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Receptor-Bound Conformation of an $\alpha_5\beta_1$ Integrin Antagonist by ^{15}N-Edited 2D Transferred Nuclear Overhauser Effects

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Integrin receptors are heterodimeric transmembrane receptors, composed of α and β noncovalently associated subunits. They mediate cell−cell adhesion and attachment of cells to the extracellular matrix. The $\alpha_5\beta_1$ integrin (fibronectin receptor) plays an important role in mediating cell attachment to fibronectin,[1] cell migration,[2] tumor invasion, and metastasis.[3] Furthermore, the recent discovery of fibrinogen as a ligand to $\alpha_5\beta_1$ on endothelial cells has suggested another biological role for $\alpha_5\beta_1$.[4] It is known that certain RGD-containing peptides can suppress metastasis in vitro by perturbing the function of $\alpha_5\beta_1$ through blocking its putative ligand from binding.[5] Therefore, it has been suggested that selective $\alpha_5\beta_1$ antagonists can lead to new cancer therapeutic agents.[6]

The RGD sequence is the recognition site for various integrin receptors, and the conformation of the RGD sequence in the individual adhesion protein or peptide is critical for the specificity of this recognition.[5c] The three-dimensional structure of $\alpha_5\beta_1$ receptor is not available. Therefore, conformational analyses and pharmacophore deductions have focused on the most stable conformations of the RGD-containing ligands in solution. For a ligand that is under fast exchange on the relaxation time scale, i.e., from association with the receptor to the free state in solution, transferred nuclear overhauser enhancement (trNOE) experiments are an attractive method[7] for studying the bound conformation of $\alpha_5\beta_1$ antagonists to its receptor. In trNOE experiments, the NOEs which build up while the peptide is associated with the receptor are transferred to the free peptide and therefore readily observed at the fast on/off exchange rate. No isotopic labeling of the peptide is required for this experiment. However, the ^{15}N-edited trNOE offers great advantages, such as the sensitivity enhancement and the utilization of ^{15}N-edited pulse sequences to suppress the spin diffusion through the ligand−receptor pathway.

The $\alpha_5\beta_1$ receptor was extracted from human placenta and purified using affinity columns. After dialysis, the $\alpha_5\beta_1$ receptor was concentrated to a final concentration of 35 mg/mL (~0.1 mM) to be used for the NMR experiments.

The partially ^{15}N-labeled $\alpha_5\beta_1$ antagonist c[Mpa^{15}*N*-Arg-^{15}*N*-Gly-^{15}*N*-Asp-^{15}*N*-Asp-^{15}*N*-Val-Cys]-NH_2 (Figure 1) was synthesized on a Novabiochem MBHA resin using the Boc solid-phase strategy. The linear peptide was cleaved from the resin by HF and the crude products were cyclized by air oxidation. Purification with RP-HPLC afforded the pure ^{15}N-labeled peptides in good overall yields. The details for the synthesis of the antagonist will be published elsewhere.

The RGD peptide used for this study is a weak binder to the $\alpha_5\beta_1$ receptor based on its IC_{50} measurement (1.2 μM). We carried out a series of HSQC titrations and determined that the RGD peptide undergoes fast exchange between free and bound states with the $\alpha_5\beta_1$ receptor. The 1D-HSQC spectra demonstrate that the chemical shifts change for the RGD sequence upon ligand binding to the $\alpha_5\beta_1$ receptor.

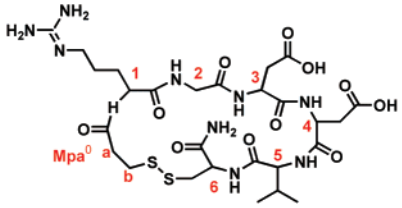

Figure 1. The structure of c[MpaRGDDVC]-NH_2.

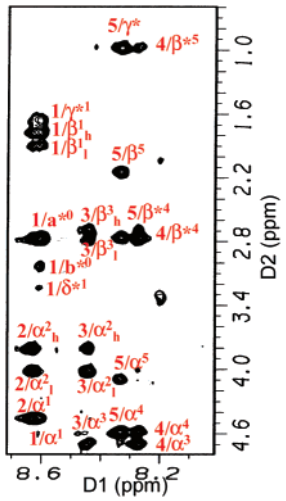

Figure 2. The ^{15}N-edited 2D-HSQC-trNOESY for bound c[MpaRGDDVC]-NH_2 at 293 K, with a mixing time at 100 ms (NH−Hα region). The asterisk denotes pseudoatoms. a*^{0} and b*^{0} refer to the Mpa^0 residue that represents −CO-CH_2(a)-CH_2(b)-SH−. As an example, 3/α^2_l refers to the cross-peak between HN of the third residue and the low-field (l) α proton of the second residue. D1 and D2 correspond to F1 and F2, respectively.

