論文の内容の要旨

論文題目

Development of artificial MET activators based on engineered self-assembling protein capsules

（自己集合タンパク質カプセルを元にした人工 MET 活性化分子の開発）

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Introduction

Hepatocyte growth factor receptor (HGFR), also called as MET, is a membrane protein that regulates a lot of downstream signaling proteins related to embryonic development, tissue regeneration and cancer metastasis. MET proteins bind to dimers of natural ligands, HGF, and are dimerized. Dimerized MET proteins automatically phosphorylate each other and promote the downstream signaling cascade (**Fig. 1a**). Because the MET signaling cascade plays an important role in organogenesis and wound healing, artificial activators of the MET cascade can be utilized for regenerative medicine. In previous researches, some artificial MET activators, such as a dimer of DNA aptamers and macrocyclic peptides have been developed. However, these molecules have complexity in chemical synthesis and limitations in further modifications.

To overcome these problems, I focused on capsids; a kind of self-assembling proteins. They can be easily prepared in bulk by *Escherichia coli* expression systems and have a lot of reaction points for further chemical modifications per one molecule such as lysine residues and cysteine residues, which can be utilized for drug conjugations and diagnostic agent conjugations. Because capsids consist of several dozens to hundreds of monomeric proteins, it is thought that capsids composed of monomeric proteins displaying the MET binding moiety on their surfaces can induce dimerization of MET proteins and activation of the MET cascade (**Fig. 1b**). To implement this system, I opted the *Aquifex aeolicus* Lumazine Synthase (AaLS) capsid as a scaffold of artificial MET activators, because it has thermal stability, hollow body and resistance against various genetic engineering.

This study aimed to develop a new artificial MET activator by using the AaLS capsid as a scaffold. I designed and displayed a MET binding peptide sequence on the outer surface of AaLS capsids and demonstrated artificial MET activations. I also modulated and enhanced the activity of obtained activator semi-quantitatively.

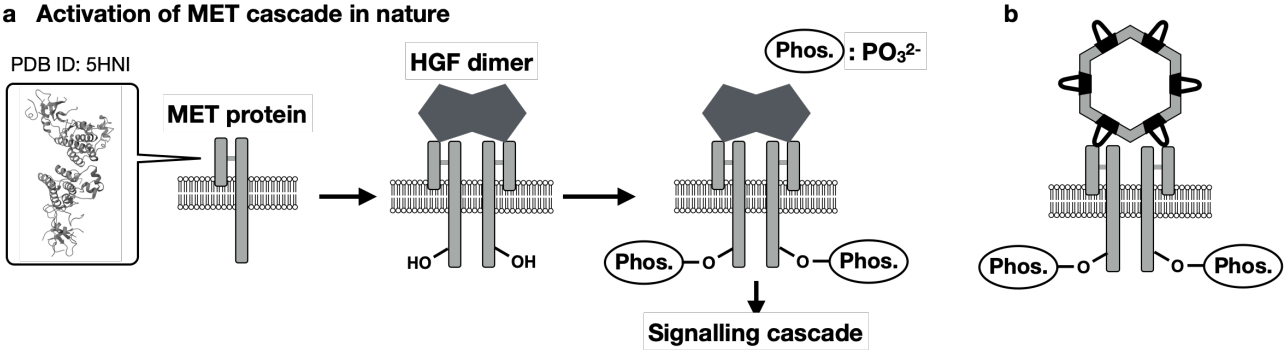

Figure 1 (a) Dimerization and autophosphorylation of MET proteins induced by HGF in nature
(b)Design of artificial MET activation in this study

Development of artificial MET activator based on AaLS capsid scaffolds

In order to dimerize MET proteins artificially, I utilized three macrocyclic peptides that strongly binds to MET (K_D = 2-19 nM), named as aMD4, aMD5 and aML5 and displayed them on AaLS monomeric proteins (capsomeres). Although these peptides are cyclized by a thioether bond between an acetyl residue of N-acetyl tyrosine and a thiol residue of a cysteine side chain, the essential peptide sequences for binding to MET consists of exclusively proteogenic amino acids, which can be expressed by *E.coli*. To display these peptides on the surface of AaLS capsids, I replaced the outer loop of circularly permutated AaLS capsomeres with the essential amino acid sequence of aMD4, aMD5 and aML5 peptide (**Fig. 2**). To do this, I prepared plasmids that encode the amino acid sequence of this engineered capsomere and co-expressed them with AaLS wild type capsomeres. The two different capsomeres automatically co-assembled to chimera capsids in *E.coli* bodies. The co-assembled capsids were then extracted from *E.coli*. After purification by affinity column chromatography and size exclusion chromatography, I analyzed their shapes and sizes by a transmission electron microscope . TEM images show engineered capsids have a diameter of 16-18 nm, which is almost the same size of AaLS wild type capsids.

