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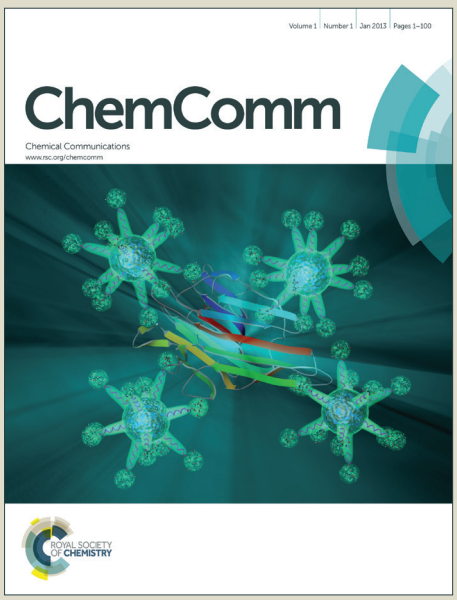

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ARTICLE TYPE

Dual binding site of angiogenin and its inhibition mechanism: the crystal structure of the rat angiogenin-heparin complex.

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The heparin complex of rat angiogenin revealed that a heparin strand is fitted into a positively charged groove formed by the dual binding site of rat angiogenin, suggesting that cell adhesion to angiogenin is facilitated by its interaction with substrates on the cell surface and can be inhibited by heparin.

Angiogenin is a well-known protein involved in angiogenesis and cell proliferation in both normal and cancer cells.[1] Recent studies reported that mutations in angiogenin are associated with amyotrophic lateral sclerosis (ALS) and Parkinson's disease.[2] Thiyagarajan et al describe the molecular mechanism of angiogenin in ALS using structural and functional studies of angiogenin-ALS variants.[3] Angiogenin is a monomeric protein and a ribonucleolytic enzyme that belongs to the pancreatic ribonuclease A (RNase A) superfamily. The angiogenic activity of angiogenin requires both the binding of the protein to endothelial cells and ribonucleolytic activity, which is weak compared to the activity of RNase A.[4] Cell adhesion to angiogenin is essential for the internalization and nuclear translocation of angiogenin as well as critical for angiogenesis; [4b, 4c, 5] however, its binding mechanism on the cell surface remains unclear because most structural studies to date have focused on the active site of angiogenin. Previous reports suggested a dual site model for angiogenin activity and that its cell-binding site is separate from the active site.[6] These studies suggested that the ^{60}KNGNPHREN^{68} motif is critical for the cell adhesion to human angiogenin (hAng). Soncin et al found that cell adhesion to angiogenin is mediated by heparan/chondroitin sulfate, which is a component in animal tissues.[4c] Heparin, which has a similar structure to heparan/chondroitin sulfate, inhibits the cell adhesion to angiogenin through interactions with angiogenin[4c] but has no effect on the ribonucleolytic activity of angiogenin.[7] The study suggested that R31, R32, R33, and R70 of hAng facilitate binding to heparin and are critical for cell adhesion.[7] Moroianu et al suggested that the putative cell-binding and nuclear translocation signal (NLS) sequence (residues ^{31}RRRGL^{35}) is also important for the internalization and nuclear translocation of hAng. [5b, 5c] They also found that heparin inhibits the internalization of angiogenin.[5c] Nevertheless, the design of an inhibitor to prevent angiogenin from binding to cells and translocating to the nucleus has been hampered due to the lack of a structure of the protein-substrate (or protein-inhibitor) complex that is involved in cell adhesion and nuclear translocation. Herein, we report the crystal structures of the uninhibited rat angiogenin (apo rAng) and a structure of the complex with the heparin inhibitor, elucidating both the inhibition by heparin and the cell adhesion mechanisms of angiogenin.

We determined the crystal structures of apo rAng and 2rAng-heparin complex (Supplementary Information for details). Heparin inhibits heparan/chondroitin-mediated cell adhesion to angiogenin.[4c, 7] Heparin and heparan/chondroitin are members of the glycosaminoglycan family of carbohydrates and consist of a variably sulfated repeating disaccharide unit. Thus, the structure of the 2rAng-heparin complex can provide insight to both the mechanism of cell adhesion to angiogenin and to its inhibition mechanisms. In the 2rAng-heparin structure, two rAng molecules (complex A and complex B) and one heparin composed of 3 repeating units are observed in an asymmetric unit (Fig. 1a). The orientation of side chain H113 in the active site of complex A is different than in complex B or in the apo rAng (Supplementary Information Fig. S3). However, in each case, there are no intramolecular interactions with H113, and two different orientations of H113 were also observed in mAng and hAng. [8-9] The different orientation of the H113 residue may affect the ribonucleolytic activity of angiogenin; however, the activity is not remarkably affected by heparin binding. [7]