To limit ligand-mediated spin-diffusion[8] and nonspecific binding effects, trNOE experiments were measured at a ligand-to-receptor ratio of 10:1. The ^{15}N-edited 2D-HSQC-trNOESY experiments were recorded with mixing times of 50 and 100 ms at 293 K (Figure 2). The buildup curve is linear up to 100 ms. No transferred NOEs were detected in the control experiments for the free ligand at the identical conditions without the presence of the $\alpha_5\beta_1$ receptor. The observation of NOEs that originate exclusively through spin diffusion via the protein is a possible pitfall in the transferred NOE experiments. By using the ^{15}N-labeled ligand and nonlabeled $\alpha_5\beta_1$ receptor, the QUIET-BIRD-NOESY[9] pulse sequence implemented

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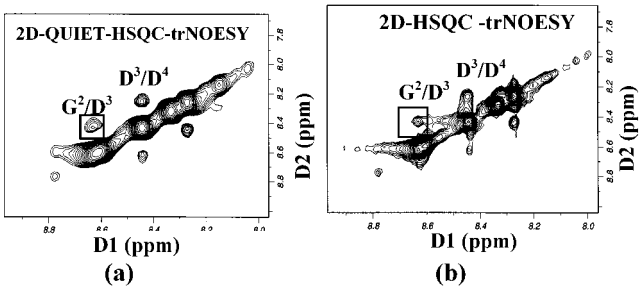

Figure 3. The ^{15}N-edited 2D-QUIET-HSQC-trNOESY (a) and ^{15}N-edited 2D-HSQC-trNOESY (b) (NH-NH region) for bound c[MpaRGDDVC]-NH_2 at 293 K, with a mixing time of 100 ms. The additional NOE cross-peak is [boxed]: GlyHN^2-AspHN^3 in the NH−NH region (a and b) appears upon ligand binding to the receptor.

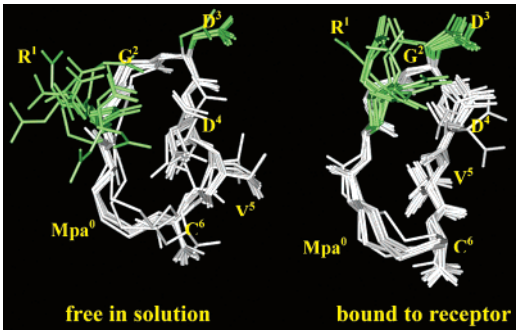

Figure 4. Superimposition of calculated families of free and bound conformations of $\alpha_5\beta_1$ antagonist c[MpaRGDDVC]-NH_2.

in 2D-QUIET-HSQC-trNOESY can, in principle, selectively excite resonances directly attached to the labeled ^{15}N and provide direct cross relaxation between them, while suppressing spin-diffusion contributions from the intervening protons. The 2D-QUIET-HSQC-trNOESY (Figure 3a) experiment confirms the results obtained from the 2D-HSQC-trNOESY experiment (Figure 3b). Both show the GlyHN^2-AspHN^3 (G^2/D^3) [boxed] cross-peak. In the 2D-HSQC NOESY experiment for the free ligand in solution obtained at 278 K and 300 ms mixing time, the additional NOE G^2/D^3 cross-peak in the NH−NH region is absent while the D^3/D^4 remains. The other NOEs for the structural calculations are included in the Supporting Information (Table 1).

Figure 4 presents the calculated free and bound conformations of c[MpaRGDDVC]-NH_2. The structural calculations were carried out by DGII and NMRchitect programs (MSI, San Diego, CA). The receptor bound conformation contains a slightly distorted type I β turn spanning Gly^2-Asp^3, supported by a medium NOE between Gly^{2}HN and Asp^{3}HN, and a medium NOE between Asp^{3}HN and Asp^{4}HN. Although the type I β turn is accessible for the free ligand, the stable conformation for the free ligand in solution contains a type II′ turn around Gly^2-Asp^3, supported by a medium NOE between Asp^{3}HN and Asp^{4}HN. Both the type I and type II′ β turns are stabilized by a hydrogen bond between the carbonyl oxygen of the Arg^1 and the amide proton of Asp^4 at the i and i+3 positions of the β turn, respectively.

When the RGD peptide binds to the $\alpha_5\beta_1$ receptor, the β-turn about Gly^2-Asp^3 changes from type II′ to type I, accompanied by the flipping of the peptide bond between Arg^1 and Gly^2. This rotation of the peptide bond changes the orientation of the side chain of Arg^1 as well. The side chain of Arg^1 rotates from a relatively equatorial position to an axial position in relation to the cyclic backbone. Consequently, this rotation brings the side chain of Arg^1 closer to Asp^3. The distance between the Arg^{1}C$_\beta$ and Asp^{3}C$_\beta$ averages 7.5 Å for the free conformation, as opposed to 5.6 Å for the bound conformation. Not only does the backbone around Gly^2 change when the ligand binds to the receptor, but the region of the ring around Val^5-Cys^6-Mpa^0 becomes narrower as well.

In conclusion, we have successfully carried out ^{15}N-edited transferred NOE experiments that provide significant insight for the elucidation of the "bioactive" (bound) conformation of the antagonist c[MpaRGDDVC]-NH_2 to the $\alpha_5\beta_1$ receptor. In general, the energy change involved in altering conformations is of a much lower order of magnitude than the binding energies (12−17 kcal/mol). Conformational changes upon receptor−ligand interaction have been described in the literature as "induced fit" phenomenon.[10] The bound structure exhibits a type I β turn around Gly^2 and Asp^3 residues which play an important role in modulating the distance between the side chains of Arg^1 and Asp^3, a key factor in the specificity of ligand binding. This shorter distance between the charged side chains of Arg^1 and Asp^3 (<6 Å) indicates that the binding pocket for the $\alpha_5\beta_1$ receptor is narrow compared to that of $\alpha_{IIb}\beta_3$.[11]

The $\alpha_5\beta_1$ integrin is involved in tumor metastasis. These results will facilitate the design of novel integrin antagonists and establish the conditions of NMR studies of the receptor-bound conformations of other integrin antagonists. This research can lead to new integrin receptor antagonists for cancer therapeutics.

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Supporting Information Available: Table 1 giving NOEs and distance restraints (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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