Integrin Cyclopeptides

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Molecular Dynamics Reveal that isoDGR-Containing Cyclopeptides Are True αvβ3 Antagonists Unable To Promote Integrin Allostery and Activation**

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Integrins are heterodimeric, transmembrane, cell-adhesion receptors regulating cellular functions crucial for the initiation, progression, and metastasis of solid tumors.[1] Specifically, integrin αvβ3 plays a key role in endothelial cell survival and migration during tumor angiogenesis.[2] It has, therefore, gained attention as an attractive therapeutic target in anti-angiogenic cancer therapy.[3] αvβ3 relays signals bi-directionally across the plasma membrane between the extracellular ligand-binding site and the cytoplasmic domains of the receptor. Signal transfer is allosterically coupled to three major equilibrium conformational states, including the 1) inactive bent state; 2) intermediate extended state with a closed headpiece, and 3) active extended form with an open headpiece[4] (Figure S1 in the Supporting Information). Although controversy remains concerning the level of complexity in integrin conformational changes, it is generally recognized that the out–in signaling, following ligand binding, and the consequent switch from the inactive to active state is accompanied by an outwards movement of the β-hybrid domain, characterized by a swing-out angle varying between 10° and 80°.[5–8] Out–in activation of αvβ3 by natural ligands occurs through the recognition of a tripeptidic motif arginine–glycine–aspartate (RGD). This sequence is therefore a lead for developing integrin antagonists.[9] The cyclopeptide isoDGR is a new αvβ3-binding motif, with potential applications in the design of integrin antagonists.[10–14] One major problem with integrin inhibitors is their potential to activate conformational changes, which can initiate unwanted signals that induce agonist-like activities and adverse paradoxical effects.[3,15,16] In this context, drug design studies aimed at developing new integrin blockers could benefit from information defining the receptor allosteric events induced by ligand binding. Therefore, in the attempt to characterize isoDGR-based αvβ3 antagonists, we exploited a combination of computational and biochemical studies to describe the dynamic changes of αvβ3 upon ligand binding. Herein, we demonstrate that isoDGR-based cyclopeptides unexpectedly inhibit receptor allosteric activation.

To characterize the interactions and the effects of RGD- and isoDGR-containing cyclopeptides on αvβ3 conformational dynamics, we performed all-atom molecular dynamics (MD) simulations of the integrin αvβ3 headpiece alone and in the presence of RGDf(NMe)V, CisoDGRC, and $_{ac}$CisoDGRC cyclopeptides (Figure 1; see Supporting Information for details). To increase the conformational sampling, we adopted a multicopy approach[17] performing three independent trajectories for each system for a total cumulative simulation time of 360 ns (Figure S2a,b and S3 in the Supporting Information). The three ligands anchor to the αv and β3 domains through an electrostatic clamp that exploits similar though not identical interaction patterns (Figure 1). On one hand, the Arg guanidinium groups of the three ligands are engaged in stable salt-bridges with the carboxylate of $D218_{\alpha v}$ and/or of $D150_{\alpha v}$. On the other hand, their Asp/isoAsp carboxylates bind to the β3 subunit, coordinating the metal ion-dependent adhesion site (MIDAS) ion through a carboxylate oxygen. Herein, we observed relevant differences in the coordination pattern of the second carboxylate oxygen: in both CisoDGRC and $_{ac}$CisoDGRC this oxygen interacts with residue $N215_{\beta3}$, which is located in the loop connecting helices α2 and α3, whereas, the very same oxygen in RGDf(NMe)V stably binds to the β1-α1 loop (residues $S121_{\beta3}$–$S123_{\beta3}$; Figure 1, Table S1, and Figure S4 in the Supporting Information). Remarkably these interactions, which play a role as the trigger of the β3 swing-out mechanism,[18] are barely present in the simulations with isoDGR-containing cyclopeptides. Thus, we wondered whether these differences in the ligand-receptor interaction patterns might induce long-distance effects on αvβ3 mobility.

