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Fragment-based methods for the discovery of inhibitors modulating lysyl-tRNA synthetase and laminin receptor interaction

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ABSTRACT

Lysyl-tRNA synthetase (KRS) is an enzyme that conjugates lysine to its cognate tRNAs in the process of protein synthesis. In addition to its catalytic function, KRS binds to the 67-kDa laminin receptor (LR) on the cell membrane and facilitates cell migration and metastasis. Modulation of this interaction by small-molecule inhibitors can be exploited to suppress cancer metastasis. In this study, we present fragment-based methods for the identification of inhibitors and monitoring protein–protein interactions between KRS and LR. First, we identified the amino acid residues, located on the KRS anticodon-binding domain, which interact with the C-terminal extension of the LR. One-dimensional (1D) relaxation-edited nuclear magnetic resonance spectroscopy (NMR) and competition experiments were designed and optimized to screen the fragment library. For screening using two-dimensional (2D) NMR, we identified the indicative signals in the KRS anticodon-binding domain and selected inhibitors that bind to KRS and compete with LR at the KRS-LR binding interface. These methods may offer an efficient approach for the discovery of anti-metastatic drugs.

1. Introduction

Aminoacyl-tRNA synthetases (ARSs) are a family of enzymes that catalyze the esterification of specific amino acids to cognate tRNAs, forming the aminoacyl-tRNA complex during protein synthesis [1]. Additionally, ARSs have non-catalytic functions that control transcription, translation, immune responses, angiogenesis, and cell migration [1–6]. Among these, the interaction between lysyl-tRNA synthetase (KRS) and the laminin receptor (LR) is an important factor for the migration of cancer cells, and recognized as an attractive target for anti-metastatic drugs [7,8].

KRS is normally expressed in the cytosol as a component of the multi-synthetase complex (MSC), and it is known to be co-localized with the laminin receptor (LR/RPSA; gene identification: 3921) on the plasma membrane after laminin stimulation [7]. The laminin-induced dissociation of KRS from MSC, and the translocation of KRS into the membrane, depends on the phosphorylation of KRS at T52 via the PI3K-p38MAPK pathway. On the plasma membrane, KRS binds to LR, and this interaction protects the laminin receptor from NEDD4 (an E3 ubiquitin-protein ligase)-mediated ubiquitination and increased cell migration [7].

The LR/ribosomal protein SA (RPSA) is a component of the ribosome; it is located in the plasma membrane in its dimeric form (p67LR) after its monomeric form (37LRP) undergoes fatty acid acylation. On cell membrane, LR has high affinity for laminin and plays an important role in tumor cell migration and invasion [9]. LR contains an N-terminal folded domain (residues 1–209), the structure of which has been determined using X-ray crystallography [10], and a metazoan-specific C-terminal extension (residues 210–295), which is intrinsically disordered [11]. Because KRS is highly overexpressed in many types of cancer cells, and the translocation of KRS leads to stabilization of p67LR on the cell membrane, modulating the interactions between KRS and LR is an attractive strategy for the discovery of anti-metastatic drugs.

Targeting protein–protein interactions (PPIs) by small-molecule inhibitors is of great interest for therapeutic purposes as well as for the identification and validation of therapeutic targets [12]. Disrupting PPIs by small-molecule inhibitors can be difficult because of large binding interfaces; therefore, it is often more feasible to modulate certain interactions such as those between the globular domain of one protein and the short peptide of its partner. For KRS and LR interaction, the binding domains for each protein have been investigated using NMR-based interaction study [11]. In our

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previous study, we found that the anticodon-binding domain of KRS binds to the C-terminal extension of LR [11]. Because the domains responsible for the interaction between KRS and LR have molecular weights of 14 and 9 kDa, respectively, it is likely that the binding interface is relatively small compared with that of the other large PPIs; this implies that it is feasible to interrupt the binding by small-molecule inhibitors. However, because most of the NMR signals from the anticodon-binding domain disappeared by LR-binding, the surface of the KRS anticodon-binding domain remains unexplored. Here we used NMR with LR C-terminal extension to identify the binding interface on the KRS anticodon-binding domain. Characterization and analysis of the binding surface provide valuable information for designing new small-molecule inhibitors.

NMR-based fragment discovery is a useful strategy for modulating many biological interactions; often, these interactions are highly dynamic with relatively weak binding affinities. The sensitivity of NMR makes it suitable for the identification of weak physical interactions. Various NMR-based screening methods have been successfully applied [13–15]. Among them, the relaxation-edited 1D NMR for identifying the binding of small molecules to target proteins is useful because it requires a relatively short measurement time and directly monitors the signals from the compounds [16]. For the discovery of compounds that bind to KRS, we improved this method by optimizing protein concentration, which enabled us to use less protein, thereby reducing non-specific binding. To identify the binding sites of the new compounds, we designed a competition experiment using known compounds. This approach enabled an efficient confirmation of the binding site of the selected compounds in very short time.

Identification of hot spot residues for PPIs is critical for designing new inhibitors. The analysis of KRS residues that interact with LR and the known inhibitors, BC-K-YH16899 and BC-K-01, provides information for a common hydrophobic pocket. In this study, we analyzed the hot spot residues that form the hydrophobic pocket and nearby cleft where fragment evolution can be applied. Among these hot spot residues, several amide (NH) groups near the pocket surface can be exploited for 2D NMR screening to find new inhibitors. Chemical shift perturbations (CSPs) for these indicative signals are monitored in compound screening [17]. Using 2D NMR screening, we can identify small-molecule inhibitors of the KRS-LR interaction. Combining 1D and 2D NMR provides an efficient method for fragment-based lead discovery modulating KRS-mediated tumor metastasis.

