Patent Literature

[0004]

Patent Literature 1: US 9428845 B

Patent Literature 2: US 2018-0164324 A

Non Patent Literature

[0005]

Non Patent Literature 1: Ulrich, H. W. et al. CANCER GENOMICS & PROTEOMICS 10: 1-18 (2013)

Non Patent Literature 2: Cabantous, S. et al. Sci Rep. 2013; 3: 2854. doi: 10.1038/srep02854

Non Patent Literature 3: Koraichi, F. et al. Journal of Cell Science 2018; 131: jcs210419 doi: 10.1242/jcs.210419

Summary ~~of Invention~~

Technical Problem

[0006]

When it is sought to obtain a molecule that forms a complex with a plurality of target molecules, possible methods include a method (i) of screening binders for each target molecule and then linking the obtained binders, and a method (ii) of obtaining a binder for one target molecule, and then contacting a complex of the target molecule and the binder with another target molecule.

[0007]

Increasing oral absorption and targeting an intracellular target requires reducing the molecular weight of candidate molecule to some extent. Thus, when aiming to obtain a candidate molecule for such purposes, the method (i) of obtaining binders for each target molecule and then linking the obtained binders will impose a significant limitation on the molecular weights of the individual binders. Contrarily, when the molecular weight is too small, limitations are imposed on the structure of the candidate molecule and the binding site of the target molecule that can be used for binding, thus the target molecules that can be applied are limited. Meanwhile, the method (ii) is a method for screening a library for a molecule having a desired property, rather than linking two molecules, and thus the first step is to select a single target molecule by mono-specificity. In this step, molecules having a predominant binding mode to the target (first target) molecule are enriched, and thus, in the next step, molecules having the desired properties are selected from a population of molecules that are predominated in the binding mode to the first target. The predominant binding mode to the first target molecule does not necessarily match the binding mode to form a complex with the plurality of

target molecules, thus the diversity of the compounds to be screened is limited. Thus, it is desired to have a screening method capable of directly selecting a molecule having a desired property from a library allowing a variety of binding modes to each target molecule.

[0008]

In addition, as more diversity of properties of the candidate molecule having not only the binding activity to a single target molecule is required, the need for screening a library containing a variety of test molecules increases. Furthermore, to form a complex with a plurality of target molecules, the candidate molecule is required to provide a complicated binding and reaction mode, which makes it difficult to predict the optimal molecular structure. Thus, a high throughput screening method that is applicable to libraries as diverse as possible and is easy to select and identify candidate molecules is required.

[0009]

Split GFP techniques is utilized for detection of protein-protein interactions (PPIs) (Non Patent Literature 2). However, since the emission of GFP is used as an indicator, the throughput capacity when applied for screening is not high. That is, in the case of fluorescence detection, a target molecule and a single candidate molecule are required to provide in each closed space such as a well, which is laborious and costly, and limits the number of test molecules that can be handled. Even if a library containing a plurality of test molecules in each confined space is prepared and screened for efficiency purposes, multiple assays are required step by step to select and identify a particular candidate molecule until fluorescence of only a single molecule can be ascertained. Thus, the throughput capacity is not high either. From these viewpoints, new methods to detect and recover divided proteins in proximity or association, instead of the method that uses the luminescence of the GFP proteins as an indicator, are required.

[0010]

The present ~~invention~~ disclosure has been made considering such circumstances, and one object of the present ~~invention~~ disclosure is to provide a method for screening for a molecule capable of forming a complex with a plurality of target molecules.

Solution to Problem

[0011]

The present inventors have considered that performing a screening of test molecules contained in a library and a plurality of target molecules which are allowed to coexist in the same system is the most effective way to produce diverse reaction modes with the target molecules. To this end, the present inventors have applied an affinity-based method for recovering candidate molecules to the techniques represented by Split GFP techniques, and have found a method for

screening for a molecule capable of forming a complex with a plurality of target molecules. It has been found in this method that a candidate molecule capable of forming a complex with a plurality of target molecules can be recovered while suppressing the recovery rate of a test molecule that forms a complex with only one target molecule.

