well as constituents of the insoluble ECM (2, 3). Human fibronectin (FN), which is typically composed of two almost identical subunits connected covalently by disulfide bonds at their C termini, is thus an abundant and ubiquitous ECM protein present in about 20 isoforms and consisting primarily of three types of repeating modules (FN-I, FN-II, and FN-III) (2–5). Distinct FN modules contain varying binding sites for several different molecules, including sulfated glycosaminoglycans, syndecans, DNA, gelatin, heparin, and fibrin (2, 3, 6). In addition, fibronectins contain binding sites for about half of the known cell surface integrin receptors (7, 8). Integrin receptors are present in many animal species, ranging from sponges to mammals (9), and play essential roles in cellular physiology (attachment, migration, proliferation, differentiation, and survival) and in disease (cancer, tumor metastasis, immune dysfunction, ischemia-reperfusion injury, viral infections, osteoporosis, and coagulopathies) (4, 10).

In this context, it is worth noting that FN-I5 (i.e. the 5th FN-I repeat) and FN-I7 modules contain a GNNGRG loop that is conserved in human, bovine, murine, rat, bird, amphibian, and fish fibronectin, suggesting that this loop is functionally important (11). In addition, we have recently shown that the deamidation of Asn263 at the Asn-Gly-Arg (NGR) sequence of FN-I5 and of peptides containing the NGR motif generates isoDGR (isoAsp-Gly-Arg), a novel cell adhesion motif binding to αvβ3 integrin (12). Consistently, cells from homozygous knock-in mice carrying the RGD → RGE mutation in fibronectin exhibited normal fibronectin fibril assembly, both in vitro and in vivo, exploiting the presence in the FN-I5 repeat of a NGR → isoDGR motif binding to αvβ3 (13). Integrin αvβ3 is a relevant receptor in tumor angiogenesis and metastasis, viral infections, inflammation, and bone resorption (8, 14), and its ligands contain the Arg-Gly-Asp (RGD) sequence (15, 16). Importantly, a cyclic β-peptide containing the CisoDGRC motif (isoDGRC-2C) is a competitive antagonist of RGD-containing ligands of αvβ3 and inhibits endothelial cell adhesion, proliferation, and tumor growth (12). Furthermore, analysis of competitive binding plots of RGD-2C and isoDGRC-2C shows that both ligands have comparable binding affinity for αvβ3, whereas DGR-2C and NGR-2C cyclopeptides show a >2 order of magnitude
lower affinity (12), pointing to stereospecific isoDGR-αβ3 interactions.

The structural determinants dictating the interaction between isoDGR and αβ3 are still unknown. To gain a structural insight into the different αβ3 recognition mechanisms, we analyzed the conformation in solution of four ligands containing either the isoDGR, RGD, NGR, or DGR motifs (Fig. 1 and supplemental Fig. S1), and we created putative binding models of the four ligands with αβ3 based on the crystallographic structure of αβ3 in its “RGD-bound” conformation (15). Consistently with functional data, our docking studies show that the isoDGR motif can perfectly mimic the canonical RGD interactions with αβ3, whereas NGR and DGR lack the stereochemical and electrostatic requirements for a correct recognition of the αβ3-binding pocket.

**EXPERIMENTAL PROCEDURES**

Cell Line and Reagents—EA.hy926 cells (human endothelial cell fused with human lung carcinoma A549) were cultured as described previously (17). Human αβ3 was from Immunological Science (Rome, Italy), and streptavidin peroxidase was from Società Prodotti Antibiotici (Milan, Italy). NGR-TNF and FN-1 were prepared as described previously (12).

Preparation and Characterization of Synthetic Peptides—CRGDCGVRY (RGD-2C), CDDRCGVR (DGR-2C), CNGRCGVR (NGR-2C), and CisoDGRCGVR (isoDGR-2C) were prepared as described (12). The biotinylated peptides CNGRCGVRSSRTPSDKYGK-bio and CARACGVRSSHRTPSDKYGK-bio (called bio-CNGRC-hTNF-(1–11) and bio-CARAC-hTNF-(1–11)) consist of CNGRCG or CARACG fused to the N-terminal sequence of human tumor necrosis factor (hTNF)-α followed by a Tyr, to enable detection, and a biotinylated Lys. These peptides were prepared and purified as described (11). Deamidation of bio-CNGRC-hTNF-(1–11) was obtained by diluting bio-CNGRC-hTNF-(1–11) in 0.1 M ammonium bicarbonate buffer, pH 8.5, and incubating for 16 h at 37 °C. The resulting β-peptide was called “heat-treated” bio-CNGRC-hTNF-(1–11). All peptides were dissolved in sterile water and stored in aliquots at −20 °C. The molecular mass of each peptide was checked by matrix-assisted laser desorption ionization-time-of-flight analysis.

Binding of Peptides to αβ3 Integrin—The binding of biotinylated peptides (bio-CNGRC-hTNF-(1–11), heat-treated bio-CNGRC-hTNF-(1–11) at 37 °C, and bio-CARAC-hTNF-(1–11)) to αβ3 integrin immobilized on microtiter plates was analyzed as described previously, using streptavidin peroxidase complexes (12). These complexes were prepared by mixing various amounts of biotinylated peptides (4 to 0.062 μg) in phosphate-buffered saline with Ca²⁺ and Mg²⁺ (DPBS, Cambrex) containing 3% bovine serum albumin with 0.03 units of streptavidin peroxidase (binding capacity 1 μg of biotin/unit of streptavidin peroxidase, final volume of 15 μl). Complexes were diluted in 3% bovine serum albumin/DPBS (1:300), added to microtiter plates coated with purified human αβ3 integrin (0.5 μg/ml), and incubated for 2 h at room temperature. After washing with DPBS, bound peroxidase was detected by chromogenic reaction with o-phenylenediamine.