Interestingly, the 2rAng-heparin complex reveals that the heparin side chains simultaneously bind to two different regions of rAng, suggesting that rAng has a dual binding mode for heparin inhibitor (Fig. 1). The first heparin-binding site (B1) surrounds the α-helix 2 (α2) of complex A (Fig. 1b, upper). In the first unit of heparin, the hydroxyl group of S28 hydrogen bonds with the hydroxyl group of ΔUA (2.94 Å). In the second unit of heparin, each of the R31, R32, and R33 side chains in the ^{31}RRRGL^{35} motif of complex A interacts with the carboxylate group of IdoA (3.03 Å for NH1-OBD and 2.94 Å for NH2-OBE), the sulfate group of $-CH_2OSO_3^-$ of GlcNS (3.44 Å for NH1-OAQ and 3.50 Å for NH2-OAQ), and the sulfate group of $-NHSO_3^-$ of GlcNS (3.51 Å for NH2-OAO and 2.68 Å for NE-OAM), respectively. In addition, the NZ of the K19 side chain weakly interacts with the sulfate oxygen (OAR) of $-CH_2OSO_3^-$ of GlcNS (4.05 Å). Thus, five amino acids are involved in the binding site B1: four positively charged amino acids (K19, R31, R32, and R33) and one hydroxyl group from S28. As mentioned above, the R31, R32, and R33 residues in hAng are known to be important

residues for heparin binding, cell adhesion, and nuclear translocation. [5b, 7] This result is consistent with the previous results. However, the K19 residue in rAng is substituted by glutamine residue (Q19) in hAng. This substitution may result in small differences in heparin binding and cell adhesion properties between rAng and hAng.

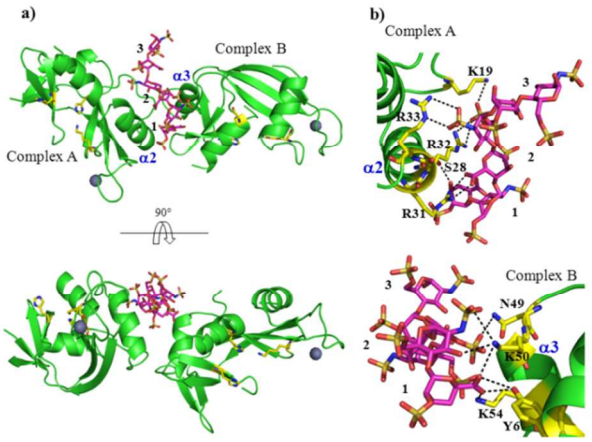

Figure 1. The 2rAng-heparin complex structure (a) and dual binding site (b). In the panel, a) yellow and magenta sticks represent the important residues for ribonucleolytic activity and the heparin backbone, respectively. Complex A and complex B show molecule 1 and molecule 2 of the two rAngs observed in the asymmetric unit, respectively. 1, 2, and 3 denote the order of the heparin molecules from the ΔUA (unsaturated uronic acid) of the first unit to the GlcNS of the third unit of the disaccharide. The first binding site (B1) on complex A (upper) and the second binding site (B2) on complex B (lower) are shown in panel b. The residues colored by yellow sticks are involved in heparin binding on sites B1 and B2.

The second binding site (B2) is observed surrounding the α-helix 3 (α3) of complex B (Fig. 1b, lower). The side chains Y6, N49, K50, and K54 in the B2 site interact with heparin. The hydroxyl group of the Y6 side chain hydrogen bonds with the oxygens (OBE and OBD) of the carboxylate group of ΔUA (2.87 Å and 3.43 Å, respectively) in the first unit of heparin. The ND2 proton of N49 shows weak hydrogen bonding with the sulfate oxygen (OBH) of IdoA (3.42 Å) in the second unit of heparin. The NZ of the K50 side chain has three interactions with the first unit of heparin: two ionic interactions with the carboxylate oxygen (OBD) of ΔUA (3.24 Å) and the sulfate oxygen (OAM) of $-NHSO_3^-$ of GlcNS (3.73 Å) and one hydrogen bond with the hydroxyl group (O3) of GlcNS (3.20 Å). The NZ of the K54 side chain also interacts with the carboxylate oxygen (OBE) of ΔUA (2.44 Å) in the first unit of heparin. These four residues are well conserved in hAng (Supplementary Information Fig. S2a). Uniquely, in the hAng, a positively charged R51 residue is located on helix 3 instead of the G51 residue that is present in rAng; this finding indicates that R51 in hAng might be involved in heparin binding and cell adhesion for internalization in humans. In fact, a previous report suggested that the K54 residue in hAng is linked to ALS in German populations and that the positively charged residues K50, R51, and K54 might interact with negatively charged molecules, such as nucleic acids or other proteins.[2d] Therefore, the structure of the complex supports previous results for hAng, suggesting that the regions of hAng corresponding to the B1 and B2 sites of rAng might be heparin binding sites in hAng. Molecular dynamics simulations suggest that complex A is more favorable (Supplementary Information for details).