[0011a] In one aspect, the present disclosure provides a method for screening for a candidate molecule capable of forming a complex with a first target molecule and a second target molecule, the method comprising steps (1) and (2) below:

(1) mixing the first target molecule linked to a first moiety, the second target molecule linked to a second moiety, and a library containing a plurality of test molecules, wherein the first moiety and the second moiety constitute a part or all of a protein; and

(2) recovering, after step (1), a complex containing the first target molecule linked to the first moiety, the second target molecule linked to the second moiety, and the candidate molecule by an affinity-based method using a third molecule capable of detecting that the first moiety and the second moiety are in proximity or association.

[0012]

The present ~~invention~~ disclosure is based on such findings, and, in one specific, non-limiting ~~aspect~~example, encompasses the following.

[1] A method for screening for a candidate molecule capable of forming a complex with a first target molecule and a second target molecule, the method comprising steps (1) and (2) below:

(1) mixing the first target molecule linked to a first moiety, the second target molecule linked to a second moiety, and a library containing a plurality of test molecules, wherein the first moiety and the second moiety constitute a part or all of a protein; and

(2) recovering, after step (1), a complex containing the first target molecule, the second target molecule, and the candidate molecule by an affinity-based method using a third molecule capable of detecting that the first moiety and the second moiety are in proximity or association.

[1.1] The method according to [1], wherein the first target molecule and the second target molecule in step (2) is the first target molecule linked to a first moiety and the second target molecule linked to the second moiety, respectively.

[2] The method according to [1] or [1.1], further comprising, after step (2), a step of identifying the candidate molecule contained in the recovered complex (step (3)).

[3] The method according to [1] or [2], wherein the third molecule is a molecule that reacts with the first moiety and/or the second moiety when the first moiety and the second moiety are in proximity or association.

[4] The method according to any one of [1] to [3], wherein the third molecule is a molecule that reacts with the first moiety and/or the second moiety when a complex containing the first target molecule, the second target molecule, and the candidate molecule is formed.

[5] The method according to any one of [1] to [4], wherein the third molecule is a molecule that does not substantially react with the first moiety and/or the second moiety when the first moiety and the second moiety are neither in proximity nor in association.

[6] The method according to any one of [1] to [5], wherein the third molecule is a molecule that reacts with the first moiety and/or the second moiety in less than one-tenth when the first moiety and the second moiety are neither in proximity nor in association, compared to when the first moiety and the second moiety are in proximity or association.

[7] The method according to any one of [1] to [6], wherein the third molecule is a molecule that reacts with the first moiety and/or the second moiety in less than one-twentieth when the first

Claims

[Claim 1]

A method for screening for a candidate molecule capable of forming a complex with a first target molecule and a second target molecule, the method comprising steps (1) and (2) below:

(1) mixing the first target molecule linked to a first moiety, the second target molecule linked to a second moiety, and a library containing a plurality of test molecules, wherein the first moiety and the second moiety constitute a part or all of a protein; and

(2) recovering, after step (1), a complex containing the first target molecule linked to the first moiety, the second target molecule linked to the second moiety, and the candidate molecule by an affinity-based method using a third molecule capable of detecting that the first moiety and the second moiety are in proximity or association.

[Claim 2]

The method according to claim 1, further comprising, after step (2), a step of identifying the candidate molecule contained in the recovered complex (step (3)).

[Claim 3]

The method according to claim 1 or 2, wherein the third molecule is a molecule that reacts with the first moiety and/or the second moiety when the first moiety and the second moiety are in proximity or association.

[Claim 4]

The method according to any one of claims 1 to 3, wherein the third molecule is a molecule that does not substantially react with the first moiety and/or the second moiety when the first moiety and the second moiety are neither in proximity nor in association.

[Claim 5]

The method according to any one of claims 2 to 4, further comprising, between steps (2) and (3), a step of eluting the candidate molecule from a complex containing the first target molecule linked to the first moiety, the second target molecule linked to the second moiety, and the candidate molecule (step 2A).

[Claim 6]

The method according to claim 5, wherein step (3) comprises determining a sequence of a polynucleotide encoding the candidate molecule eluted in step (2A).

[Claim 7]

The method according to any one of claims 1 to 6, comprising a step of repeating steps (1) and (2) a plurality of times.

[Claim 8]

The method according to any one of claims 1 to 7, wherein the method is performed in vitro.

[Claim 9]

The method according to any one of claims 1 to 8, wherein the test molecule is a molecule encoded by a polynucleotide.