Cell Adhesion Assay—The effect of peptides on EA.hy926 cell adhesion was investigated by seeding EA.hy926 cells (7.5 × 10⁴) in 96-well flat bottom plates in the presence of increasing concentration of ligands (NGR-2C, RGD-2C, isoDGR-2C, and DGR-2C). After 4 h of incubation at 37 °C, nonadherent cells were washed out. The amount of adherent cells was measured.
NMR Experiments and Structure Calculations—For each ligand NMR spectra of an ∼5 mM sample (90% H2O, 10% D2O) at pH 3 were recorded at 280 K on a Bruker Avance-600 spectrometer (Bruker BioSpin) equipped with a triple-resonance TCI cryoprobe with an x, y, z shielded pulsed-field gradient coil. The experiments were performed at acidic pH to avoid both signal loss because of high exchange rates at neutral pH and deamidation of the asparagine in the NGR-2C peptide (12). Proton resonances were assigned by conventional two-dimensional experiments as follows: total correlation spectroscopy ($t_{mix} = 60$ ms), nuclear Overhauser effect spectroscopy (NOESY), and rotational nuclear Overhauser effect spectroscopy ($t_{mix} = 100–400$ ms) (19). Cross-peaks intensities were measured from NOESY spectra at 200 ms. No differences were observed in the experiments at higher mixing times. Water proton signals were suppressed with excitation sculpting sequence (20).

All resonances of the four ligands have been assigned with the only exception of the amide proton of C1, which has a higher exchange rate with the solvent even at low pH (Biological Magnetic Resonance Data Bank accession codes: RGD, 1928339, isoDGR, 38926841; NGR, 32415285; and DGR, 52915018). The $^3$J$_{HN-HA}$ coupling constants were obtained directly from the resolved amide proton resonances of well digitized mono-dimensional spectrum. The temperature coefficients of the amide protons were obtained from linear fits of the chemical shift data from mono-dimensional spectra acquired in a temperature range from 280 to 300 K (5 K-increasing steps). Data were processed with NMRPipe (21) and analyzed using the NMRView software (22).

Structure Calculations—Structures were calculated using ARIA 1.2, ambiguous restraints for iterative assignment (23), in combination with CNS 1.2, crystallography, and NMR systems (24) using only manually assigned NOEs as experimental restraints. Coupling constants that were in the range between 6 and 8.5 Hz were not used in calculations because their values were suggestive of rapidly interconverting conformers coexisting in solution. To avoid bias in the calculations, hydrogen bonds inferred from temperature coefficients data were not included. These data were only used for structure validation. Calculations were carried out in the simplified all-hydrogen PARALLEDG5.3 force field with nonbonded interactions modeled by PROLSQ force field (22). Parameters for isoaspartic residue were derived from the aspartic residue applying appropriate dihedrals and improper angles. A total of eight iterations (200 structures per iteration) were performed. The ARIA default water refinement was performed on the 30 best structures of the final iteration. The stereochemical quality of the structures was assessed with PROCHECK-NMR program (25).

In the case of isoDGR-2C, residue 2 was excluded from torsion angle check. However, its torsion angle compared well with $\varphi$ and $\psi$ angles of isoaspartic residues measured in deposited x-ray structures (Protein Data Bank codes 1AT6, 1DY5, 1RTU, 2FI5, 2FTM, 1C9P, 1LSQ, 2FI4, and 2FTL).

Molecular Dynamics Simulations—Simulations were performed on the lowest energy NMR structures of each ligand using the GROMACS 3.3.1 package (26) with the optimized parameters for liquid simulation (OPLS) force field (27). All trajectories were calculated in periodic cubic boxes (5 × 5 × 5 nm) of explicit SPC water molecules (28).

The system was neutralized by 1–2 chloride ions according to the charge of the ligand. Bond lengths were constrained using the LINCS algorithm (29), and Lennard-Jones interactions were calculated with a 0.9-nm twin-range cutoff. Full electrostatic potentials were computed using the PME method (30) with a cutoff of 0.9 nm. The system was first minimized using steepest descent algorithm and then equilibrated at 300 K for 100 ps under NPT (number of molecules, volume, temperature) and periodic boundary conditions. REMD simulations were performed with 16 replicas run in parallel at the following temperatures from 293 to 353 K with 4 K-increasing steps. The temperatures were chosen so as to maintain an exchange rate of 5–10%. Transitions between adjacent temperatures were attempted every 500 MD steps (1 ps) using a Metropolis transition probability (31), which gives a probability of exchange between two replicas $i$ and $j$: $P(i,j) = \exp(-(\beta_i - \beta_j)(E_i - E_j))$, where $\beta = 1/k_BT$; $E$ is the potential energy of the system; $T$ is the absolute temperature, and $k_B$ is Boltzmann’s constant. Each replica was simulated for 2 ns, with an integration time step of 0.002 fs under NVT (number of molecules, volume, temperature) conditions, yielding a total sampling time of 32 ns. Configurations were saved prior to every attempted transition, leading to an ensemble at each temperature containing 2000 structures. The full trajectories were clustered over the backbone atoms of the macrocycle and the disulfide bridge using the GROMOS algorithm g_cluster (32) as implemented in the GROMACS 3.3.1 package with a cutoff of 0.07 nm.

Electrostatic Surface Potential Calculations—The electrostatic potential of the four ligands has been calculated using the adaptive Poisson-Boltzmann solver program (APBS 0.5) (33). The charges (Q) and radii (R) of atoms in the PQR file required by APBS were taken from the OPLS force field. Electrostatic potential was visualized using the PyMOL program (Delano Scientific LLC) with positive potential in blue and negative potential in red in a range between $-5$ and $+5$ kT/e.

