

[DESCRIPTION]

[Title of Invention] METHOD FOR DETECTING PROTEIN-PROTEIN INTERACTION

[Technical Field]

5 The present invention relates to a method for detecting a protein-protein interaction, applications thereof, and a kit for use in the method.

[Background Art]

10 Methods for detecting a protein-protein interaction can be roughly categorized into two groups. One is a method characterized by using a protein having been separated from living cells. Examples of such a method include surface plasmon resonance, protein mass spectroscopy, and anisotropy measurements. However, these methods have
15 difficulty detecting an interaction in an environment similar to an actual intracellular environment.

20 Then, as the second method, a method has been developed, in which a protein-protein interaction is detected using living cells. Typical methods thereof are a yeast two hybrid system which detects a transcriptional activity of a reporter, and modified methods thereof. Besides, another method has been also developed, which utilizes reconstitution of enzymes such as β -galactosidase and dihydrofolate reductase (DHFR).

25 Nevertheless, these methods have problems that they are incapable of detecting a position where a

protein-protein interaction has taken place (positional information on the protein-protein interaction), as well as a period until a protein-protein interaction takes place, a period until the interaction ends, a duration of the interaction, and the like (temporal information on the protein-protein interaction).

Meanwhile, the method for detecting a protein-protein interaction using living cells also includes a method utilizing reconstitution of a fluorescent protein. Nevertheless, once reconstituted, the fluorescent protein does not dissociate. Accordingly, this method has a problem that it is incapable of detecting a period until a protein-protein interaction ends, a duration of the interaction, and the like. Further, there is another problem that a period until a protein-protein interaction takes place and the like cannot be detected because emission of fluorescence requires a certain time after a protein-protein interaction takes place.

Furthermore, there is also a method utilizing a luciferase reconstitution technique. In such a method, a luciferase is reversibly reconstituted and dissociated. However, since the luminescent signal emitted by a reconstituted luciferase is weak, the exposure time has to be a long in order to obtain intracellular positional information, and positional information and temporal information on a protein-protein interaction with high

turnover rate cannot be obtained.

Additionally, in the methods utilizing the reconstitution of a fluorescent protein, a luciferase, or the like, a signal can be detected only after such a reconstitution. This also brings about such a problem that it is difficult to trace, for example, both before and after a protein-protein interaction takes place, proteins which are located at different positions by the interaction.

On the other hand, as a method for detecting a protein-protein interaction in living cells, fluorescence resonance energy transfer (FRET) has been developed, which detects energy transfer dependent on a distance between molecules. This method has an advantage of obtaining positional information and temporal information on where and when a protein-protein interaction takes place. Nevertheless, since a positional relation between a donor fluorescent protein and an acceptor fluorescent protein used in the method is important to detect the protein-protein interaction, the method involves a complicated step of investigating the optimization of a linker (spacer) connecting these fluorescent proteins to a detection-target protein, so that such a system has been difficult to construct. Further, it has also been difficult to analyze the result due to cross excitation by which an acceptor fluorescent protein is excited, and to bleed-through in which fluorescence of a donor

fluorescent protein bleeds through a filter (absorption filter) set for detecting fluorescence of an acceptor fluorescent protein. Moreover, use of fluorescent proteins of two colors (donor fluorescent protein and acceptor fluorescent protein) also brings about a problem that only limited fluorescent proteins are usable in order to detect information other than a detection-target protein.

Recently, Tobias Meyer et al. have reported a method for detecting a protein-protein interaction by utilizing intracellular localization (translocation) (PTL 1). In this method, one of proteins subjected to interaction detection is fused to a protein that specifically binds to a particular site in a cell, while the other of the proteins subjected to interaction detection is fused to a fluorescent protein or the like. Then, these fusion proteins were expressed in a cell, and the protein-protein interaction is detected on the basis of a signal of the fluorescent protein or the like at the particular site in the cell.

In addition, Nibert et al. have reported a method for detecting a protein-protein interaction, using a fusion protein in which one of proteins subjected to interaction detection is fused to a protein for forming a viral inclusion body, and using, as an indicator, accumulation of the other of the proteins subjected to interaction detection in the

viral inclusion body (PTL 2).

However, in these methods for detecting a protein-protein interaction by utilizing intracellular localization, one of proteins subjected to interaction detection is forcibly (artificially) translocated and confined at a particular site in a cell. Accordingly, the detection is impossible at a site where a protein-protein interaction naturally takes place, that is, in an intracellular environment unique to the protein-protein interaction, which brings about a problem that positional information on the protein-protein interaction cannot be obtained, and other similar problems. Moreover, it is also impossible to detect the interaction between proteins localized in a natural state at the same site as the site of the intracellular localization.

Against this problem, Sara Peterson Bjorn et al. have reported a method for detecting a protein-protein interaction (redistribution-trap method), in which proteins are allowed to interact with each other in an intracellular environment where the proteins naturally function, and then the cells are stimulated with a drug or the like to thereby induce aggregate formation from the interacting proteins, the aggregate formation being indicative of the interaction (PTL 3).

However, this method needs to stimulate cells at certain time so that the aggregate formation can be induced,

and also needs to remove the drug or the like used for the stimulation to detect the presence or absence of an interaction subsequently after the stimulation. Hence, the method has problems such as being incapable of obtaining temporal information on when the protein-protein interaction takes place, and incapable of detecting a protein-protein interaction that changes (takes place, ends, takes place again, and so forth) for a certain period and at a certain position.

[Citation List]

[Patent Literature]

[PTL 1] International Publication No. W02000/017221

[PTL 2] International Publication No. W02006/099486

[PTL 3] United States Patent No. 7282347

[Summary of Invention]

[Technical Problem]

The present invention has been made in consideration of the above-described problems in the conventional techniques. An object of the present invention is to provide a method capable of detecting a protein-protein interaction in a cell in an intracellular environment unique to the protein-protein interaction, and also capable of detecting positional information and temporal information on the protein-protein interaction.

[Solution to Problem]

The present inventors have come up with an idea of

utilizing, in detecting an interaction between two proteins (a first protein and a second protein), a first fusion protein comprising the first protein and an association-inducing protein, and a second fusion protein comprising the second protein and a fluorescent protein having a multimerization ability. Specifically, the inventors have come up with a construction of a system as follows. When the two fusion proteins are expressed in a cell, an interaction if any between the first protein and the second protein induces an association action between the association-inducing protein and another association-inducing protein. Thereby, the fusion proteins autonomously form an assembly, and the fluorescent protein contained in the fusion protein is detected as a fluorescent focus (see Figs. 1 and 2). Further, the inventors have come up with the utilization, as the association-inducing protein, of a protein having such natures of: being present in a dispersed manner in a cell when fused to monomeric Azami Green 1 (mAG1), one of monomeric fluorescent proteins; and forming a fluorescent focus (assembly) in a cell when fused to a fluorescent protein having a multimerization ability.

Hence, the present inventors, first, expressed in cells candidate proteins fused to mAG1 or fluorescent proteins having a multimerization ability, and screened for association-inducing proteins on the basis of

fluorescent focus formation (see Figs. 3 and 4).

The screening result has revealed that a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota, a SAM domain of TEL, a SAM domain of DGK delta, and a SAM domain of Tankyrase-1 are usable as the association-inducing protein.

Then, the present inventors have revealed that the use of these identified proteins as association-inducing proteins in combination with fluorescent proteins having a multimerization ability indeed enables detection of certain protein-protein interactions on the basis of fluorescent foci.

This method is capable of detecting a protein-protein interaction in an intracellular environment unique to the protein interaction, and also capable of detecting positional information and temporal information on the protein-protein interaction. Moreover, it is also possible to identify an amino acid residue involved in the protein-protein interaction, and to screen for a substance modulating the protein-protein interaction.

Thus, the present invention relates to a method for detecting a protein-protein interaction, applications thereof, and a kit for use in the method. More specifically, the present invention provides the following inventions.

(1) A method for detecting an interaction between a first protein and a second protein, the method comprising the

steps of:

expressing in a cell a first fusion protein comprising the first protein and an association-inducing protein, and a second fusion protein comprising the second protein and a fluorescent protein having a multimerization ability;

detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus.

(2) The method according to (1), wherein the fluorescent focus is detected to detect the interaction taking place or ending, a period until the interaction takes place or ends, or a duration of the interaction.

(3) The method according to (1), wherein the fluorescent focus is detected to detect the interaction taking place or ending in response to a particular stimulus, a period until the interaction takes place or ends, or a duration of the interaction.

(4) The method according to any one of (1) to (3) for screening for a protein interacting with a particular protein, wherein

one of the first protein and the second protein is the particular protein, while the other is a test protein, and

a protein interacting with the particular protein is selected according to the detection of the fluorescent focus.

(5) The method according to any one of (1) to (3) for identifying any one of an amino acid residue in the first protein and an amino acid residue in the second protein, which are involved in the interaction, wherein

in a case where a protein in which a mutation is introduced is used as any one of the first protein and the second protein, if a fluorescence intensity of the fluorescent focus is reduced in comparison with a protein in which no mutation is introduced, the amino acid residue in which the mutation is introduced is determined to be involved in the interaction.