Next, in order to measure activities of engineered capsids, MET phosphorylation level induced by engineered capsids was quantified by western blotting . As the result, capsids displaying aMD5 and aML5 don't exhibit significant activities of MET dimerization and phosphorylation. However, the capsid displaying aMD4 successfully induced phosphorylation of MET in a concentration dependent manner.

Semi-quantitative modulation and improvement of activity of the developed MET activator

To confirm that obtained capsids comprise both of AaLS wild type capsomeres and engineered capsomere, I analyzed them by SDS-PAGE. The upper band of the SDS-PAGE image correspond to the engineered capsomere and the lower band correspond to the AaLS wild type capsomere, which indicates that purified capsids are composed of both capsomeres. The results also show that the ratio of AaLS wild type capsomeres and engineered capsomeres can be

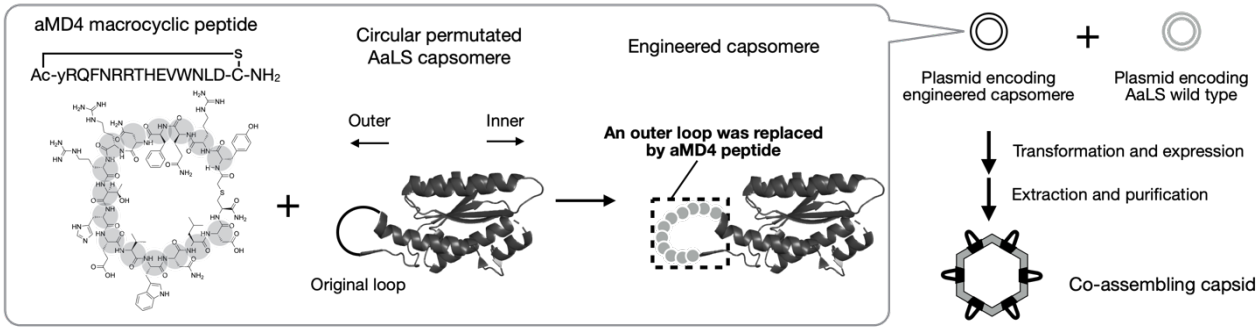

Figure 2 Design and strategy of expression of engineered capsids. As a representative example, the sequence of aMD4 is shown. MET binding motif of the aMD4 macrocyclic peptide (marked by gray circules) was embedded in the original outer loop of circularly permutated AaLS capsomere. Engineered capsomeres were expressed with AaLS wild type capsomeres by using two kinds of plasmids.

controlled semi-quantitatively by regulating the expression level of engineered capsomeres. When expression of engineered capsomere is induced by 1.0 μg/mL, 0.5 μg/mL and 0.1 μg/mL of tetracycline, the ratio of engineered capsomeres in co-assembling capsids was 50.0 %, 37.8 % and 30.6 % respectively. The activities of these engineered capsids were measured. As the ratio of capsomere displaying aMD4 peptide increased, the dimerization activity improved. This result indicates that not only the ratio of composing capsomere but also the activities of capsids can be modulated.

To maximize the activity of engineered capsids, I engineered the cpAaLS-aMD4 capsomere and introduced cysteines at both of front and rear positions of aMD4 peptide. I hypothesized that these cysteines form a disulfide bond and assist aMD4 to form its original conformation. I expressed this new capsomere, named as cpAaLS-aMD4C, with AaLS wild type capsomeres and obtained co-assembling capsids with a ratio of engineered capsomeres of 49.6%. MET activation assay of these capsids show the introduction of disulfide bonds improved the activity of AaLS-based MET activators and a stronger artificial MET activator was obtained.

Conclusion

New artificial MET activators based on self-assembling protein capsules were developed by utilizing a macrocyclic peptide and AaLS capsid scaffolds. The activity can be controlled by modulating the displaying ratio of macrocyclic peptides and further enhanced by inducing disulfide bonds at the base of macrocyclic moieties. It is known that MET activation also induces internalization of MET proteins and binding ligands into the endosome and lysosome. The developed system is applicable for not only artificial MET activation and regenerative medicine, but also specific drug delivery to cells expressing MET on their surfaces.