To gain insights into long-range communications among αvβ3 domains in the different systems, we calculated the correlation matrix $Corr_{ij}$ that describes linear correlations between any pairs of Cα atoms as they move around their average position during the dynamics (Figure 2a–d). This approach allows identification of pairs/groups of residues with

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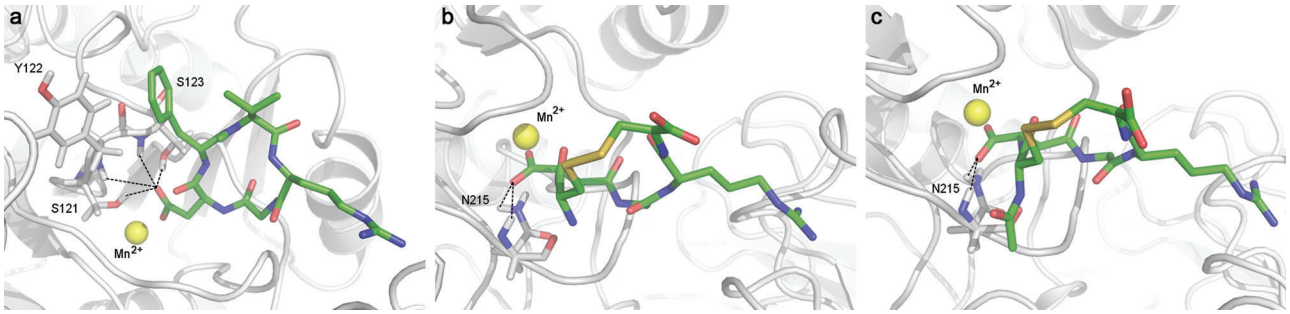

Figure 1. RGDf(NMe)V, CisoDGRC, and $_{ac}$CisoDGRC exploit similar though not identical interaction patterns to bind αvβ3. αvβ3 binding pocket (gray) of a) RGDf(NMe)V, b) CisoDGRC, and c) $_{ac}$CisoDGRC (representative snapshots). αvβ3 residues (one-letter code) directly coordinating the carboxylate groups of Asp/isoAsp residue of the ligands are represented with sticks, Yellow sphere = MIDAS cation (Mn^{2+}); green = cyclopeptide ligands; blue = nitrogen; red = oxygen; yellow = sulfur. Dotted lines highlight the different ligand-receptor interaction patterns.