(6) A method for screening for a substance modulating an interaction between a first protein and a second protein, the method comprising the steps of:

expressing in a cell a first fusion protein comprising the first protein and an association-inducing protein, and a second fusion protein comprising the second protein and a fluorescent protein having a multimerization ability, in presence of a test compound;

detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

selecting the test compound as a substance inducing

the interaction if a fluorescence intensity of the fluorescent focus is higher than a fluorescence intensity of a fluorescent focus formed in absence of the test compound, and selecting the test compound as a substance suppressing the interaction if the fluorescence intensity of the fluorescent focus is lower than the fluorescence intensity of the fluorescent focus formed in the absence of the test compound.

(7) The method according to any one of (1) to (6), wherein the association-inducing protein is at least one protein selected from the group consisting of a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKC δ , a SAM domain of TEL, a SAM domain of DGK δ , and a SAM domain of Tankyrase-1.

(8) A method for screening for an association-inducing protein, the method comprising the steps of:

(a) expressing in a cell a fusion protein comprising a test protein and mAG1;

(b) expressing in a cell a fusion protein comprising the test protein and a fluorescent protein having a multimerization ability; and

(c) selecting the test protein as an association-inducing protein if a fluorescent focus is not detected in step (a) but a fluorescent focus is detected in step (b).

(9) The method according to any one of (1) to (8), wherein

the fluorescent protein having a multimerization ability is at least one fluorescent protein selected from the group consisting of monomeric Kusabira-Orange 2, Azami-Green, Kusabira-Orange 1, dimeric Keima-Red, Kikume Green-Red, monomeric Keima-Red, monomeric Midoriishi-Cyan1, monomeric Kusabira-Orange 1, monomeric Kikume Green-Red1, Midoriishi-Cyan1, Kusabira-Cyan1, dimeric Azami-Green (AB), dimeric Azami-Green (AC), TGuv, Momiji, COR3.01, COR5, and DsRed2.

(10) A kit for use in the method according to any one of (1) to (9), the kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (j):

(a) a vector comprising a DNA encoding the association-inducing protein and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the association-inducing protein when expressed;

(b) a vector comprising a DNA encoding the fluorescent protein having a multimerization ability and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the fluorescent protein when expressed;

(c) a vector comprising a DNA encoding mAG1 and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein

is fused to the mAG1 when expressed;

(d) a vector encoding the first fusion protein;

(e) a vector encoding the second fusion protein;

(f) a vector set comprising the vector according to
5 any one of (a) and (d) and the vector according to any one
of (b) and (e);

(g) a vector set comprising the vector according to
(b) and the vector according to (c);

(h) a transformed cell comprising a vector encoding
10 the first fusion protein;

(i) a transformed cell comprising a vector encoding
the second fusion protein; and

(j) a transformed cell comprising a vector encoding
the first fusion protein and a vector encoding the second
15 fusion protein.

[Advantageous Effects of Invention]

The present invention makes it possible to detect
a protein-protein interaction in an intracellular
environment unique thereto, and to detect positional
20 information and temporal information on the
protein-protein interaction.

[Brief Description of Drawings]

[Fig. 1] Fig. 1 is a diagram for illustrating a concept
of a method for detecting a protein-protein interaction
25 of the present invention. Specifically, the diagram
illustrates that when a first fusion protein comprising

a first protein (B) and an association-inducing protein and a second fusion protein comprising a second protein (A) and a fluorescent protein having a multimerization ability are expressed in a cell, an interaction between the first protein (B) and the second protein (A) can be determined according to the detection of a fluorescent focus formed by assembly formation between the first fusion protein and the second fusion protein in the cell.

[Fig. 2] Fig. 2 is a diagram for illustrating the concept of the method for detecting a protein-protein interaction of the present invention. Specifically, the diagram illustrates that in a case where a first protein (C) does not interact with a second protein (A), even if a first fusion protein comprising the first protein (C) and an association-inducing protein and a second fusion protein comprising the second protein (A) and a fluorescent protein having a multimerization ability are expressed in a cell, the first fusion protein and the second fusion protein are not associated with each other, and are present in a dispersed manner in the cell, so that no fluorescent focus is detected.

[Fig. 3] Fig. 3 is a diagram for illustrating a concept of a screening method for an association-inducing protein according to the present invention. Specifically, the diagram illustrates that an association-inducing protein according to the present invention is capable of forming

an assembly (fluorescent focus) in a cell, when fused to a fluorescent protein having a multimerization ability.

[Fig. 4] Fig. 4 is a diagram for illustrating the concept of the screening method for an association-inducing protein according to the present invention. Specifically, the diagram illustrates that the association-inducing protein according to the present invention is present in a dispersed manner in a cell, when fused to monomeric Azami Green 1 (mAG1).

[Fig. 5] Fig. 5 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a protein (mAG1-p62(PB1)) composed of mAG1 fused to a PB1 domain of p62 (p62(PB1)), and a protein (AG-p62(PB1)) composed of p62(PB1) fused to Azami Green (AG) serving as the fluorescent protein having a multimerization ability.

[Fig. 6] Fig. 6 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a fusion protein composed of a FRB domain of a mTOR protein and an AG protein, and a fusion protein composed of p62(PB1) and FKBP12. Note that a FRB domain of a mTOR protein (mTOR(FRB)) and a FKBP12 protein are known to interact with each other in the presence of rapamycin.

[Fig. 7] Fig. 7 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent

focus formation by expressing in cultured cells a fusion protein composed of a FRB domain of a mTOR protein and p62(PB1), and a fusion protein composed of an AG protein and FKBP12. Note that, in the figure, the scale bars at the lower right portions represent 5 μ m.

[Fig. 8] Fig. 8 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells the fusion protein composed of a FRB domain of a mTOR protein and p62(PB1), and a fusion protein composed of a mAG1 protein and FKBP12. Note that, in the figure, the scale bars at the lower right portions represent 5 μ m.

[Fig. 9] Fig. 9 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a fusion protein (mTOR-AG) composed of a FRB domain of a mTOR protein and an AG protein, and a fusion protein (p62(PB1)-FKBP12) composed of a p62(PB1) protein and FKBP12, in the absence (-) or presence (+) of rapamycin.

[Fig. 10] Fig. 10 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a fusion protein (mTOR-mAG1) composed of a FRB domain of a mTOR protein and a mAG1 protein, and the fusion protein (p62(PB1)-FKBP12) composed of a p62(PB1) protein and FKBP12, in the absence (-) or presence (+) of rapamycin.

[Fig. 11] Fig. 11 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells the fusion protein (mTOR-AG) composed of a FRB domain of a mTOR protein and an AG protein, and a fusion protein (p62(PB1_{nc})-FKBP12) composed of a p62(PB1) protein mutant having lost no homomultimerization ability and FKBP12, in the absence (-) or presence (+) of rapamycin.

[Fig. 12] Fig. 12 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a protein composed of a mAG1 protein or an AG protein (fluorescent protein having a multimerization ability) fused to a PB1 domain of MEK5 (MEK5(PB1)) or a PB1 domain of Nbr1 (Nbr1(PB1)).

[Fig. 13] Fig. 13 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a protein composed of a mAG1 protein or an AG protein fused to a PB1 domain of PKCιota (PKCιota(PB1)) or a PB1 domain of TFG (TFG(PB1)).

[Fig. 14] Fig. 14 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a protein composed of a mAG1 protein or an AG protein fused to a SAM domain of TEL (TEL(SAM)) or a SAM domain of DGK delta (DGKd)

(DGK delta(SAM)).

[Fig. 15] Fig. 15 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a protein composed of a mAG1 protein or an AG protein fused to a SAM domain of Tankyrase (Tankyrase(SAM)) or a SAM domain of EphB2 (EphB2(SAM)).

[Fig. 16] Fig. 16 shows micrographs for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells the following fusion-protein combinations, in the presence of rapamycin: a combination of a fusion protein (mTOR(FRB domain)-AG) composed of a FRB domain of a mTOR protein and an AG protein with a fusion protein (FKBP12-TFG(PB1)) composed of a FKBP12 protein and TFG(PB1); a combination of mTOR(FRB domain)-AG with a fusion protein (FKBP12-TEL(SAM)) composed of a FKBP12 protein and TEL(SAM); a combination of mTOR(FRB domain)-AG with a fusion protein (FKBP12-DGKd(SAM)) composed of a FKBP12 protein and DGK delta(SAM); and a combination of mTOR(FRB domain)-AG with a fusion protein (FKBP12-Tankyrase(SAM)) composed of a FKBP12 protein and Tankyrase(SAM).

[Fig. 17] Fig. 17 shows micrographs for illustrating the result of analyzing whether or not it is possible to detect a rapamycin-dependent interaction between mTOR(FRB) and a FKBP12 protein according to fluorescent focus formation

by using K01, dKeima, KikGR, or AG as the fluorescent protein having a multimerization ability according to the present invention.

[Fig. 18] Fig. 18 shows a micrograph for illustrating the result of analyzing the presence or absence of fluorescent focus formation by expressing in cultured cells a protein composed of monomeric Kusabira-Orange 2 (mK02) fused to p62(PB1).