2. Materials and methods

2.1. Preparation of protein samples

The N-terminal fragment of the human lysyl-tRNA synthetase (residues 1–207; KRS_{1-207}) with the N-terminal hexa-histidine tag was expressed under the control of the T7 promoter in an expression vector pET28a (Novagen) using *Escherichia coli* BL21 (λDE3) as described previously. To obtain a uniformly labeled ^{15}N KRS_{1-207}, the bacterial cells were grown in M9 minimal medium containing $^{15}NH_4Cl$ as the sole source of nitrogen (99% ^{15}N; Cambridge Isotope Laboratories). After induction with 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at a cell density (OD_{600}) of 0.6, the cells were further grown for 3 h at 37 °C, then harvested and resuspended in lysis buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF] at pH 7.0). Cells were then disrupted by sonication on ice, and cell lysates were centrifuged at 32,500*g* for 60 min at 4 °C. The supernatant was loaded into a 5-ml Ni-NTA (HisTrap; GE Healthcare) column pre-equilibrated with buffer A (20 mM HEPES, 500 mM NaCl, 10% glycerol, 1 mM PMSF, and 50 mM imidazole at pH 7.0). KRS_{1-207} was eluted out of the column by using buffer A containing 1 M imidazole. The hexa-histidine tag was cleaved by subjecting it to an overnight incubation with thrombin at 4 °C during dialysis against buffer A without PMSF. After cleavage, proteins were obtained from the flow-through of the Ni-NTA column. After dialysis against 20 mM HEPES, 100 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM PMSF at pH 7.0, proteins were concentrated by ultrafiltration (Millipore, 3000 MWCO) and loaded onto a Superdex S200 gel-filtration column (16 mm/60 cm, GE healthcare) pre-equilibrated with 20 mM HEPES, 100 mM NaCl, 1 mM PMSF, and 1 mM DTT at pH 7.0.

The C-terminal fragment of the human laminin receptor (residues 210–295, $LR_{210-295}$; LR/RPSA; gene identification: 3921) construct was made by inserting the LR sequence into the pET-28a (Novagen) vector after the SUMO sequence. His-SUMO-$LR_{210-295}$ were overexpressed in *E. coli* BL21 (λDE3) by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at a cell density (OD_{600}) of 0.6 at 18 °C. Cells were then cultured overnight at 18 °C, harvested by centrifugation at 2970*g* for 30 min at 4 °C, and resuspended in lysis buffer (20 mM Tris-Cl [pH 8.0], 300 mM NaCl, 10% glycerol, and 1 mM PMSF). Next, cells were disrupted by sonication on ice, and cell lysates were centrifuged at 32,500*g* for 60 min at 4 °C. The supernatant was loaded into a 5-ml Ni-NTA (HisTrap; GE Healthcare) column pre-equilibrated with buffer B (20 mM Tris-Cl [pH 8.0], 500 mM NaCl, 10% glycerol, 1 mM PMSF, and 50 mM imidazole). His-SUMO-$LR_{210-295}$ was eluted out from the column with buffer B containing 500 mM imidazole. The SUMO tag was cleaved by subjecting to an overnight incubation with Ulp (ubiquitin-like-specific protease 1, kindly provided by Dr. Song at KAIST, Korea) at 4 °C during dialysis against buffer B without PMSF. Tag-free $LR_{210-295}$ was obtained from the flow-through of the Ni-NTA column after SUMO cleavage. After dialysis against 20 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM DTT, and 1 mM PMSF, tag-free $LR_{210-295}$ was concentrated by ultrafiltration (Millipore, 3000 MWCO) and loaded onto a Superdex S200 gel-filtration column (16 mm/60 cm, GE healthcare) pre-equilibrated with 20 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM PMSF, and 1 mM DTT.

A glutathione-S-transferase-fused 37LRP (residues 1–295; GST-LR) construct was made by inserting the laminin receptor sequence into the pGEX-4T-1 (Novagen) vector after the GST sequence. GST-LR was overexpressed in *E. coli* BL21 (λDE3) by the addition of 0.5 mM IPTG at a cell density (OD_{600}) of 0.5 at 18 °C. Cells were cultured overnight at 18 °C, harvested by centrifugation at 2970*g* for 30 min at 4 °C, and resuspended in lysis buffer (PBS [pH 7.4] and 1 mM PMSF). Then, cells were disrupted by sonication on ice and cell lysates were centrifuged at 32,500*g* for 60 min at 4 °C. The supernatant was loaded onto a 5-ml glutathione sepharose (GSTrap; GE Healthcare) column pre-equilibrated with phosphate buffered saline (pH 7.4). GST-LR was eluted out from the column with buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM reduced glutathione, and 1 mM PMSF. After concentration by ultrafiltration (Millipore, 30,000 MWCO), the GST-LR was loaded onto a Superdex S200 gel-filtration column (16 mm/60 cm, GE healthcare) pre-equilibrated with 20 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM PMSF, and 1 mM DTT.

2.2. NMR spectroscopy

All NMR measurements were performed with an Avance 600 MHz NMR spectrometer equipped with a triple-resonance, pulsed field gradient probe (Bruker, Germany). All spectra were measured at 298 K. Data processing and analysis were conducted using Topspin 3.1 program (Bruker, Germany).

The pulse sequence used to record the ^{1}H-^{15}N transverse relaxation optimized spectroscopy (TROSY) experiments was essentially