Molecular Docking Calculations—Docking calculations of the four ligands on the globular head of the extracellular part of $\alpha v \beta 3$ in its ligand-bound conformation (Protein Data Bank code 1L5G), have been performed using the docking program HADDOCK2.0 (34, 35).

For each ligand an ensemble of the best 30 NMR structures in terms of energy was docked onto $\alpha v \beta 3$. The protocol follows a three-stage docking procedure, which includes the following: (a) randomization of orientations and rigid body minimization, (b) simulated annealing in torsion angle space, and (c) refinement in Cartesian space with explicit water. Ambiguous interaction restraints ($\alpha v \beta 3$: Asp$^{130}$, Asp$^{218}$, Tyr$^{122}$, Arg$^{214}$, Asn$^{215}$ and Arg$^{216}$, ligand, residues 2–4) (35) were derived from the known interactions of the RGD motif of the cyclic pentapeptide in the Protein Data Bank structure 1L5G. OPLS force field was used. During the rigid body docking step 1000 structures were calculated, allowing the ligand to explore solutions rotated by 180°, thus increasing the sampling of the solutions. The best 200 solutions in terms of intermolecular energies were selected for
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a semiflexible simulated annealing in which the side-chains of avβ3 and of the cyclic peptides located at the binding interface (avβ3, residues 148–152, 216–220 on the αv domain and 118–120, 124–126 on β3 domain; ligand, residues 1–9) were allowed to move in a semi-rigid body docking protocol to search for conformational rearrangements. The models were then subjected to a water refinement step (TIP3P model). Backbone and side-chain of amino acids 6–9 were fully flexible in iteration 1 and in the water refinement step. Analogous calculations have been performed using for each ligand an ensemble of conformers composed by the centroids representing the highest populated clusters (80%) identified in the clusters of REMD trajectories (4, 23, 10, and 5 structures for RGD, isoDGR, NGR, and DGR, respectively).

The analysis of the simulations was performed applying in-house Python and Tcl scripts. Root mean square deviation (r.m.s.d.) values were calculated using the ProFit program (available on line). The fitting of the protein was performed on the flexible residues (Table 3) using the McLachlan algorithm (36). The r.m.s.d. values of the cyclopeptides were calculated on the backbone of residues 1–5 and on the sulfur and Cβ atoms of residues 1 and 5. The final r.m.s.d. matrix was then clustered using the algorithm described in Daura et al. (32), where a cluster is defined as an ensemble of at least two conformations displaying a r.m.s.d. smaller than 1 Å. In the case of NGR-2C, the cut off was increased to 1.3 Å.

The final structures after water refinement were clustered and scored using a combination of energy terms defined as follows: 1.0 × E_vdw + 1.0 × E_elec + 0.1 × E_AIR + 1.0 × E_desolv, the subscripts are as follows: vdw, van der Waals energy; elec, the electrostatic energy; AIR, the ambiguous interaction restraint energy; and desolv, the desolvation energy calculated using the atomic desolvation parameters of Fernandez-Recio and colleagues (37).

RESULTS

Unlike NGR-2C and DGR-2C, isoDGR-2C and RGD-2C Can Efficiently Bind avβ3 Integrin—We have shown previously that peptides containing the NGR motif, such as CNGRC and FN-I5, can rapidly deamidate (half-life, 2–3 h) when incubated for 16 h at pH 8.5 and 37 °C. This reaction leads to the generation of species containing the DGR and isoDGR motifs (12). To assess the effect of NGR deamidation on avβ3 binding, we have analyzed the interaction of a biotinylated NGR peptide (bio-CNGRC-hTNF-(1–11)) to avβ3 integrin before and after incubation at 37 °C. To this aim, we prepared complexes of biotinylated peptides and streptavidin-peroxidase, and we analyzed their binding to avβ3 integrin (Fig. 2A). Similar results were observed also with a FN-I5 peptide (data not shown). This suggests that peptide deamidation is very critical for avβ3 binding.

Given that NGR deamidation can lead to the formation of isoDGR and DGR, we performed additional assays to assess which of these molecular species was responsible for the increase in binding after heat treatment. To this aim we compared the capability of synthetic DGR-2C, isoDGR-2C, and NGR-2C to compete the binding of heat-treated bio-CNGRC-hTNF-(1–11) to avβ3 integrin adsorbed on microtiter plates. The RGD-2C peptide was analyzed in parallel as a positive control. The results showed that isoDGR-2C and RGD-2C could inhibit the binding of heat-treated bio-CNGRC-hTNF-(1–11) to avβ3 with similar potency, whereas >100-fold higher concentration of DGR and NGR were necessary to induce partial competition (Fig. 2B). These results confirm the hypothesis that isoDGR, unlike NGR and DGR, can efficiently mimic RGD in binding avβ3.

To assess whether these ligand-integrin interactions can also occur in living cells, we investigated the effect of RGD-2C,
DGR-2C, isoDGR-2C, and NGR-2C on the adhesion of EA.hy926 cells. As expected both RGD-2C and isoDGR-2C, but not DGR-2C, efficiently inhibited cell adhesion (Fig. 2C). Furthermore, a 10-fold higher concentration of NGR-2C was necessary to compete to comparable levels. Considering that assay incubation was 3.5 h and that the half-life of NGR deamidation in cell culture medium is 2–3 h (12), it is very likely that NGR competition was actually related to isoDGR formation during assay incubation.

Taken together, these results suggest that isoDGR, unlike DGR and NGR, can functionally mimic RGD in the interaction with adhesion receptors, such as DGR and NGR, can functionally mimic RGD in the interaction with adhesion receptors, such as DGR and NGR, can functionally mimic RGD in the interaction with adhesion receptors, such as DGR and NGR.