[Fig. 19] Fig. 19 shows micrographs for illustrating that the result of analyzing whether or not a fluorescence intensity of a fluorescent focus formed by an association between mTOR(FRB domain)-AG and FKBP12-p62(PB1) is dependent on the rapamycin concentration.

[Fig. 20] Fig. 20 is a graph for illustrating that the result of analyzing whether or not the fluorescence intensity of the fluorescent focus formed by the association between mTOR(FRB domain)-AG and FKBP12-p62(PB1) is dependent on the rapamycin concentration.

[Fig. 21] Fig. 21 is a graph for illustrating that the result of analyzing whether or not a fluorescence intensity of a fluorescent focus formed by an association between mTOR(FRB domain)-AG and p62(PB1)-FKBP12 is dependent on the rapamycin concentration.

[Fig. 22] Fig. 22 is a graph for illustrating the result of analyzing whether or not the fluorescence intensity of

the fluorescent focus formed by the association between mTOR(FRB domain)-AG and p62(PB1)-FKBP12 in the presence of rapamycin is suppressed in a manner dependent on the concentration FK506. Note that FK506 competitively
5 inhibits between an interaction between a FKBP12 protein and rapamycin, thereby inhibiting an interaction between the FKBP12 protein and a FRB domain of a mTOR protein (mTOR(FRB)).

[Fig. 23] Fig. 23 shows micrographs for illustrating the
10 result of analyzing a fluorescence intensity of a fluorescent focus in the presence of Nutlin-3 by expressing in cultured cells a fusion protein (p62(PB1)-p53) composed of p62(PB1) and a portion of p53, and a fusion protein (AG-MDM2) composed of an AG protein and MDM2. Note that
15 Nutlin-3 is known as an inhibitor against an interaction between a p53 protein and an MDM2 protein. Moreover, in the figure, the scale bars at the lower left portions represent 10 μ m.

[Fig. 24] Fig. 24 is a graph for illustrating the result
20 of analyzing whether or not the fluorescence intensity of the fluorescent focus formed by the association between p62(PB1)-p53 and AG-MDM2 is suppressed in a manner dependent on the Nutlin-3 concentration.

[Fig. 25] Fig. 25 is a graph for illustrating the result
25 of analyzing whether or not the fluorescence intensity of the fluorescent focus formed by the association between

p62(PB1)-p53 and AG-MDM2 is suppressed in a manner dependent on the Nutlin-3 concentration.

[Fig. 26] Fig. 26 shows micrographs for illustrating the result of observing cells expressing p62(PB1)-p50 and AG-p65 (I κ B α (-)), and cells expressing p62(PB1)-p50, AG-p65, and I κ B α (I κ B α (+)). Note that p50 and p65 form a heterodimer, which is localized in the nucleus, and also localized in the cytoplasm by the interaction with I κ B α . Moreover, in the figure, "AG" shows the result of detecting AG-derived fluorescence. "Immunostaining with anti-I κ B α antibody" shows the result of observing cells subjected to this immunostaining. "Merging" shows the result of merging the "AG", the "immunostaining with anti-I κ B α antibody," and the result of observing cells whose nuclei were stained with Hoechst 33342.

[Fig. 27] Fig. 27 is a graph for illustrating the result of analyzing whether or not intracellular localization of a fluorescent focus formed by an association between p62(PB1)-p50 and AG-p65 is changed in accordance with the amount of I κ B α introduced to the cells (the amount of pI κ B α added).

[Fig. 28] Fig. 28 shows micrographs for illustrating the result of observing cells co-expressing p62(PB1)-CDK4 and AG-p21 (AG-p21+PB1-CDK4), and cells expressing p62(PB1)-CDK4, AG-p21, and Cyclin D1 (AG-p21+PB1-CDK4+Cyclin D1). Note that p21 interacts with

a complex composed of CDK4 and Cyclin D1. Moreover, in the figure, "AG" shows the result of detecting AG-derived fluorescence. "Immunostaining with anti-Cyclin D1 antibody" shows the result of observing cells subjected to this immunostaining. "Merging" shows the result of merging the "AG" and the "immunostaining with anti-Cyclin D1 antibody."

[Fig. 29] Fig. 29 shows micrographs for illustrating the result of analyzing a fluorescence intensity and localization of a fluorescent focus by expressing in cultured cells the following fusion-protein combinations. Note that, in the figure, the scale bars at the lower right portions represent 20 μ m: a combination of a fusion protein (p62(PB1)-Sec5) composed of p62(PB1) and a portion of a Sec5 protein with a fusion protein (AG-RalB(WT)) composed of an AG protein and a RalB protein (wildtype); a combination of p62(PB1)-Sec5 with a fusion protein (AG-RalB(S28N)) composed of an AG protein and a RalB protein (inactive mutant); and a combination of p62(PB1)-Sec5 with a fusion protein (AG-RalB(Q72L)) composed of an AG protein and a RalB protein (active mutant). Note that a Sec5 protein is known to interact with a RalB protein in a GTP-activated form. Moreover, it is known that the interaction is reduced with an inactive mutant RalB(S28N) of RalB, but enhanced with an active mutant RalB(Q72L) of RalB. Further, it has also been revealed that a RalB protein is localized at the

cell membrane by palmitoylation of the C-terminus thereof.

[Fig. 30] Fig. 30 shows micrographs for illustrating the result of detecting fluorescence only in the vicinity of the cell membrane by expressing in cultured cells the following fusion-protein combinations: a combination of p62(PB1)-Sec5 with AG-RalB(WT); a combination of p62(PB1)-Sec5 with AG-RalB(Q72L); a combination of p62(PB1)-Sec5 with AG-RalB(S28N); and a combination of p62(PB1) with AG-RalB(WT).

[Fig. 31] Fig. 31 shows micrographs for illustrating the result of observing cells (WT) co-expressing p62(PB1)-p53 and AG-MDM2, and cells (W23L) co-expressing p62(PB1)-p53_W23L and AG-MDM2. Note that the amino acid at position 23 of p53 is located at an interaction interface site between p53 and MDM2, and a W23L mutation of p53 reduces the interaction.

[Fig. 32] Fig. 32 shows micrographs for illustrating the result of observing formation and extinction of a fluorescent focus formed by an association between a fusion protein (Calmodulin-AG) composed of a calmodulin protein and an AG protein, and a fusion protein composed (M13peptide-p62(PB1)) of a partial sequence (M13 peptide) of myosin light chain kinase 2 and p62(PB1), before histamine addition, 90 seconds after the addition, and 620 seconds after the addition. Note that, in the figure, the scale bars at the lower right portions represent 5 μ m.

Moreover, it has been revealed that an interaction between calmodulin and an M13 peptide takes place in response to a transient increase in intracellular calcium ion concentration that occurs when a G protein-coupled receptor (GPCR) receives a ligand (for example, histamine).

[Fig. 33] Fig. 33 shows micrographs for illustrating the result of observing localization of a fluorescent focus formed by an association between FKBP12-p62(PB1) and a fusion protein (mTOR(FRB domain)-AGNLS) composed of mTOR(FRB domain), an AG protein, and a nuclear localization signal (NLS). Note that, from the left, the first panel shows a fluorescence image of cells before rapamycin addition, the second panel shows a fluorescence image of the cells after the rapamycin addition, and the third panel shows the result of merging superimposing the fluorescence image and bright-field image of the cells after the rapamycin addition. Moreover, in the figure, the scale bars at the lower right portions represent 5 μ m.

[Fig. 34] Fig. 34 shows a micrograph for illustrating the result of observing localization of a fluorescent focus formed by an association between a fusion protein (AG-cRaf) composed of an AG protein and a cRaf protein, and a fusion protein (p62(PB1)-HRas) composed of p62(PB1) and an HRas protein, the p62(PB1)-HRas having a prenylated sequence at the C-terminus. Note that, in the figure, the scale bar at the lower right portion represents 5 μ m.

[Fig. 35] Fig. 35 shows a micrograph for illustrating the result of observing localization of a fluorescent focus formed by an association between a fusion protein (Smac-p62(PB1)) composed of a portion of a Smac protein and p62(PB1), and a fusion protein (XIAP-AG) composed of a portion of a XIAP protein and an AG protein. Note that, in the figure, the scale bar at the lower right portion represents 5 μ m.

[Fig. 36] Fig. 36 shows a micrograph for illustrating the result of observing localization of a fluorescent focus formed by an association between a fusion protein (p62(PB1)-BclX(L)) composed of p62(PB1) and a portion of a BclX(L) protein, and a fusion protein (AG-BAD) composed of an AG protein and a portion of a BAD protein. Note that, in the figure, the scale bar at the lower right portion represents 5 μ m.

[Fig. 37] Fig. 37 shows a micrograph for illustrating that a fluorescent focus formed by an association between a fusion protein (AG-Rac1) composed of an AG protein and a Rac1 protein, and a fusion protein (p62(PB1)-PBD) composed of p62(PB1) and a p21 binding domain is localized in the nucleus. Note that, in the figure, the scale bar at the lower right portion represents 5 μ m. Moreover, guanine nucleotide exchange factors (GEFs) convert a Rac1 protein to an active form. Further, it is known that an active Rac1 protein and PBD interact with each other.