[Claim 10]

The method according to any one of claims 1 to 8, wherein the test molecule is a peptide.

[Claim ~~9~~11]

The method according to any one of claims 1 to ~~8~~10, wherein the library is a display library or a DNA encoding library.

[Claim ~~10~~12]

The method according to any one of claims 1 to ~~9~~11, wherein the library is a library having a diversity of 1×10^4 or more.

[Claim 13]

The method according to any one of claims 1 to 12, wherein the candidate molecule is a peptide.

[Claim 14]

The method according to any one of claims 1 to 13, wherein the complex which the candidate molecule is capable of forming with the first target molecule and the second target molecule is a complex of three components.

[Claim 15]

The method according to any one of claims 1 to 14, wherein the first moiety and the first target molecule and the second moiety and the second target molecule are each linked by a linker.

[Claim ~~11~~16]

The method according to any one of claims 1 to ~~10~~11, wherein the protein is GFP, luciferase, ubiquitin, or a SNAP tag.

[Claim 17]

The method according to any one of claims 1 to 16, wherein the first moiety and the second moiety are GFP10 and GFP11, respectively, or GFP11 and GFP10, respectively, and the third molecule is a protein containing GFP1-9.

[Claim 18]

The method according to any one of claims 1 to 16, wherein the first moiety and the second moiety are an N-terminal fragment of a SNAP tag and a C-terminal fragment of a SNAP

tag, respectively, or a C-terminal fragment of a SNAP tag and an N-terminal fragment of a SNAP tag, respectively, and the third molecule is a SNAP biotin.

[Claim 19]

The method according to any one of claims 1 to 16, wherein the first moiety and the second moiety are an N-terminal fragment of ubiquitin and a C-terminal fragment of ubiquitin, respectively, or a C-terminal fragment of ubiquitin and an N-terminal fragment of ubiquitin, respectively, and the third molecule is an anti-ubiquitin antibody.

[Claim 20]

The method according to any one of claims 1 to 16, wherein the first moiety and the second moiety are Nanoluc-9 and Nanoluc-10, respectively, or Nanoluc-10 and Nanoluc-9, respectively, and the third molecule is a protein containing Nanoluc1-8.

[Claim 21]

The method according to any one of claims 1 to 20, wherein the first target molecule is a protein belonging to an immunophilin family.

[Claim 22]

The method according to any one of claims 1 to 21, wherein the first target molecule is selected from the group consisting of a FK506 binding protein (FKBP), cyclophilin, FRB, calcineurin, and Ras proteins such as Kras, Nras, and Hras.

[Claim ~~12~~23]

The method according to any one of claims 1 to ~~11~~22, wherein the affinity-based method is a pull-down method.

[Claim ~~13~~24]

The method according to any one of claims 5 to ~~12~~23, wherein the eluting in step (2A) is performed with an enzyme or by heat.

[Claim ~~14~~25]

The method according to any one of claims 5 to ~~13~~24, wherein the eluting in step (2A) is performed by cleaving a first linker located between the first moiety of the protein and the first target molecule and a second linker located between the second moiety of the protein and the second target molecule.

[Claim ~~15~~26]

The method according to any one of claims 1 to ~~14~~25, wherein the protein providing the first moiety and the second moiety contains an amino acid sequence selected from the group consisting of (a) to (c) below;

(a) an amino acid sequence represented by SEQ ID NO: 71, 75, 78, or 82;

(b) the amino acid sequence represented by SEQ ID NO: 71, 75, 78, or 82 in which one or more amino acids are deleted, inserted, substituted, and/or added; and

(c) an amino acid sequence having 80% or more sequence identity to the amino acid sequence represented by SEQ ID NO: 71, 75, 78, or 82.

[Claim 27]

The method according to any one of claims 1 to 26, wherein the third molecule is derived from the protein that provides the first moiety and the second moiety.

[Claim 28]

The method according to any one of claims 1 to 27, further comprising the following steps of:

(4) mixing the first target molecule fixed on a solid phase support and a library containing a plurality of test molecules, and recovering a test molecule capable of binding to the first target molecule; and/or

(5) mixing the second target molecule fixed on a solid phase support and a library containing a plurality of test molecules, and recovering a test molecule capable of binding to the second target molecule.