**Conformational Analysis of RGD-2C, isoDGR-2C, DGR-2C, and NGR-2C Macrocycles in Solution**—To investigate the structural determinants at the basis of the different biological activities of RGD-2C, isoDGR-2C, NGR-2C, and DGR-2C, their conformational properties in the unbound state have been characterized by standard two-dimensional solution NMR methods and molecular dynamics simulations.

**NMR Studies Indicate Flexibility of RGD-2C, isoDGR-2C, DGR-2C, and NGR-2C Macrocycles**—Analysis of two-dimensional NOE and rotational nuclear Overhauser effect spectroscopy spectra of the free ligands revealed the presence of only intra-residue and sequential nuclear Overhauser effect (NOE) and rotational nuclear Overhauser effect patterns with very few medium range contacts (Table 1), suggesting conformational variability for the ligands. The four ligands showed similar number of NOEs (~90) with the exception of isoDGR-2C, which had the lowest number of NOEs (~60). The latter suggests that the β-bond of the isoaspartic acid induces a greater conformational flexibility than that observed in the other three macrocycles.

Furthermore, the J_{HN-HA} couplings inside the macrocycles were all comprised in values ranging between 6 and 8.5 Hz (supplemental Table S1), indicating averaging between rapidly interconverting conformers. In addition, some residues outside the ring presented higher coupling constants (e.g. Tyr^{n} of RGD-2C $J_{HN-HA}$ = 8.7 Hz), suggestive of a more extended conformation for residues inside the tail.

Finally, the temperature coefficients $\langle\delta\Delta/\Delta T\rangle > 3.5$ ppb/K of the amide protons of the ligands inside the macrocycle (from residue 1 to 4) indicated partially or fully solvent exposure, consistent with macrocycle flexibility (supplemental Table S1) (20). In a different way, residue Cys^{5} showed lower temperature coefficients, suggesting a certain degree of solvent protection probably because of intra-molecular hydrogen bonds (supplemental Table S1).

**Solution Structures of the Four Macrocycles Indicate That isoDGR-2C Displays the Highest Conformational Heterogeneity**—Using the available short and medium range NOEs, solution structures of the four macrocycles have been calculated with the program CNS 1.2 (24), interfaced with ARIA 1.2 (23). For each ligand, we selected the 30 conformers with lowest energy for detailed structural analysis. The calculated structures were consistent with the experimental restraints, with no NOE distance violations greater than 0.4 Å and with all residues in the allowed regions of the Ramachandran plot (Table 1). The final ensembles of low energy structures of the four macrocycles are illustrated in Fig. 3, A–D. The C-terminal tails (residues 6–9) are highly disordered, whereas the macrocycles adopt a more ordered conformation with r.m.s.d. values ranging between 0.9 and 1.1 Å, calculated over both the backbone atoms and the disulfide bridge. In contrast, isoDGR-2C displays the highest conformational heterogeneity (r.m.s.d. ~ 1.3 Å), consistently with the lower number of NOEs (Table 1).

The dynamic behavior of the four macrocycles was further investigated by REMD, performed on the lowest energy conformer of each bundle of structures. The methodology is based on the parallel tempering Monte Carlo method (31, 38, 39), where multiple copies (or replicas) of identical systems are simulated in parallel at different temperatures, thus enabling an enhanced conformational sampling. We ran 16 replicas, for a total simulation length of 32 ns, over a temperature range between 293 and 353 K. For each ligand, we performed cluster analysis to separate the pool of conformations produced by the REMD simulations into families of structures of similar geo-
metric properties. Then we used these families of structures to identify the main structural features of the systems, focusing on the few structures (centroids) that are representative of the clusters of each macrocycle. We applied a root mean square deviation criterion of 0.7 Å over the heavy atoms of the macrocycle during pairwise comparisons of individual structures. Clustering yielded 20, 32, and 45 clusters for RGD-2C, DGR-2C, and NGR-2C, respectively. Notably, applying the same cut-off for isoDGR clustering, we identified 89 clusters, suggesting a larger conformational variability for the isoDGR macrocycle, in agreement with experimental NMR results. For each macrocycle, the bundle of centroids was compared with its lowest energy NMR conformer, yielding an r.m.s.d. of 1.75 Å over the backbone atoms and the disulfide bond, thus confirming the flexibility of the macrocycles (supplemental Table S2).

Backbone Torsion-Angle Analysis Reveals That an Inverse γ-Turn Conformation Is Highly Populated in DGR-2C and NGR-2C—The bundles of centroids representing the conformations of the four macrocycles were consistent with the corresponding NOE distance restraints, with all residues in the allowed regions of the Ramachandran plot (supplemental Table S2).

We then inspected these bundles for the presence of elements of secondary structure. Analysis of both φ and ψ angles and of hydrogen bonds provides support for the presence of an inverse γ-turn centered on residue 4 of each ligand. The inverse γ-turn was stabilized by the formation of an hydrogen bond between the amide proton of residue 5 and the carbonyl of residue 3. In addition, this inverse γ-turn recurred more frequently in both NGR-2C and DGR-2C than in RGD-2C and isoDGR-2C (~30–40 versus 10%), in agreement with the lower temperature factors of amide proton Cys measured in NGR-2C and DGR-2C as compared with those observed in RGD-2C and isoDGR-2C (supplemental Table S1). Of note, consistent results have been obtained in the NMR solution structure analyses.