Furthermore, GEFs are localized differently depending on the type. For this reason, an interaction between a Rac1 protein and PBD takes place in intracellular regions in accordance with the GEFs localized differently depending on the type.

[Fig. 38] Fig. 38 shows micrographs for illustrating that a fluorescent focus formed by an association between AG-Rac1 and p62(PB1)-PBD is localized at the border of cells. Note that, in the figure, the lower panel is obtained by enlarging a region surrounded by the white line in the upper panel. Moreover, the scale bar at the lower right portion of the upper panel represent 5 μm , and the scale bar at the upper left portion of the lower panel represents 1 μm .

[Fig. 39] Fig. 39 shows micrographs for illustrating that when AG-Rac1 and p62(PB1) are expressed in cells, these proteins do not interact with each other, so that no fluorescent focus is detected at the border and so on of a cell. Note that, in the figure, the lower panel is obtained by enlarging a region surrounded by the white line in the upper panel. Moreover, the scale bar at the lower right portion of the upper panel represents 5 μm , and the scale bar at the upper left portion of the lower panel represents 1 μm .

[Fig. 40] Fig. 40 shows micrographs for illustrating the result of observing cells expressing AG-Rac1 and p62(PB1)-PBD in the absence (-) or presence (+) of an

inhibitor mevastatin against geranylgeranyl group modification. Note that if geranylgeranyl group modification is inhibited, Rac1 is localized in the nucleus. Moreover, the figure shows the results of observing the same cells; "A" is observed using a normal inverted epifluorescence microscope; and "B" is observed using a total internal reflection fluorescence microscopy system with arc lamp source capable of exciting only the vicinity of the cell membrane.

[Fig. 41] Fig. 41 shows micrographs for illustrating the result of observing cells expressing AG-Rac1 and RhoGDI-p62(PB1) in the absence (-) or presence (+) of an inhibitor mevastatin against geranylgeranyl group modification. Note that a Rac1 protein interacts with RhoGDI via a geranylgeranyl group of the Rac1 protein. Moreover, the figure shows the results of observing the same cells; "A" is observed using a normal inverted epifluorescence microscope; and "B" is observed using a total internal reflection fluorescence microscopy system with arc lamp source capable of exciting only the vicinity of the cell membrane.

[Fig. 42] Fig. 42 shows micrographs for illustrating the result of detecting only fluorescence in the vicinity of the cell membranes of cells(WT) expressing p62(PB1)-KRas(WT) and AG-cRaf(R59A), and cells (G12D) expressing p62(PB1)-KRas(G12D) and AG-cRaf(R59A), after

EGF addition (+) or no addition (-). Note that KRas activated in a manner dependent on EGF interacts with cRaf. Moreover, this protein-protein interaction changes the localization of cRaf from the cytoplasm to the cell
5 membrane.

[Fig. 43] Fig. 43 is a graph for illustrating a change over time in a total fluorescence intensity of fluorescent foci in cells co-expressing p62(PB1)-BclX(L) and Bak-AG after ABT-737 addition. Note that although BclX(L) and
10 Bak interact with each other via a BH3 domain, this protein-protein interaction is competitively inhibited by ABT-737 (BH3 mimetic).

[Fig. 44] Fig. 44 is a graph for illustrating a change over time in a total fluorescence intensity of fluorescent foci in cells co-expressing p62(PB1)-BclX(L) and AG-Bax, after ABT-737 addition. Note that although BclX(L) and
15 Bax interact with each other via a BH3 domain, this protein-protein interaction is competitively inhibited by ABT-737.

[Fig. 45] Fig. 45 is a graph for illustrating a change over time in a total fluorescence intensity of fluorescent foci in cells stably expressing p62(PB1)-p53 and AG-MDM2, before and after Nutlin-3 addition. Note that, in the graph, the x axis represents time (minutes), provided that time
20 when Nutlin-3 was added is 0.

[Fig. 46] Fig. 46 is a graph for illustrating a change

over time in a total fluorescence intensity of fluorescent foci in cells stably expressing mTOR(FRB domain)-AG and p62(PB1)-FKBP12, before and after rapamycin addition. Note that, in the graph, the x axis represents time (minutes), provided that time when rapamycin was added is 0.

[Fig. 47] Fig. 47 is a graph for illustrating a change over time in a total fluorescence intensity of fluorescent foci in cells expressing p62(PB1)-ERK_substrate and AG-Pin1(ww)-NES, before and after EGF and U0126 addition. Note that when ERK in cells is activated by an EGF stimulus, the ERK substrate (ERK_substrate) is phosphorylated; as a result, the ERK_substrate and a ww domain of a Pin1 protein (Pin1(ww)) interact with each other. Further, if a MEK inhibitor U0126 is added, the ERK activity is decreased; as a result, the ERK substrate is dephosphorylated, terminating the interaction between the ERK substrate and Pin1(ww). In the graph, the x axis represents time (minutes), provided that time when EGF was added is 0. Additionally, U0126 was added to the cells, 14 minutes after the EGF addition.

[Fig. 48] Fig. 48 is a graph for illustrating a change over time in a total fluorescence intensity of fluorescent foci in cells expressing p62(PB1)-HRas(WT) and AG-cRaf(R59A), before and after EGF addition. Note that, in the graph, the x axis represents time (minutes), provided that time when EGF was added is 0.

[Fig. 49] Fig. 49 shows micrographs for illustrating the result of observing cells expressing AG-mCAB, p62(PB1)-FKBP12, and mTOR(FRB)-K01, in the absence (-) or presence (+) of rapamycin, or in the absence (-) or presence (+) of FK506. Note that rapamycin binds to a FKBP12 protein, forming a complex, and further that this complex binds to a FRB domain of a mTOR protein (mTOR(FRB)). Moreover, mCAB (protein composed of a portion of calcineurin A fused to a portion of calcineurin B) interacts with a FKBP12 protein via FK506. In the figure, "AG" and "K01" show the results of detecting fluorescences derived from AG and K01, respectively.

[Description of Embodiments]

<Method for Detecting Protein-Protein Interaction>

A method for detecting a protein-protein interaction of the present invention is a method for detecting an interaction between a first protein and a second protein, the method comprising the steps of:

expressing in a cell a first fusion protein comprising the first protein and an association-inducing protein, and a second fusion protein comprising the second protein and a fluorescent protein having a multimerization ability;

detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

determining an interaction between the first protein

and the second protein according to the detection of the fluorescent focus.

In the present invention, the term "protein" means a molecule in which 2 or more amino acids are linked by a peptide bond(s), and modified products thereof. Thus, the term is a concept including not only full-length proteins, but also so-called oligopeptides and polypeptides. Examples of the modification of the protein include phosphorylation, glycosylation, palmitoylation, prenylation (for example, geranylgeranylation), methylation, acetylation, ubiquitination, SUMOylation, hydroxylation, and amidation.

As the "first protein" and the "second protein" according to the present invention, it is possible to use desired proteins intended for detection of interaction.

The "interaction between the first protein and the second protein" according to the present invention includes not only direct interactions, but also indirect interactions such as an interaction for forming a complex in which another molecule (protein, nucleic acid, sugar, lipid, low-molecular-weight compound, or the like) is interposed between the first protein and the second protein.

The "fluorescent protein having a multimerization ability" according to the present invention is a fluorescent protein capable of forming a fluorescent focus,

when fused to a PB1 domain of p62 (p62(PB1)) and expressed in a cell, as a result that the fusion proteins with the p62(PB1) are associated with each other. Thus, the "fluorescent protein having a multimerization ability" includes not only fluorescent proteins capable of forming a homomultimer in a cell without fusing with p62(PB1), but also fluorescent proteins such as mK02 generally believed to be monomeric fluorescent proteins, as described in Examples later. Examples of such a "fluorescent protein having a multimerization ability" include Midoriishi-Cyan1 (MiCy1), Kusabira-Orange 1 (K01), dKeima570 (dimeric Keima570), dimeric Keima-Red (dKeima, dKeima-Red), Azami-Green (AG), Kaede, Kikume Green-Red (KikGR, KikGR1), monomeric Kusabira-Orange 1 (mK01), monomeric Kusabira-Orange 2 (mK02), TurboGFP, TurboYFP, ZsGreen, DsRed, HcRed, eqFP578, eqFP611, EosFP, FP484, Renilla GFP, Dendra, IFP1.4, iRFP, monomeric Keima-Red (mKeima, mKeima-Red), monomeric Midoriishi-Cyan1 (mMiCy1), monomeric Kikume Green-Red1 (mKikGR1), Kusabira-Cyan1 (KCy1), dimeric Azami-Green (AB) (dAG (AB)), dimeric Azami-Green (AC) (dAG (AC)), TGuv, Momiji, COR3.01, COR5, and DsRed2. Preferable are mK02, mKeima, mMiCy1, mK01, mKikGR1, MiCy1, KCy1, K01, dKeima, dAG (AB), dAG (AC), TGuv, Momiji, KikGR, AG, COR3.01, COR5, and DsRed2. Moreover, from the viewpoint of facilitating detection of a clear fluorescent focus in the method of the present invention,

more preferable are fluorescent proteins capable of forming a homotetramer, such as TGuv, Momiji, AG, KikGR, COR3.01, COR5, and DsRed2, and particularly preferable is AG.