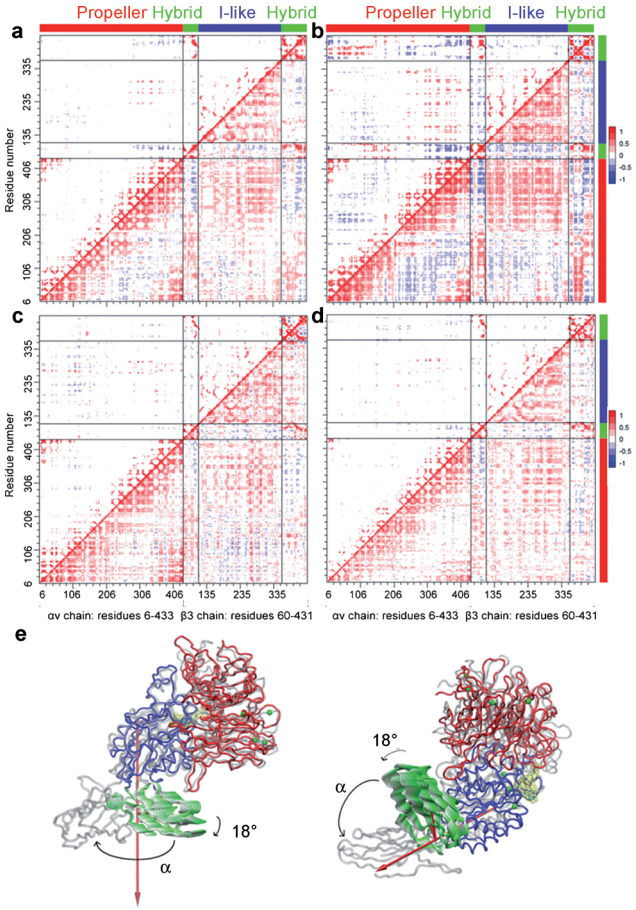

Figure 2. RGDf(NMe)V and isoDGR-containing cyclopeptides differently affect $\alpha v\beta_3$ motions. Correlation maps of a) free $\alpha v\beta_3$, and bound to b) CisoDGRC, c) $_{ac}$CisoDGRC, d) RGDf(NMe)V(yellow surface in (e)). Correlated and anti-correlated motions between atom pairs are represented in red and blue, respectively. Above the diagonal of the matrices only $|Corr_{ij}| > 0.5$ are reported. e) The motion described by the third eigenvector of αvβ3-RGDf(NMe)V complex is shown in two different orientations. The swing-out movement of the hybrid domain is illustrated as linear interpolation between the extreme projections (represented in green and white) of MD structures onto the third eigenvector. The red arrow represents the rotation axes of the hybrid domain towards the open conformation, represented in gray by the crystallographic structure of αIIbβ3 (PDB: 3FCU).

correlated (in the same direction) and anti-correlated (in opposite directions) motions. The comparison between free and bound αvβ3 shows that intrinsic intra- and inter-domain correlations are differently modulated by the three ligands. In particular, binding of RGDf(NMe)V enhances long-range correlated and anti-correlated motions within residues of the hybrid domain (L357–F431), and among the hybrid, the propeller and the I-like domains. Overall, RGDf(NMe)V activates an interaction network inside αvβ3 that induces more concerted motions in the ligand-bound integrin as compared to its free form. In contrast, both integrin-bound CisoDGRC and $_{ac}$CisoDGRC reduce αvβ3-concerted dynamics, as compared to those induced by RGDf(NMe)V (Figure 2a–d). Herein, root mean squared fluctuations (RMSF; Supporting Results and Figure S3 in the Supporting Information) are consistent with the fact that isoDGR-containing cyclopeptides affect αvβ3 flexibility and long-range rearrangements differently as compared to RGDf(NMe)V.

In such complex systems, it is crucial to filter out the motions that most affect the overall dynamics. Thus, we applied essential dynamics (ED), which is an approach known to highlight functionally relevant collective motions in a reduced dimensionality space, to interpret experimental conformational variations, and to understand the motions that are relevant for protein activity.[19] In the four systems we studied, most of the total variance is captured by a relatively small number of independent variables (eigenvectors) that represent the collective atom movements. The cumulative total variance captured by the first three eigenvectors is around 50% for the αvβ3, αvβ3-CisoDGRC, and αvβ3-$_{ac}$CisoDGRC simulations, whereas the cumulative value of the total variance is 70% for the αvβ3-RGDf-(NMe)V complex (Table 1). This is consistent with the fact that RGDf(NMe)V increases the correlated αvβ3-motions, unlike isoDGR-containing ligands. In addition, although in the four systems the main movements described by the first three eigenvectors involve primarily the hybrid domain, their ED subspaces are different (Figure S5 and Table S2 in the Supporting Information). This is in agreement with the notion that different ligands can variably modulate the αvβ3-correlated motions.

Table 1: Proportion of variance captured by the first six eigenvectors derived from ED analysis of MD ensembles.[a]

Eigenvector index	αvβ3 variance [%]	αvβ3-RGDf(NMe)V variance [%]	αvβ3-CisoDGRC variance [%]	αvβ3-$_{ac}$CisoDGRC variance [%]
1	25.5 (25.5)	44.4 (44.4)	32.5 (32.5)	30.9 (30.9)
2	18.1 (43.6)	20.9 (65.3)	22.1 (54.6)	20.5 (51.4)
3	10.8 (54.4)	5.1 (70.4)	9.7 (64.3)	8.6 (60.0)
4	6.6 (61.0)	2.6 (73.0)	5.8 (70.1)	4.2 (64.2)
5	4.8 (65.8)	2.2 (75.2)	3.3 (73.4)	4.0 (68.2)
6	4.0 (69.8)	2.0 (77.2)	2.5 (75.9)	2.6 (70.8)

[a] In parentheses, cumulative value of the total variance captured by the first six eigenvectors.

We next wondered whether some eigenvectors were able to capture the swing-out movement of the hybrid domain.[5–8] Notably, only the third eigenvector of αvβ3-RGDf(NMe)V is able to reproduce the hybrid motion on the pathway to opening, with a swing-out angle α of 18° (Figure 2e, Figure S6, and Video S1 in Supporting Information). In contrast, none of the essential movements assessed for αvβ3, either free or bound to isoDGR-containing ligands, matches the hybrid characteristic swing-out movement. Therefore, we conclude that isoDGR-containing cyclopeptides, unlike RGDf-(NMe)V, do not induce the αvβ3 headpiece-opening.