NGR-2C Has a Different Electrostatic Surface as Compared with RGD-2C, isoDGR-2C, and DGR-2C—During REMD simulations, both RGD-2C and isoDGR-2C adopted an extended conformation with the basic and the acidic moieties pointing in opposite directions, thus creating a highly polarized structure with the positive and negative poles located at a distance of ~13–14 Å (Fig. 3, E–H, and supplemental Table S3). Likewise, residues 2 and 4 of DGR-2C and NGR-2C pointed in opposite directions, although their distances were slightly shorter (~12–13 Å). Most importantly, the presence of the amino group instead of the carboxylic moiety in NGR-2C markedly changes the electrostatic surface strongly reducing the negative electrostatic contribution (Fig. 3H).

Overall, NMR methods and molecular dynamics simulations indicate that there are important differences among the four macrocycles. First, isoDGR-2C has the greatest conformational heterogeneity induced by the β-bond of the isoaspartic acid. Second, NGR-2C, presenting a prevalent positively charged surface electrostatic potential, lacks the characteristic dipolar anchor points that represent a crucial pharmacophoric requirement for αβ3 recognition (40–45).
Docking Models of RGD-2C, isoDGR-2C, DGR-2C, and NGR-2C onto αvβ3—To gain a structural insight into the different binding affinities of the four macrocycles for αvβ3, we created putative binding models of the four ligands using the docking program HADDOCK2.0 (34, 35), based on the crystallographic structure of αvβ3 in complex with RGDf(NMV), a cyclic RGD pentapeptide (15, 16).

HADDOCK2.0 relies on a data-driven docking protocol consisting of a rigid-docking step followed by a semi-flexible step, which includes side-chain flexibility for both the receptor and the ligand. To account for the degree of conformational heterogeneity of a ligand, the protocol also allows for the docking of an ensemble of structures during the same run. Thus, for each ligand, the bundle of the 30 lowest energy NMR structures was docked onto the x-ray structure of αvβ3 following removal of the ligand. The program was first successfully tested for its ability to model the experimental αvβ3-RGDf(NMV) interactions, being able to reproduce the crystallographic binding mode (15) with an r.m.s.d. of ~0.5 Å, calculated on the heavy atoms of the RGD sequence (supplemental Fig. S2A).

Docking calculations are in agreement with data describing the different biological activity of the four macrocycles. RGD-2C and isoDGR-2C converged to unique low energy docking poses, contained in the mostly populated cluster (Fig. 4, A and B, and Table 2). Conversely, DGR-2C and NGR-2C failed to find highly populated binding solutions with a favorable HADDOCK score (Fig. 4, C and D, and Table 3). Noteworthy, the program generated essentially the same results when we used a bundle of structures containing conformers representative of the highest populated clusters produced by REMD simulations, thus confirming the reliability of the obtained models (supplemental Fig. S3). For RGD-2C and isoDGR-2C, HADDOCK calculations identified a highly populated cluster (~15 structures) with a consistent set of interactions with the receptor, showing a favorable HADDOCK score (~450 a.u.) along with an r.m.s.d. of...
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Distances are reported as mean ± S.D. over the solutions of each cluster.

| TABLE 2 | Statistics of the clusters obtained during HADDOCK calculations |
|----------|---------------------------------------------------------------|
|          | HADDOCK energy | r.m.s.d. | BSA | Number of structures |
| RGD-2C   | a.u. | Å | Å |   |
| Cluster 1 | 450 ± 27 | 0.8 ± 0.3 | 949 ± 96 | 15 |
| Cluster 2 | 163 ± 34 | 7.0 ± 0.4 | 973 ± 105 | 9 |
| Cluster 3 | 398 ± 48 | 2.7 ± 0.3 | 1045 ± 91 | 6 |
| Cluster 4 | 100 ± 42 | 7.6 ± 0.1 | 839 ± 49 | 6 |
| isoDGR-2C| a.u. | Å | Å |   |
| Cluster 1 | 414 ± 32 | 0.9 ± 0.4 | 970 ± 118 | 13 |
| Cluster 2 | 378 ± 65 | 2.8 ± 0.2 | 998 ± 130 | 6 |
| Cluster 3 | 401 ± 21 | 1.6 ± 0.3 | 902 ± 89 | 6 |
| Cluster 4 | 368 ± 35 | 3.7 ± 0.3 | 740 ± 119 | 6 |
| NGR-2C   | a.u. | Å | Å |   |
| Cluster 1 | 289 ± 26 | 3.9 ± 0.4 | 973 ± 123 | 7 |
| Cluster 2 | 286 ± 66 | 0.8 ± 0.5 | 800 ± 219 | 6 |
| Cluster 3 | 185 ± 62 | 2.9 ± 0.6 | 803 ± 164 | 5 |
| Cluster 4 | 163 ± 20 | 7.3 ± 0.4 | 613 ± 11 | 5 |
| DGR-2C   | a.u. | Å | Å |   |
| Cluster 1 | 193 ± 42 | 9.6 ± 0.2 | 754 ± 145 | 14 |
| Cluster 2 | 150 ± 27 | 9.5 ± 0.3 | 728 ± 106 | 7 |
| Cluster 3 | 377 ± 21 | 2.8 ± 0.2 | 755 ± 87 | 5 |

* HADDOCK energy includes the following terms: intermolecular van der Waals and electrostatic energies, empirical desolvation energy, and the native contact analysis (34, 35). Energies are reported as mean ± S.D. over the solutions of each cluster.

* a.u. Å Å 2

* BSA means buried surface area. Values are reported as mean ± S.D. over the solutions of each cluster.