Note that, typically, mK02, AG, K01, dKeima, KikGR, mKeima, mMiCy1, mK01, mKikGR1, MiCy1, KCy1, dAG (AB), dAG (AC), TGuv, Momiji, COR3.01, DsRed2, and COR5 are respectively a protein having the amino acid sequence of SEQ ID NO: 133, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB107915, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB128820, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB209968, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB193293, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB209969, a protein having the amino acid sequence of SEQ ID NO: 137, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB128821, a protein having the amino acid sequence of SEQ ID NO: 139, a protein having the amino acid sequence specified under Genbank ACCESSION No: AB128822, a protein having the amino acid sequence of SEQ ID NO: 141, a protein having the amino acid sequence of SEQ ID NO: 143, a protein having the amino acid sequence of SEQ ID NO: 145, a protein having the amino acid sequence of SEQ ID NO: 147, a protein having the amino acid sequence of SEQ ID NO: 149, a protein having the amino acid

sequence of SEQ ID NO: 151, a protein having the amino acid sequence of SEQ ID NO: 153, and a protein having the amino acid sequence of SEQ ID NO: 172.

5 The amino acid sequences of these fluorescent proteins may be mutated naturally (i.e., non-artificially). Moreover, a mutation can also be introduced artificially. Such a mutant can also be used in the present invention, as long as it can emit fluorescence and form a homomultimer in a cell.

10 The "association-inducing protein" according to the present invention is a protein allowing a fluorescent focus to be detected when fused to a fluorescent protein having a multimerization ability and expressed in a cell, as a result that such fusion proteins are associated with each other, and being present in a dispersed state in a cell
15 when fused to monomeric Azami Green 1 (mAG1) and expressed, as illustrated in Examples and Figs. 3 and 4 later.

The "association-inducing protein" according to the present invention is preferably a PB1 domain of p62, a PB1
20 domain of TFG, a PB1 domain of PKC ι , a SAM domain of TEL, a SAM domain of DGK delta, and a SAM domain of Tankyrase-1. From the viewpoint of facilitating detection of a fluorescent focus in the method of the present invention, more preferable are a PB1 domain of p62, a PB1 domain of
25 TFG, a SAM domain of TEL, a SAM domain of DGK delta, and a SAM domain of Tankyrase-1.

In addition, typically, a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota, a SAM domain of TEL, a SAM domain of DGK delta , and a SAM domain of Tankyrase-1 are respectively a protein having the amino acid sequence specified under SEQ ID NO: 4, a protein having the amino acid sequence specified under SEQ ID NO: 12, a protein having the amino acid sequence specified under SEQ ID NO: 10, a protein having the amino acid sequence specified under SEQ ID NO: 14, a protein having the amino acid sequence specified under SEQ ID NO: 18, and a protein having the amino acid sequence specified under SEQ ID NO: 20.

The amino acid sequences of these "association-inducing proteins" may be mutated naturally (i.e., non-artificially). Moreover, a mutation can also be introduced artificially. Such a mutant having no association ability by itself can also be used in the present invention, as long as it has such a nature of forming an assembly (fluorescent focus) when fused to a fluorescent protein having a multimerization ability.

In the "first fusion protein" according to the present invention, the association-inducing protein may be fused on either the N-terminus side or the C-terminus side of the first protein. Further, the association-inducing protein may be fused to the first protein directly, or may be fused indirectly via a spacer protein. Furthermore, the "first fusion protein" according to the present

invention may be fused to another functional protein. In this case, the other functional protein may be fused on one or both of the N-terminus side and the C-terminus side of the fusion protein, or may be fused directly or indirectly between the association-inducing protein and the first protein. The other functional protein is not particularly limited, and selected as appropriate depending on a function desirably provided to the fusion protein according to the present invention. Examples of a functional protein used to facilitate purification of the fusion protein include a Myc-tag protein, a His-tag protein, a hemagglutinin (HA)-tag protein, a FLAG-tag protein (registered trademark, Sigma-Aldrich Co.), a glutathione S-transferase (GST) protein, and also a fluorescent protein that exhibits wavelength characteristics different from those of the fluorescent protein having a multimerization ability in the second fusion protein.

In the "second fusion protein" according to the present invention, the fluorescent protein having a multimerization ability may be fused on either the N-terminus side or the C-terminus side of the second protein, as in the case of the "first fusion protein." Moreover, the fluorescent protein having a multimerization ability may be fused to the second protein directly, or may be fused indirectly via the spacer protein. Furthermore, the "second fusion protein" according to the present invention

may be fused to the above-described other functional protein. In this case, the other functional protein may be fused on one or both of the N-terminus side and the C-terminus side of the fusion protein, or may be fused directly or indirectly between the fluorescent protein having a multimerization ability and the second protein, as in the case of the "first fusion protein."

The "cell" according to the present invention are not particularly limited, and may be an eukaryotic cell, or may be a prokaryotic cell. Examples of the "cell" include an animal cell (HeLaS3 cell, U2OS cell, and the like), an insect cell (Sf9 cell, and the like), a plant cell, yeast, and *Escherichia coli*. Moreover, such cells may be in a state of being cultured *in vitro* (for example, cells grown in a medium or on a medium), or in a state of being present *in vivo* (for example, cells in a transgenic animal in which a DNA encoding the first fusion protein and a DNA encoding the second fusion protein are introduced).

The expression of the fusion proteins in the cell according to the present invention may be a transient expression or a constitutive expression, depending on the purpose. The fusion proteins in the cell can be expressed by introducing into the cell a vector according to the present invention, which will be described later. Examples of known techniques for introducing the vector

into the cell include, in the case of an animal cell, a lipofection method, an electroporation method, a phosphate calcium method, a DEAE-dextran method, and methods utilizing a virus (adenovirus, lentivirus, adeno-associated virus, or the like). Moreover, in the case of an insect cell, the examples include methods utilizing a baculovirus. Further, in the case of a plant cell, the examples include an *Agrobacterium* method, an electroporation method, a lithium acetate method, and the like. In addition, in the case of yeasts, the examples include a lithium acetate method, an electroporation method, and a spheroplast method. Furthermore, in the case of *Escherichia coli*, the examples include a heat shock method (for example, a calcium chloride method, a rubidium chloride method), an electroporation method, and the like.

A "fluorescent focus" to be detected in the present invention is formed by an association between the first fusion protein and the second protein. Typically, the "fluorescent focus" has a fluorescence intensity in a region of 0.2 to 5 μm , the fluorescence intensity being higher than a fluorescence intensity of a fluorescent protein having a multimerization ability, which is present in a dispersed state (see Examples described later and Figs. 1 and 2).

The "detection of the fluorescent focus" can be carried out, for example, through observation using a

fluorescence microscope including an excitation filter and an absorption filter corresponding to a fluorescent protein having a multimerization ability, and analysis using an imaging cytometer such as IN Cell Analyzer (manufactured
5 by GE Healthcare).

In the method of the present invention, if the fluorescent focus is detected in the cell, it can be determined that the first protein and the second protein interact with each other; meanwhile, if the fluorescent
10 focus is not detected, it can be determined that the first protein and the second protein do not interact with each other.

<Screening Method for Association-Inducing Protein>

The association-inducing protein according to the
15 present invention can be selected, as described in Examples later, by a screening method comprising the following steps (a) to (c):

(a) expressing in a cell a fusion protein comprising a test protein and mAG1;

20 (b) expressing in a cell a fusion protein comprising the test protein and a fluorescent protein having a multimerization ability; and

(c) selecting the test protein as an association-inducing protein if a fluorescent focus is not
25 detected in step (a) but a fluorescent focus is detected in step (b).

The "test protein" according to the present invention is not particularly limited, and it is possible to use desired proteins intended for detection of association-inducing ability.

5 Note that the "mAG1" (monomeric Azami Green 1) is typically a protein having the amino acid sequence of SEQ ID NO: 135. Moreover, the amino acid sequence of a protein may be mutated naturally (i.e., non-artificially). Further, a mutation can also be introduced artificially.
10 Such a mutant can also be used in the present invention, as long as it can emit fluorescence and can be present in a monomeric form in the cell.

 The fluorescent protein having a multimerization ability used in the screening method for an
15 association-inducing protein of the present invention is as described above.

 In the "fusion protein comprising the test protein and mAG1" or the "fusion protein comprising the test protein and the fluorescent protein having a multimerization
20 ability" according to the present invention, the mAG1 or the fluorescent protein having a multimerization ability may be fused on either the N-terminus side or the C-terminus side of the test protein. Moreover, the mAG1 or the fluorescent protein having a multimerization ability may
25 be fused to the test protein directly, or may be fused on indirectly via the spacer protein. The "test protein"

according to the present invention may be fused to the above-described other functional protein. In this case, the other functional protein may be fused on one or both of the N-terminus side and the C-terminus side of the fusion protein, or may be fused directly or indirectly between the mAG1 or the fluorescent protein having a multimerization ability and the test protein.

In the screening of the present invention, the test protein is selected as an association-inducing protein if a fluorescent focus is not detected when fused to the mAG1 and expressed in the cell but a fluorescent focus is detected when fused to the fluorescent protein having a multimerization ability and expressed in the cell.