Pursuing this hypothesis, we performed gel-filtration experiments to monitor possible αvβ3 conformational changes upon addition of RGDf(NMe)V, CisoDGRC, and $_{ac}$CisoDGRC. The complete recombinant purified αvβ3 ectodomain ($_{tt}$αvβ3) was analyzed by size-exclusion chromatography before and after mixing with saturating concentrations of RGDf(NMe)V, CisoDGRC, and $_{ac}$CisoDGRC. Notably, unlike RGDf(NMe)V, isoDGR-containing cyclopeptides did not induce a shift in the elution volume of $_{tt}$αvβ3 (Figure 3a). Accordingly, the derived Stokes radii for integrin $_{tt}$αvβ3, either free or in complex with isoDGR-containing cyclopeptides, were similar (about 7.55 nm), consistent with a lack of a conformational rearrangement following ligand binding. In contrast, binding of RGDf(NMe)V increased Stokes radius of $_{tt}$αvβ3 up to 7.89 nm, which is compatible with a swing-out motion of the β3-hybrid domain in the open-extended conformation[20] (Figure 3a and Figure S1 in the Supporting Information). Overall, these results confirm that αvβ3 in solution remains in an inactive conformation after treatment with CisoDGRC and $_{ac}$CisoDGRC.

We further investigated whether isoDGR-containing cyclopeptides could also act as inhibitors of αvβ3 allostery when the receptor is bound to the cell surface. It is known that receptor activation and/or ligand binding change the conformation of the β3 subunit, resulting in the exposure of new epitopes called ligand-induced binding sites (LIBS), which can be detected by LIBS-specific monoclonal antibodies (mAbs), such as AP5 mAb.[21] Consistent with our MD and gel-filtration results, CisoDGRC and $_{ac}$CisoDGRC fail to induce exposure of the β3-LIBS epitope recognized by the AP5 mAb on human umbilical vein endothelial cells (HUVEC; Figure 3b). In contrast, RGDf(NMe)V induces a significant exposure of LIBS, as shown by AP5 staining,