### TABLE 3

Summary of the distances (Å) between the ligands and αvβ3 observed in the highest populated clusters (cluster 1)

Distances are reported as mean ± S.D. calculated over the solutions belonging to cluster 1.

| Ligand | αvβ3 | RGD x-ray | RGD x-ray dock | RGD-2C | isoDGR-2C | NGR-2C | DGR-2C |
|--------|-------|-----------|----------------|--------|-----------|--------|--------|
| C-NTerm | Tyr122 | 2.1 ± 0.1 | 2 ± 0.1 | 1.8 ± 0.1 | 2.2 ± 0.1 | 1.9 ± 0.2 | 2.0 ± 0.2 |
| D-OX/LAS-OX | Tyr122, Asp111 | 1.9 | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.3 ± 0.2 | 1.9 ± 0.1 | 2.0 ± 0.1 |
| D-OX/LAS-OX | Gln180 | 2.7 | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.8 ± 0.1 | 1.8 ± 0.1 | 2.0 ± 0.3 |
| D-OX | Arg214 | 3.0 | 2.0 ± 0.1 | 1.9 ± 0.1 | 2.3 ± 0.1 | 1.9 ± 0.2 | 1.9 ± 0.2 |
| D-OX/Asp218 | Arg214 | 2.7 | 2.4 ± 0.1 | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.0 ± 0.2 |
| D-OX | Tyr142 | 2.1 | 2.1 ± 0.2 | 1.9 ± 0.2 | 1.9 ± 0.1 | 1.9 ± 0.2 | 2.0 ± 0.3 |
| D-OX | Asp218 | 2.9 | 2.2 ± 0.2 | 1.9 ± 0.2 | 1.9 ± 0.1 | 1.9 ± 0.2 | 2.0 ± 0.3 |
| D-OX | Glu214 | 2.7 | 1.9 ± 0.1 | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.8 ± 0.1 | 2.3 ± 0.1 |
| D-OX | Asp218 | 1.9 | 1.7 ± 0.1 | 1.8 ± 0.2 | 2.0 ± 0.4 | 2.0 ± 0.3 | 2.0 ± 0.2 |
| D-OX | Asp216 | 1.8 | 1.8 ± 0.1 | 2.3 ± 0.1 | 2.3 ± 0.1 | 2.4 ± 0.1 |

* HNTerm denotes N-terminal protons.
* OX denotes carbonyl oxygen.
* LX denotes backbone oxygen.
* HX denotes amide proton.
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FIGURE 5. HADDOCK models of ligand-integrin-binding site. Surface representation of αβ3 binding pocket in complex with RGD-2C (A), isoDGR-2C (B), DGR-2C (C), and NGR-2C (D), with the α- and β-subunits represented in pink and pale cyan, respectively. In each panel, the side-chains of the ligand interacting with the receptor are shown in green, with nitrogen, oxygen, and sulfur atoms in blue, red, and yellow, respectively; the disulfide bond is represented with gray sticks; the MIDAS cation is represented with a red sphere. The side chains of both αβ3 and the ligand directly involved in the binding are labeled with the three- and one-letter code, respectively. Red and blue dotted lines denote the hydrogen bonds of the ligand with α- and β-subunits, respectively. The models (A–C) of the binding site correspond to the best (lowest energy) HADDOCK score structures of the highest populated cluster (see corresponding cluster 1 in each panel of Fig. 4). D, because HADDOCK calculations on NGR-2C produced two clusters with both similar score and populations (cluster 1 and 2 in Fig. 4D), the best structure of cluster 2 (thin sticks with NGR residues in magenta) is superimposed on the best structure of cluster 1 to allow comparison. E, model of DGR-2C in complex with αβ3 as defined in cluster 3 of Fig. 4. The pose is rotated by 180° with respect to cluster 1, and it is poorly populated and lacks the fundamental stabilizing interactions with the metal ion. F, superposition of HADDOCK structures of RGD-2C (yellow, cluster 1), isoDGR-2C (green, cluster 1), NGR-2C (purple, cluster 1), and DGR-2C (red, cluster 3) in complex with αβ3. The side chains of both RGD-2C and isoDGR-2C coordinate the metal ion, whereas the carboxyl- and amino-group of DGR-2C and NGR-2C, respectively, move away from the MIDAS site.

receptor-bound isoDGR-2C does not adopt an inverse γ-turn conformation to interact with αβ3.

It is worth noting that isoDGR motif displays some additional interactions with αβ3 as compared with RGD sequence. First, the isoDGR complex is also stabilized by an hydrogen bond between the backbone amide of Gly3 and the carbonyl of Arg216 (Fig. 5B and Table 3). As a matter of fact, the isoDGR central glycine contributes to the recognition of the receptor via polar interactions, while both in the crystallographic structure and in the αβ3-RGD-2C model the RGD central glycine interacts with the protein only through weak hydrophobic interactions (Fig. 5A and Table 3). Second, the reversed orientation inside the binding pocket of isoDGR allows the formation of an additional interaction between the carbonyl of Tyr122 and the N terminus of Cys1 (Fig. 5B and Table 3). Yet this interaction is missing both in the crystallographic structure and in the αβ3-RGD-2C model, suggesting that the N-terminal residue flanking the isoDGR motif might confer binding specificity.

Our model clearly shows that isoDGR-2C fulfills the pharmacophoric requirements for an αβ3 ligand, adopting both the proper distance (~13.7 Å) (supplemental Table S3) and the correct orientation between the two charged groups, which can simultaneously bind the α- and β-subunits. In this context, it is of note that the β-bond of the isoaspartic residue, with the consequent insertion of an additional torsion angle (Fig. 1B and supplemental Fig. S1) inside the main-chain, conforms to the backbone and the side-chains of the ligand the necessary flexibility to adapt well inside the crevice, thus optimizing the backbone and side-chain interactions with the receptor.