<Method for Obtaining Temporal Information and the like on Protein-Protein Interaction>

As described in Examples, particularly Example 12, later, the method of the present invention is capable of detecting not only a protein-protein interaction taking place, but also a protein-protein interaction ending, on the basis of the presence or absence of the fluorescent focus according to the present invention. Moreover, as described in Examples such as Examples 19, 24 to 28, it is also possible to trace occurrence or the like of such a protein-protein interaction over time. Further, as described in Examples such as Examples 20 to 22, the present invention is also capable of detecting a protein-protein

interaction in any region in a cell without being influenced by localization of an association-inducing protein and a fluorescent protein having a multimerization ability, and so forth.

5 Thus, the present invention can provide a method, wherein the fluorescent focus according to the present invention is detected to detect an interaction taking place or ending, a period until the interaction takes place or ends, or a duration of the interaction.

10 In detecting the "interaction taking place or ending" in the manner described above, the present invention is also capable of specifying an intracellular region where the protein-protein interaction takes place as described particularly in Example 21 later.

15 Additionally, as described in Examples such as Examples 19, 23, 27 to 29 later, according to the present invention, detecting the "interaction taking place or ending" makes it possible to detect a signal transduction occurring and ending, in which the protein-protein
20 interaction is involved, a period until the signal transduction occurs or ends, and a duration of the signal transduction, and also to specify an intracellular region where the signal transduction occurs.

25 Moreover, as described in Examples later, the present invention is capable of detecting the interaction between the first protein and the second protein, even if the

interaction takes place or ends in response to a particular stimulus. Thus, the present invention can also provide a method for detecting the fluorescent focus according to the present invention, wherein the fluorescent focus is
5 detected to detect the interaction taking place or ending in response to a particular stimulus, a period until the interaction takes place or ends, or a duration of the interaction.

It is only necessary that the "particular stimulus" according to the present invention be a stimulus capable
10 of directly or indirectly inducing or inhibiting a protein-protein interaction. Moreover, the "particular stimulus" may be a stimulus attributable to an endogenous factor produced in a cell (for example, increase or decrease
15 in intracellular calcium ion concentration, activation or inactivation of an enzyme), or may be a stimulus applied to a cell from the outside (for example, administration of a ligand (agonist or antagonist) to a receptor in a cell).

Further, as particularly described in Examples 19,
20 24 to 28 later, such a method of the present invention is also capable of detecting a particular stimulation starting or ending, a period until the stimulation starts or ends, or a duration of the stimulation, by detecting the fluorescent focus according to the present invention.

Furthermore, as described in Examples such as
25 Examples 11 and 13 later, the method of the present invention

is also capable of detecting an increase or decrease of a protein-protein interaction in accordance with a degree of the particular stimulus (for example, in a case where the particular stimulus is a drug, its concentration).

5 Thus, in the case where the particular stimulus is a drug, the 50% effective concentration (EC50) and the 50% inhibitory concentration (IC50) of the drug against a protein-protein interaction can be determined by the present invention.

10 In addition, as described in Example 29 later, the method of the present invention is capable of distinguishing and detecting, in a single cell, multiple types of protein-protein interactions, multiple types of protein-protein interactions dependent respectively on
15 particular stimuli, and eventually a signal transduction in which these protein-protein interactions are involved.

<Screening Method for Protein Interacting with Particular Protein>

20 As described in Examples, particularly Example 30, later, the present invention makes it possible to detect any protein-protein interaction. Thus, the present invention can provide a method for screening for a protein interacting with a particular protein, wherein

25 one of the first protein and the second protein is the particular protein, while the other is a test protein, and

a protein interacting with the particular protein is selected according to the detection of the fluorescent focus according to the present invention.

The "test protein" according to the present invention is not particularly limited. Protein groups encoded by cDNA libraries can be suitably used from the viewpoint that it is possible to comprehensively and efficiently select proteins interacting with particular proteins.

<Method for Identifying Amino Acid Residue Involved in Protein-Protein Interaction>

As described in Examples later, the fluorescence intensity of a fluorescent focus and a strength of a protein-protein interaction correlate with each other in the present invention. Thus, the present invention can provide a method for identifying any one of an amino acid residue in the first protein and an amino acid residue in the second protein, which are involved in the protein interaction, wherein

in a case where a protein in which a mutation is introduced is used as any one of the first protein and the second protein, if an intensity of the fluorescent focus is reduced in comparison with a case of using a protein in which no mutation is introduced, the amino acid residue in which the mutation is introduced is determined to be involved in the interaction.

The "fluorescence intensity of the fluorescent focus"

according to the present invention includes not only a fluorescence intensity of a single fluorescent focus, but also a total fluorescence intensity of fluorescent foci present in a certain region(for example, in one cell, in one field of view and in one fluorescence image observed with a fluorescence microscope).

Those skilled in the art can prepare the "protein obtained by introducing a mutation into the first protein and the like" by selecting known techniques as appropriate. An example of such known techniques includes site-directed mutagenesis.

< Screening Method for Substance Capable of Modulating Protein-Protein Interaction>

As described above, in the method of the present invention, a strength of a protein-protein interaction can be grasped on the basis of the fluorescence intensity of the fluorescent focus. Thus, the present invention can provide a method comprising the steps of:

expressing in a cell a first fusion protein comprising a first protein and an association-inducing protein, and a second fusion protein comprising a second protein and a fluorescent protein having a multimerization ability, in presence of a test compound;

detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

selecting the test compound as a substance inducing the interaction if an intensity of the fluorescent focus is higher than an intensity of a fluorescent focus formed in absence of the test compound, or selecting the test compound as a substance suppressing the interaction if the intensity of the fluorescent focus is lower than the intensity of the fluorescent focus formed in the absence of the test compound.

The test compound used in the screening method of the present invention is not particularly limited. Examples thereof include an expression product from a gene library, a synthetic low-molecular-weight compound library, a peptide library, an antibody, a substance released by a bacterium, a liquid extract and a culture supernatant of cells (microorganisms, plant cells, animal cells), a purified or partially purified polypeptide, an extract derived from a marine organism, plant, or animal, soil, and a random phage peptide display library.

Moreover, examples of a state of being in the presence of the test compound include a state where the cell according to the present invention are in contact with the test compound by addition or the like of the test compound to a medium, and a state where the test compound is introduced in the cell according to the present invention.

<Kit for Use in Methods of the present invention>

The present invention can provide a kit for use in

the above-described methods. The kit of the present invention is a kit comprising an instruction and at least one substance selected from the group consisting of the following (a) to (j):

5 (a) a vector comprising a DNA encoding the association-inducing protein and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the association-inducing protein when expressed;

10 (b) a vector comprising a DNA encoding the fluorescent protein having a multimerization ability and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the fluorescent protein when expressed;

15 (c) a vector comprising a DNA encoding mAG1 and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the fluorescent protein when expressed;

(d) a vector encoding the first fusion protein;

20 (e) a vector encoding the second fusion protein;

(f) a vector set comprising the vector according to any one of (a) and (d) and the vector according to any one of (b) and (e);

(g) a vector set comprising the vector according to
25 (b) and the vector according to (c);

(h) a transformed cell comprising a vector encoding

the first fusion protein;

(i) a transformed cell comprising a vector encoding the second fusion protein; and

(j) a transformed cell comprising a vector encoding the first fusion protein and a vector encoding the second fusion protein.

It is only necessary that the vectors according to the present invention comprise a regulatory sequence necessary for an expression (transcription and translation) of the inserted DNA in the cell according to the present invention. Examples of such a regulatory sequence include a promoter, an enhancer, a silencer, a terminator, a poly(A) tail, and a ribosomal binding site (Shine-Dalgarno (SD) sequence). Further, the vectors according to the present invention may comprise a selection marker (such as a drug resistance gene), and a reporter gene (such as a luciferase gene, a β -galactosidase gene, a chloramphenicol acetyltransferase (CAT) gene). Moreover, examples of a type of such vectors according to the present invention include a plasmid vector, an episomal vector, and a viral vector.

The proteins encoded by the vectors according to the present invention are, as described above, a protein having an association-inducing ability, a fluorescent protein having a multimerization ability, mAG1, and fusion proteins with these proteins. From the viewpoint of further

improving the efficiency of expressing a DNA encoding such a protein, a DNA having codons optimized in accordance with the species of a cell expressing the protein (for example, humanized-codon DNA) may be inserted in the vectors according to the present invention.

Examples of the "cloning site allowing an insertion of a DNA encoding a certain protein" in (a), (b), and (c) above include a multiple cloning site containing one or more restriction-enzyme recognition sites, a TA cloning site, and a GATEWAY(registered trademark) cloning site.

To a preparation of the vectors according to the present invention, other components such as a buffer, a stabilizer, a preservative, and an antiseptic may be added.

The transformed cell according to the present invention can be prepared, as described above, by introducing the vectors according to the present invention into a cell. Moreover, to a preparation of the transformed cell according to the present invention, a medium necessary for storage and culturing of the cell and other components such as a stabilizer, a preservative, and an antiseptic may be added or attached.