The double mutants R31A/R32S and K50L/K54Q of rAng were prepared to examine the effects on both the enzymatic activity and nuclear translocation of rAng. The double R31A/R32S mutant has the same enzymatic activity as the native rAng, but the double mutant K50L/K54Q has a slightly lower activity than that of the native rAng (Supplementary Information Fig. S4). This result indicates that the deletion of positively charged K50 and K54 has some effects on the enzymatic activity of rAng. In contrast, the double mutants R31A/R32S and K50L/K54Q are not translocated into the nucleolus in HeLa cells after 30 min (Fig. 2). These results are consistent with the crystal structure of the 2rAng-heparin complex reported in this study. Together with the previous study[4c, 5b, 5c, 7] and our the 2rAng-heparin complex structure and in vivo studies, the results suggest that the blockage of the dual binding site by heparin is important for the inhibition of the nuclear translocation through the prevention of the cell adhesion to rAng.

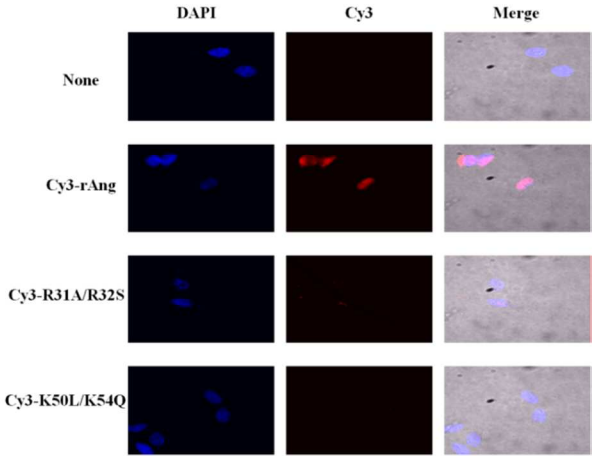

Figure 2. Nuclear translocation of the native rat angiogenin (rAng) versus the double mutants R31A/R32S and K50L/K54Q in HeLa cells. HeLa cells were incubated with Cy3-labeled native rAng (Cy3-rAng) or Cy3-labeled double mutants (Cy3-R31A/R32S and Cy3-K50L/K54Q) of rAng (5 μg/ml) for 30 min at 310 K, and examined for fluorescence using fluorescence microscopy. DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) was used for blue fluorescent nuclear stain. Dual stain on the merged fluorescence image of DAPI and Cy3 shows nuclear translocated the native rat angiogenin in HeLa cells whereas single blue strain shows no nuclear translocation of the double mutants. Original magnification, 60X.

This heparin binding mechanism could facilitate cell adhesion and internalization of hAng through interactions with heparan/chondroitin sulfate on the cell surface because most of the residues participating in heparin binding in sites B1 and B2 are conserved in hAng (Supplementary Information Fig. S2a). The B1 and B2 sites in hAng are positively charged and similar to those of apo rAng (Fig. 3a-b), whereas the surface charges of sites B1 and B2 of RNase A are not positively charged (Fig. 3c),

indicating that RNase A does not have heparin binding sites. RNase A also showed a weak binding to heparin,[7] most likely because heparin interacts weakly with the positively charged residues surrounding the active site of RNase A (Fig. 3c). The surface potential of the 2rAng-heparin complex shows that sites B1 and B2 form a positively charged groove and two units of heparin fit well into the positively charged groove (Fig. 3d). Fig. 3e-f shows the surface charges of apo rAng and hAng aligned to the 2rAng-heparin complex. hAng shows a similar surface potential compared to apo rAng surrounding the heparin binding site because of the high conservation of the positively charged residues. Thus, the data suggest that hAng has a dual cell-binding site, forming a groove induced by heparan/chondroitin sulfate on the cell surface. It appears that the interactions between two apo rAng molecules are not sufficient to stabilize the groove in the absence of the heparin molecule because the groove does not form in the crystal structure of apo rAng. Apo rAng exists solely in the monomeric form in solution and its crystal structure shows a different orientation and different crystal contacts compared to the structure of the complex (Supplementary Information Fig. S1 and S5).