DGR-2C Fails to Identify a Favorable Position Inside the Binding Pocket—HADDOCK calculations performed on DGR-2C failed to generate highly populated low energy solutions (Fig. 4C and Table 2), clearly indicating the inability of DGR-2C to favorably interact with αβ3 (Fig. 5C and Fig. 5F). The highest populated cluster (cluster 1) displays higher HADDOCK energy than clusters found in both RGD-2C and isoDGR-2C (approximately ~200 a.u.), and it differs by more than 9 Å from the lowest energy structure. Remarkably, in cluster 1, DGR-2C was docked onto the receptor with an opposite orientation as compared with isoDGR-2C, thus losing all the important stabilizing interactions of the complex, including the metal ion coordination (Table 3). In agreement with its unfavorable HADDOCK score, DGR-2C creates only superficial interactions as indicated by its
lower buried surface area (~780 Å²) in comparison with the isoDGR and RGD-2C ones (~950 Å²). In attempting the binding with αβββ, DGR-2C maintains the inverse γ-turn conformation observed in NMR and molecular dynamics (MD) studies along with the proper distance (~14 Å) between the charged groups. However, the reduced number of interactions with the receptor suggests that the macrocycle backbone conformation and the side-chain orientations are not appropriate to accommodate in the binding crevice of αβββ.

Furthermore, we identified few poses (rotated by 180° with respect to cluster 1) in cluster 3 where the ligand penetrates inside the binding crevice orienting Asp² and Arg⁴ toward the β-subunit and the α-subunit, respectively (Fig. 5, E–F). However, this cluster differed by 2.5 Å from the lowest energy complex structure and was poorly populated (five structures), suggesting that this binding mode has low probability to occur. In fact, in this cluster, the macrocycle has to change its backbone conformation to fit inside the binding pocket, breaking the γ-turn and strongly reducing the distance between Asp² and Arg⁴ (~11 Å). Therefore, these data provide further support for the hypothesis that DGR-2C does not have the correct stereochemistry and the necessary flexibility to accommodate inside αβββ.

Overall, our results from both cell-adhesion experiments and docking performed on DGR-2C are consistent with previous binding and conformational studies performed on retro-peptides. The latter clearly indicated a dramatic decrease in activity for the DGR sequence as compared with the parent compound structure containing the RGD motif, ascribing this decrease in activity to the inappropriate amide bond directions and side-chain topology adopted by the retro-molecules (46).

**NGR-2C Fails to Converge to Unique Binding Solutions—**
HADDOCK calculations on NGR-2C failed to identify unique docking solutions inside αβββ, clearly showing poor convergence of the docking poses, with significantly higher energies (~200 a.u.) than those calculated for RGD-2C and isoDGR-2C (Fig. 4D and Table 2). Because we could not identify any cluster with a 1-Å cut-off, we increased it up to 1.3 Å, obtaining only poorly populated clusters (less than seven poses per cluster, see Table 2) with two opposite orientations inside the binding pocket (Fig. 4D, clusters 1–3 versus cluster 4). Moreover, in the first two clusters, displaying both similar HADDOCK energy and populations, the macrocycle adopted different backbone conformations inside the binding pocket (r.m.s.d. ~ 3.5 Å), providing further support for the inability of the NGR motif to specifically recognize αβββ (Fig. 5, D and F). Consistently, the poses contained in clusters 1 and 2 (Fig. 4D) displayed a reduced pattern of interactions, although adopting the same orientation as isoDGR-2C inside the binding pocket. On one end, the Arg⁴ guanidinium group maintains its interactions with Asp¹ and Asp²¹⁸. On the other end, the positive electrostatic surface of the N-2 amino group causes repulsive interactions with the metal ion and with the side-chains of both Asn²¹⁵ and Arg²¹⁴, thus forcing the Asn² amino group to move away from the MIDAS site (Fig. 5D), strongly destabilizing the NGR-2C-receptor complex.

Similarly to what observed for DGR-2C, in cluster 4 (Fig. 4D) the macrocycle does not maintain the γ-turn when bound to αβββ and the distance between Asn² and Arg⁴ is significantly reduced (~11 Å) as compared with the distances measured in RGD-2C and isoDGR-2C (supplemental Table S3). This lends support to the hypothesis that the macrocycle backbone conformation and the side-chain orientations are not appropriate to optimize the ligand-receptor interactions. Therefore, our data provide a structural rationale to explain the inability of NGR-2C to dock onto αβββ.

**DISCUSSION**

The main finding of this work is that isoDGR-2C has the ability to properly interact with αβββ, both recapitulating the canonical RGD-αβββ contacts and establishing additional polar interactions, as demonstrated by both functional and structural studies, thus defining a new natural αβββ recognition motif that does not comply with the strict RGD sequence requirement rule.

To this extent, it is well established that αβββ integrin binds RGD motif-containing proteins, such as fibronectin, vitronectin, fibrillin-1, von Willebrand factor, osteopontin, and echistatin (47–52). Generally, the integrin recognition mechanism follows strict sequence and stereospecific requirements, as characterized in detail throughout both functional and structural studies (15, 52–54). In contrast, here we show that this strict RGD sequence requirement rule can be bent. In fact, our biological data indicate that isoDGR-2C and RGD-2C bind to αβββ with similar affinity, and that these interactions can occur also in living cells, because isoDGR-2C and RGD-2C are also able to inhibit cell adhesion of endothelial cells. Consistently, we have recently shown that fibronectin can interact with αβββ not only via the classical RGD mechanism but also through an isoDGR site in the FN-I₅ module, generated by the deamidation of an NGR sequence (12). Likewise, cells from homozygous knock-in mice carrying the RGD → RGE mutation in fibronectin can be assembled into fibrils in vivo and in vitro via αβββ binding to the isoDGR motif in FN-I₅, generated by NGR deamidation (13).