The "instruction" according to the present invention is an instruction for utilizing the vectors or the transformed cell in the methods of the present invention. The instruction may comprise, for example, experimental techniques and experimental conditions for the methods of

the present invention, and information on the preparation of the present invention (for example, information such as a vector map indicating the base sequence, cloning site, and the like of the vectors, information on the origin and nature of the transformed cell, culture conditions of the cell, and so forth).

[Examples]

Hereinafter, the present invention will be described more specifically based on Examples. However, the present invention is not limited to the following Examples.

(Example 1)

<Screening 1 for Association-Inducing Protein>

For constructing a system for detecting a protein-protein interaction, proteins were searched for, which functioned as the "association-inducing protein" according to the present invention, on the basis of the concept illustrated in Figs. 1 and 2 and by the method illustrated in Figs. 3 and 4. Specifically, screened for were proteins, which were present in a dispersed manner in a cell when fused to monomeric Azami Green 1 (mAG1) (see Fig. 4), while being capable of forming a fluorescent focus (assembly) in a cell when fused to a fluorescent protein having a multimerization ability (see Fig. 3).

As such screening targets, first of all, attention was focused on a PB1 (Phox and Bem1p) domain. A protein composed of mAG1 fused to a PB1 domain of p62 (hereinafter,

also referred to as "p62(PB1)"), and a protein composed of p62(PB1) fused to Azami Green (AG) serving as the fluorescent protein having a multimerization ability were expressed in cultured cells. Then, an association between the fusion proteins and eventually the presence or absence of formation of a fluorescent focus formed by the assembly formation were examined by a method described below. Note that AG has been known to form a homotetramer. Fig. 5 shows the obtained result.

(Preparation of Plasmid DNAs)

As a plasmid DNA for fusing mAG1, phmAG1-MCLinker (manufactured by limited company Amalgaam Co., Ltd.) was used.

Meanwhile, in preparing a plasmid for fusing AG (phAG-MCLinker), first, a humanized-codon Azami Green (AG) gene (DNA encoding a region having the amino acid sequence of SEQ ID NO: 2 (the DNA had the base sequence of SEQ ID NO: 1) was artificially synthesized. PCR amplification was carried out using the artificially synthesized humanized-codon AG (hAG) gene as a template, and the following primer set:

hAG	forward	primer	1;
5'-CTAGCTAGCATTGCCACCATGGTGAGCGTGATCAAGCCCGAG-3' (SEQ ID NO: 57), and			
hAG	reverse	primer	1;
5'-ACTACCGGTCTTGGCCTGGCTGGGCAGCATGCTGTACC-3' (SEQ ID NO:			

58).

Then, the amplification product thus obtained was cleaved with NheI and AgeI, and inserted into phmAG1-MCLinker having been treated with the same restriction enzymes. Thus, phAG-MCLinker was prepared.

Further, in preparing phmAG1-p62(PB1) and phAG-p62(PB1), first, a DNA encoding a PB1 domain of p62 (region having the amino acid sequence of SEQ ID NO: 4) (the DNA had the base sequence of SEQ ID NO: 3) was amplified from a cDNA library of HeLaS3 cells by PCR using the following primer set:

p62(PB1) forward primer 1;
5'-AAGAATTCGATGGCGTCGCTACCGTGAAGGCCTACCTTCTGGGC-3'

(SEQ ID NO: 59), and

p62(PB1) reverse primer 1;
5'-AATTGGCGGCCGCTTATTTCTCTTTAATGTAGATTCGGAAGATGTC-3'

(SEQ ID NO: 60).

Then, the amplification product thus obtained was cleaved with EcoRI and NotI, and inserted into phAG-MCLinker and phmAG1-MCLinker having been treated with the same restriction enzymes; thus, phAG-p62(PB1) and phmAG1-p62(PB1) were prepared, respectively.

(Transfection into Cultured Cells)

HeLaS3 cells were used as cultured cells into which the phAG-p62(PB1) and the phmAG1-p62(PB1) were introduced. Note that HeLaS3 cells were cultured in DMEM Low glucose

(manufactured by SIGMA ALDRICH CO.) containing 10% FBS (manufactured by Equitech-Bio Inc.). Moreover, on the day before the transfection, the HeLaS3 cells were seeded onto a 35-mm glass base dish (manufactured by Asahi Glass Co., Ltd.). Further, at the time of the transfection, 1 µg of the phAG-p62(PB1) or phMAG1-p62(PB1) was diluted with OptiMEM (manufactured by Life Technologies Corporation), and 10 µl of PolyFect(R) Transfection Reagent (manufactured by QIAGEN N.V.) was added thereto and stirred. Then, the resultant was further mixed with 600 µl of the culture solution, subsequently added to the HeLaS3 cells, and observed 22 hours thereafter.

(Observation of Transfected Cells)

After the transfection treatment, the HeLaS3 cells were observed in a buffer at pH 7.4 containing Hanks' Balanced Salt Solutions (manufactured by Life Technologies Corporation) and 20 mM HEPES (manufactured by Dojindo Laboratories), using an IX-71 inverted fluorescence microscope (manufactured by Olympus Corporation), a U-MGFPHQ filter (manufactured by Olympus Corporation), and an ORCA-ER digital camera (manufactured by Hamamatsu Photonics K. K.).

As apparent from the result shown in Fig. 5, p62(PB1) was present in a dispersed state in the cells when fused to MAG1. On the other hand, when p62(PB1) was fused to AG, a fluorescent protein having a multimerization ability,

fluorescent foci were detected in the cells, revealing that fusion proteins composed of p62(PB1) and AG were associated with each other, forming the fluorescent foci. Thus, it was revealed that the PB1 domain of p62 itself did not have an association ability, but had a nature of forming an assembly (fluorescent focus) when fused to the fluorescent protein having a multimerization ability, suggesting that the PB1 domain of p62 was suitably usable as the association-inducing protein according to the present invention.

(Example 2)

<Detection 1 of Protein-Protein Interaction>

In order to verify that the PB1 domain of p62 was suitably usable as the association-inducing protein according to the present invention, in other words, to verify that the PB1 domain of p62 was applicable to the model illustrated in Figs. 1 and 2, a test was conducted by a method described below using proteins whose interaction was inducible by adding a drug. Figs. 6 to 8 show the obtained results.

Note that a FRB domain of a mTOR protein (also referred to as "mTOR(FRB)" or "mTOR(FRB domain)") and a FKBP12 protein used in Example 2 have been known to interact with each other in the presence of rapamycin (see Chen J et al., Proc Natl Acad Sci U S A., May 23, 1995, vol. 92, no. 11, pp. 4947 to 4951).

(Preparation of Plasmid DNAs)

In preparing a plasmid for fusing AG (phAG-MNLinker), first, a humanized-codon Azami Green (AG) gene was amplified by PCR using phAG-MCLinker as a template and the following primer set:

hAG forward primer 2;
5'-GGACCGGTATGGTGAGCGTGATCAAGCCCGAG-3' (SEQ ID NO: 61),
and

hAG reverse primer 2;
5'-TTTCTAGATCACTTGGCCTGGCTGGGCAGCATGC-3' (SEQ ID NO: 62).

Then, the amplification product thus obtained was cleaved with AgeI and XbaI, and inserted into phmAG1-MNLinker (manufactured by limited company Amalgaam Co., Ltd.) having been treated with the same restriction enzymes. Thus, phAG-MNLinker was prepared.

Meanwhile, in preparing a plasmid for fusing p62(PB1) (pp62(PB1)-MNLinker), first, the DNA encoding a PB1 domain of p62 (region having the amino acid sequence of SEQ ID NO: 4) (the DNA had the base sequence of SEQ ID NO: 3) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

p62(PB1) forward primer 2;
5'-GGGACCGGTATGGCGTCGCTACCGTGAAGGCCTACCTTC-3' (SEQ ID NO: 63), and

p62(PB1) reverse primer 2;
5'-ACCTCTAGATTATTTCTCTTTAATGTAGATTCGGAAGATG-3' (SEQ ID

NO: 64).

Then, the amplification product thus obtained was cleaved with AgeI and XbaI, and inserted into phmAG1-MNLinker having been treated with the same restriction enzymes. Thus, pp62(PB1)-MNLinker was prepared.

Further, in preparing the plasmid for fusing p62(PB1) (pp62(PB1)-MCLinker), first, the DNA encoding a PB1 domain of p62 (region having the amino acid sequence of SEQ ID NO: 4) (the DNA had the base sequence of SEQ ID NO: 3) was amplified by PCR using the pp62(PB1)-MNLinker as a template and the following primer set:

p62(PB1) forward primer 3;
5'-TAGCGCTAGCATTGCCACCATGGCGTCGCTCACCGTGAAGGCCTACCTTC-
3' (SEQ ID NO: 65), and

p62(PB1) reverse primer 3;
5'-AAAACCGGTTTTCTCTTTAATGTAGATTCGGAAGATG-3' (SEQ ID NO: 66).

Then, the amplification product thus obtained was cleaved with NheI and AgeI, and inserted into phmAG1-MCLinker having been treated with the same restriction enzymes. Thus, pp62(PB1)-MCLinker was prepared.