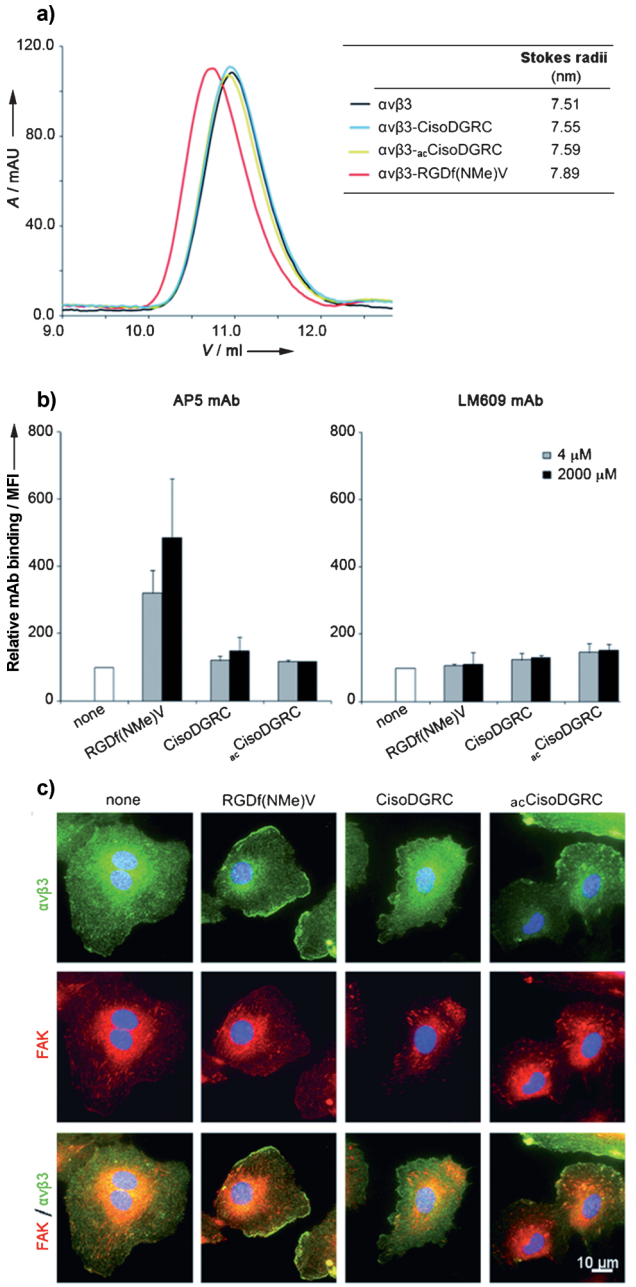

Figure 3. Ligand effects on αvβ3 conformation and localization. a) Gel-filtration profiles of αvβ3 (black) alone or with saturating amounts of RGDf(NMe)V (red), CisoDGRC (blue), or $_{ac}$CisoDGRC (yellow) with their corresponding Stokes radii. b) HUVEC were exposed to RGDf-(NMe)V, CisoDGRC, or $_{ac}$CisoDGRC (4 and 2000 μM), stained by immunofluorescence for β3 LIBS and total αvβ3 expression (with AP5 and LM609 mAbs, respectively), and analyzed by flow cytometry. c) Localization of αvβ3 integrin (green) and FAK (red) was monitored by immunofluorescence staining. HUVEC were plated on fibronectin and treated with saturating amounts of RGDf(NMe)V, CisoDGRC, or $_{ac}$CisoDGRC. Only RGDfNMeV caused disappearance of αvβ3 from focal adhesion and promoted relocalization at the cell periphery.

owing to conformational changes that occur following binding to the ligand. In conclusion, computational analysis along with biochemical and phenotypic characterization indicate that isoDGR-containing cyclopeptides inhibit integrin acti-

vation unlike RGDf(NMe)V, which induces integrin activation.

We next wondered whether blockage of the allosteric movement of αvβ3 could have a functional relevance, influencing the pathways usually triggered by ligand-induced αvβ3 activation. We therefore compared the effects of RGDf(NMe)V and isoDGR-containing cyclopeptides in αvβ3-expressing HUVEC under conditions of β1 integrin-mediated adhesion. Herein, αvβ3 was present at focal adhesions (stained with focal adhesion kinase (FAK) antibodies); notably, RGDf(NMe)V activated αvβ3 and promoted the redistribution of αvβ3 from focal adhesions to the cell periphery,[22] an event critical for cell migration.[23] In contrast, both $_{ac}$CisoDGR and CisoDGRC did not induce accumulation of αvβ3 at the cell border, further supporting the hypothesis that isoDGR-containing cyclopeptides compete with ligand binding without inducing integrin activation (Figure 3c).[24]

In conclusion, computational and biochemical studies show that isoDGR-containing cyclopeptides act as true antagonists of αvβ3 integrin, thus defining a new class of ligands, which block the ligand binding site and inhibit receptor allosteric activation. Computational results, in agreement with previous studies on αIIβ3 inhibitors,[18] suggest that subtle differences in ligand/αvβ3 interactions involving the β1-α1 loop differently affect the allosteric response of the receptor to ligand binding. Unlike RGDf-(NMe)V, isoDGR-containing cyclopeptides, which barely interact with the β1-α1 loop, fail to induce the swing-out of the hybrid domain. In addition, gel filtration studies and a lack of LIBS-exposure upon binding confirm that binding of isoDGR-containing cyclopeptides does not induce αvβ3 conformational changes, different from what was observed for RGDf(NMe)V. Finally, isoDGR-containing cyclopeptides inhibit integrin accumulation at the cell edge, whereas RGDf(NMe)V has activating properties.[22] RGDf(NMe)V induced αvβ3 relocalization at the cell edge, thus resulting in the conversion of an inactive integrin into a multifaceted signaling machine that induces undesirable effects including receptor clustering, activation, and redistribution. In contrast, the intrinsic ability of the isoDGR motif to block receptor allosteric activation holds promise for drug development. Conceivably, isoDGR-based drugs could replace the current generation of integrin-binding compounds, representing a promising solution to designing integrin antagonists, devoid of intrinsic paradoxical activation effects.

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