Our three-dimensional model of isoDGR-αβββ interactions indicates that this binding is dictated by a novel and stereospecific recognition pattern, offering a structural rationale for αβββ binding specificity of deamidated-Asn²⁶³ FN-I₅. Here, a key finding of our structural studies is that isoDGR docks onto αβββ in an inverted orientation as compared with the RGD ligand. This orientation allows isoDGR to anchor the α and β domains like an electrostatic clamp through the isoaspartic and arginine side-chains, reproducing the canonical interactions characterizing the αβββ recognition via RGD (15). Furthermore, the acidic and basic residues of isoaspartate and arginine are at the correct distance (13.72 Å, supplemental Table S3) and orientation to engage stabilizing interactions with the polar region of the receptor, which is a crucial pharmacophoric requirement of an αβββ ligand. Most importantly, the reversed orientation of isoDGR-2C inside the binding pocket allows for additional interactions as compared with RGD-2C and RGDf(NMV), involving the N-terminal cysteine flanking the isoDGR sequence and the central glycine (Table 3). In particular, isoDGR-2C cysteine 1 points toward the binding site contributing to receptor recognition, whereas in RGD-2C it faces
out from the receptor. Furthermore, the backbone atoms of isoDGR-2C central glycine engage additional polar interactions, whereas in the RGD motif the backbone adopts an inverse $\gamma$-turn conformation centered on the glycine, thus contributing to $\alpha\beta\beta$ recognition only through weak hydrophobic contacts.

In this context, it is worth noting that the presence of the $\beta$-bond in isoDGR-2C confers to the macrocycle a high degree of backbone flexibility, as shown by both NMR analysis and MD simulations. Therefore, the ligand can easily accommodate inside the shallow crevice of the receptor orienting the isoaspartate toward the MIDAS cation, which is a central anchor point for the ligand, thus completing its coordination sphere. Under this view, the greatest conformational heterogeneity of isoDGR-2C induced by the $\beta$-bond of the isoaspartic acid is conceivably a favorable feature. As a matter of fact, the entropic penalty paid by the flexible isoDGR macrocycle upon binding to $\alpha\beta\beta$ is largely compensated by the stabilizing backbone and side-chains interactions engaged with the receptor. Notably, our results are in agreement with rational drug design, because peptidomimetics employing our results are in agreement with rational drug design, because peptidomimetics employing $\beta$-amino acids mimicking the aspartate residue are potent antagonists of $\alpha\beta\beta$ (45, 55), thus suggesting that the conformational plasticity of the $\beta$-bond favors the binding to the receptor exploiting the adaptation ability of ligand-receptor interaction.

Both NMR and MD studies on DGR-2C provide further support for the importance of the $\beta$-bond in contributing to ligand binding. As a matter of fact, DGR-2C has a reduced conformational freedom of the cycle and does not have the correct stereochemistry to interact with the receptor. Thus, to fit inside the receptor binding groove DGR-2C should reduce the distance between the arginine and the aspartic residues breaking the stable inverse $\gamma$-turn, and therefore paying a highly unfavorable energy penalty. These results along with data from both binding and cell adhesion experiments are consistent with previous binding and conformational studies performed on retropeptides, indicating a dramatic decrease in activity for the DGR sequence as compared with the parent compound structure containing the RGD motif (46). This decrease in activity can be ascribed to the inappropriate amide bond directions and side-chain topology adopted by the retro-molecules, consistently with the strict RGD sequence requirement rule.

Another important finding of this work is that NGR-2C is not an $\alpha\beta\beta$ recognition motif because it lacks the fundamental pharmacophoric features for high receptor affinity, as shown by both NMR and MD studies in agreement with functional results from binding and cell adhesion experiments. Similarly to DGR-2C, NGR-2C adopts an unfavorable backbone conformation to accommodate inside the binding groove, and most importantly it misses the acidic group, which completes the coordination of the divalent metal ion of the MIDAS site. In addition, NGR-2C presents a prevalent positively charged surface electrostatic potential and thus lacks the characteristic dipolar anchor points that represent a crucial pharmacophoric requirement for $\alpha\beta\beta$ recognition (40 – 45). Altogether, these data provide a structural rationale to explain the inability of NGR-2C to bind to $\alpha\beta\beta$. Consistently, in agreement with previous reports (12, 13), functional studies, based on inhibition of endothelial cell adhesion and in vitro binding of peptides to purified $\alpha\beta\beta$ integrin, suggest that NGR neither affects cell adhesion nor competes the binding to $\alpha\beta\beta$, whereas the heat-induced NGR → isoDGR deamidation causes a marked increase in binding (>100-fold).

In this context, we therefore argue that NGR → isoDGR transition may represent an important physiological mechanism able to work as a molecular timer for activating latent integrin-binding sites in fibronectin (12). Furthermore, the NGR motif by itself binds to aminopeptidase N (CD13) (56, 57), which affects major biological events, including cell proliferation, secretion, invasion, and angiogenesis, thus participating in the regulation of tumorigenesis. In particular, aminopeptidase N is specifically expressed in endothelial and subendothelial cells in angiogenesis and in various types of tumors (56 – 59). An interesting possibility is that the NGR motif contained in fibronectin interacts with CD13 on one hand, and on the other hand gives origin to a natural recognition motif (isoDGR) of $\alpha\beta\beta$ integrin, conferring to fibronectin multifunctionality and plasticity in mediating a variety of physiological and pathological processes, including development, angiogenesis, inflammation, wound repair, and tumor growth.

In conclusion, our data provide the structural basis to define isoDGR as a new natural recognition motif of $\alpha\beta\beta$, which indicates that NGR lacks the necessary pharmacophoric requirements to bind $\alpha\beta\beta$, thus helping to explain the functional differences of fibronectin NGR and isoDGR sequences.

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