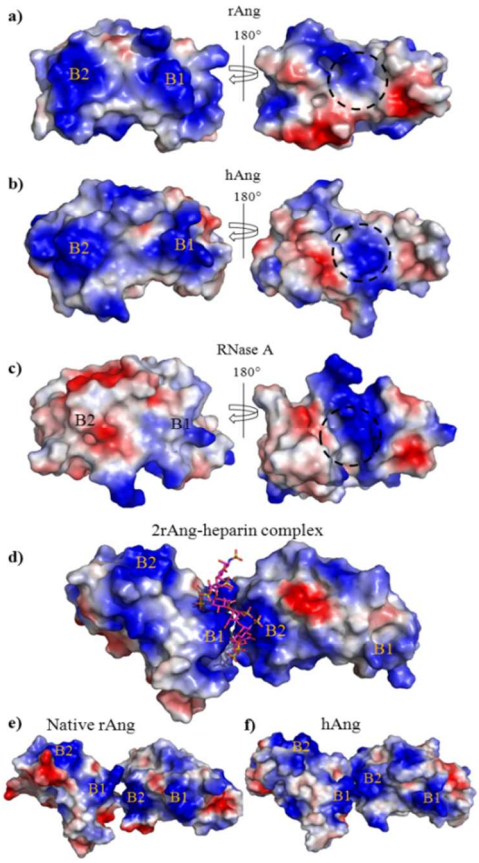

Figure 3. Surface charges for rAng (a, complex B), hAng (b, PDB: 1A4Y), RNase A (c, PDB: 7RSA), and the 2rAng-heparin complex (d). The B1 and B2 denoted on hAng and RNase A correspond to sites B1 and B2 of the rAng complex. The dotted circle represents the active site of each protein. The surface charges of apo rAng (apo A) and hAng are shown in panels e and f, respectively, following structural alignment with the 2rAng-heparin complex.

In addition to sites A and B, the previous report suggested that the ^{60}KNGNPHREN^{68} motif and R70 connect helix 3 to the β-strand 2 of hAng and are responsible for cell adhesion and heparin binding to hAng.[6b, 7] This region shows a highly divergent sequence when compared to rAng (Supplementary Information Fig S2a). In hAng, this region is positively charged because the A59 and G65 residues in rAng are replaced with K60 and R66 residues in the hAng, respectively. Thus, the positively charged region in hAng may facilitate heparin binding and cell adhesion as third binding site in hAng.

In conclusion, our data suggests that a dual cell-binding site of angiogenin is involved in cell adhesion through interactions with heparan/chondroitin sulfate on the cell surface, which supports the findings by previous reports. The heparin molecule blocks the binding sites of angiogenin, resulting in the prevention of cell adhesion, which is an essential step for the internalization and subsequent nuclear translocation of angiogenin. The nuclear translocation of angiogenin is one of the most important steps in angiogenesis because angiogenin enhances ribosomal RNA transcription and activates angiogenic factors in the nucleus.[10] Thus, inhibiting the internalization and the nuclear translocation of angiogenin is critical for the prevention of angiogenesis in pathological conditions. In this report, the structure of the 2rAng-heparin complex shows that the heparin molecule binds to the NLS sequence (^{31}RRRGL^{35}) on site B1 and to the Y6, N49, K50, and K54 residues in site B2, suggesting that heparin inhibits cell adhesion to angiogenin by inhibition of the dual binding site and nuclear translocation by blocking the internalization of angiogenin. Our findings provide a foundation for designing new and specific inhibitors of angiogenesis without causing a loss of ribonucleolytic activity in angiogenin or RNase A. Recent studies revealed that the antibiotic neomycin inhibited PC-3 cell tumor growth in athymic mice by blocking the nuclear translocation of angiogenin and decreased both cancer cell proliferation and angiogenesis.[10c] Based on our results, glycosaminoglycan polymer mimetics,[11] neomycin, and neomycin sulfate could be useful templates for designing inhibitors for cancer therapy.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental section, Tables S1, Figures S1−S5, and supporting references. See DOI: 10.1039/b000000x/

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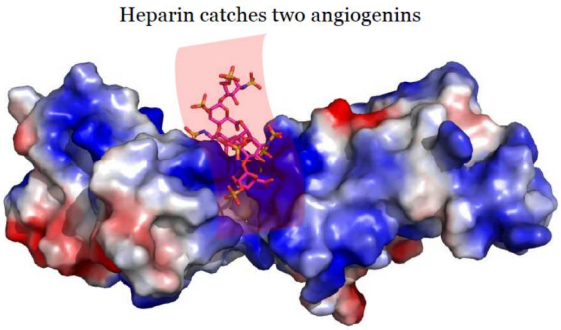

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A negative heparin strand is well fitted into the positive groove formed by the dual heparin-binding site of the angiogenin.