Moreover, in preparing pmTOR(FRB domain)-hAG, first, a DNA encoding a FRB domain of mTOR (region having the 2025th to 2114th amino acids of the mTOR protein, the region had

the amino acid sequence of SEQ ID NO: 22) (the DNA had the base sequence of SEQ ID NO: 21) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

5 mTOR(FRB) forward primer;
5'-GCCGAATTCGGCCACCATGGAGATGTGGCATGAAGGCCTGGAAGAGGCATC
TCG-3' (SEQ ID NO: 67), and
 mTOR(FRB) reverse primer;
5'-GGGCTCGAGCCCTGCTTTGAGATTCGTCGGAACACATGATAATAGAGGTCC
10 C-3' (SEQ ID NO: 68).

Then, the amplification product thus obtained was cleaved with EcoRI and XhoI, and inserted into pHAG-MNLinker having been treated with the same restriction enzymes. Thus, pmTOR(FRB domain)-hAG was prepared. Note
15 that the pmTOR(FRB domain)-hAG encodes a fusion protein composed of mTOR(FRB) and an AG protein (the fusion protein may also be referred to as "mTOR(FRB)-AG").

Further, in preparing pp62(PB1)-FKBP12, first, a DNA encoding FKBP12 (full length, region having the amino acid
20 sequence of SEQ ID NO: 24) (the DNA had the base sequence of SEQ ID NO: 23) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

 FKBP12 forward primer;
5'-GCCGAATTCGATGGGAGTGCAGGTGGAAACC-3' (SEQ ID NO: 69),
25 and
 FKBP12 reverse primer;

5'-GGGCTCGAGTTATTCCAGTTTTAGAAGCTCCA-3' (SEQ ID NO: 70).

Then, the amplification product thus obtained was cleaved with EcoRI and XhoI, and inserted into pp62(PB1)-MCLinker having been treated with the same restriction enzymes. Thus, pp62(PB1)-FKBP12 was prepared. Note that the pp62(PB1)-FKBP12 encodes a fusion protein composed of p62(PB1) and a FKBP12 protein (the fusion protein may also be referred to as "p62(PB1)-FKBP12").

Meanwhile, in preparing phAG-FKBP12, first, phAG-MCLinker was cleaved with NheI and AgeI to prepare a hAG1 gene. Then, the hAG1 gene was inserted into pp62(PB1)-FKBP12 having been treated with the same restriction enzymes to cut out a p62(PB1) region therefrom. Thus, phAG-FKBP12 was prepared.

Further, in preparing phmAG1-FKBP12, first, phmAG1-MCLinker was cleaved with NheI and AgeI to prepare a hmAG1 gene. Then, the hmAG1 gene was inserted into pp62(PB1)-FKBP12 having been treated with the same restriction enzymes to cut out a p62(PB1) region therefrom. Thus, phmAG1-FKBP12 was prepared. Note that the phmAG1-FKBP12 encodes a fusion protein composed of a mAG1 protein and a FKBP12 protein (the fusion protein may also be referred to as "mAG1-FKBP12").

Furthermore, in preparing pmTOR(FRB domain)-p62(PB1), first, pp62(PB1)-MNLinker was cleaved with AgeI and XbaI to prepare a p62(PB1) gene. Then, the

gene was inserted into pmTOR(FRB domain)-hAG having been treated with the same restriction enzymes to cut out a hAG region therefrom. Thus, pmTOR(FRB domain)-p62(PB1) was prepared. Note that the pmTOR(FRB domain)-p62(PB1) encodes a fusion protein composed of mTOR(FRB) and p62(PB1) (the fusion protein may also be referred to as "mTOR(FRB)-p62(PB1)").

(Transfection into Cultured Cells, and Observation of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 1:

a combination of pmTOR(FRB domain)-hAG with pp62(PB1)-FKBP12;

a combination of phAG-FKBP12 with pmTOR(FRB domain)-p62(PB1); and

a combination of phmAG1-FKBP12 with pmTOR(FRB domain)-p62(PB1).

Moreover, the transfected cells were observed also by the same method as that described in Example 1. Then, fluorescence images were captured before 100 nM rapamycin (manufactured by Merck KGaA) was added and 300 seconds after the addition.

As apparent from the result shown in Fig. 6, mTOR(FRB)-AG was present in a dispersed manner before rapamycin was added (see the left panel in Fig. 6); meanwhile,

fluorescent foci were detected in the cells after the addition (see the right panel in Fig. 6). Moreover, as apparent from the result shown in Fig. 7, AG-FKBP12 was present in a dispersed manner before rapamycin was added (see the upper panel in Fig. 7); meanwhile, fluorescent foci were detected in the cells after the addition (see the lower panel in Fig. 7). Thus, it was revealed that by the rapamycin-dependent interaction between mTOR(FRB) and the FKBP12 protein, mTOR(FRB)-AG was autonomously associated with p62(PB1)-FKBP12, and AG-FKBP12 was autonomously associated with mTOR(FRB)-p62(PB1), thereby both forming fluorescent foci.

On the other hand, as apparent from the result shown in Fig. 8, in the case where mAG1 was used as the fluorescent protein, no fluorescent focus was detected in the cells after rapamycin was added, and no rapamycin-dependent assembly formation was observed. Thus, it was revealed that if the fluorescent protein having a multimerization ability and the association-inducing protein were not used in combination, no assembly was formed by the protein-protein interaction and no fluorescent focus was detected, verifying that the model illustrated in Figs. 1 and 2 was practicable.

(Example 3)

<Detection 2 of Protein-Protein Interaction>

In order to verify that the PB1 domain of p62 was

applicable to the model illustrated in Figs. 1 and 2, a test was conducted by a method described below using the FRB domain of mTOR and FKBP12 described above. Figs. 9 to 11 show the obtained results.

5 (Transfection into Cultured Cells, and Observation of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 2.
10 Then, fluorescence images were captured before 100 nM rapamycin (manufactured by Merck KGaA) was added and 300 seconds after the addition.

A combination of pmTOR(FRB domain)-hAG with pp62(PB1)-FKBP12;

15 a combination of pmTOR(FRB domain)-hmAG1 with pp62(PB1)-FKBP12; and

a combination of pmTOR(FRB domain)-hAG with pp62(PB1_nc)-FKBP12.

(Preparation of Plasmid DNAs)

20 By the same method as that described in Example 2, pmTOR(FRB domain)-hmAG1 was prepared by cutting out a DNA encoding hAG from pmTOR(FRB domain)-hAG, and inserted a DNA encoding hmAG1 into that position instead.

Moreover, pp62(PB1_nc)-FKBP12 was prepared by
25 introducing a mutation using pp62(PB1)-FKBP12 as a template, and AMAP(TM) Multi Site-directed Mutagenesis Kit

(manufactured by limited company Amalgaam Co., Ltd.) according to the attached instruction, with the following primer:

a primer having the DNA sequence of SEQ ID NO: 154:

5'-GCTTCCAGGCGCACTACCGCGCTGAGCGCGGGGACTTGGTTGCCTTTTC-3'

Note that p62(PB1_nc) is a mutant obtained by introducing a 2-amino acid mutation to an interface where p62(PB1) interacts with another p62(PB1), so that the mutant has no association-inducing ability.

As apparent from the result shown in Fig. 9, similarly to the result described in Example 2, mTOR(FRB)-AG was present in a dispersed manner before rapamycin was added (see the upper panel in the figure); meanwhile, fluorescent foci were detected in the cells after the addition (see the lower panel in the figure).

On the other hand, as apparent from the result shown in Fig. 10, similarly to the result described in Example 2, in the case where mAG1 was used as the fluorescent protein, no fluorescent focus was detected in the cells even after rapamycin was added, and no rapamycin-dependent assembly formation was observed.

Further, as apparent from the result shown in Fig. 11, in the case where the mutant p62(PB1_nc) no longer capable of forming a homomultimer was used in place of p62(PB1) also, no fluorescent focus was detected in the cells even after rapamycin was added, and no

rapamycin-dependent assembly formation was observed.

Thus, it was revealed that if the fluorescent protein having a multimerization ability and the association-inducing protein were not used in combination,
5 no assembly was formed by the protein-protein interaction and no fluorescent focus was detected, verifying that the model illustrated in Figs. 1 and 2 was practicable.

It should be noted that the method for detecting a protein-protein interaction of the present invention is
10 a method totally different from conventional methods for detecting a protein-protein interaction in that fluorescent foci are autonomously formed only when a protein-protein interaction takes place.

(Example 4)

15 <Screening 2 for Association-Inducing Protein>

In order to find out association-inducing proteins having natures similar to those of p62(PB1), screening was carried out by the same method as that described in Example 1.

20 As such screening targets other than the PB1 domain, attention was focused on a SAM domain. A protein composed of mAG1 fused to a PB1 domain or a SAM domain derived from proteins, or a protein composed of AG serving as the fluorescent protein having a multimerization ability fused
25 to a PB1 domain or a SAM domain derived from proteins was expressed in cultured cells. Then, an association between

the fusion proteins and eventually the presence or absence of formation of a fluorescent focus formed by the association were examined by a method described below. Figs. 12 to 15 show the obtained results.

(Preparation of Plasmid DNAs)

To fuse the PB1 domain or the SAM domain derived from the proteins on the C-terminus side of a fluorescent protein via a flexible linker for the expression, phmAG1-MCLinker was used as a plasmid for fusing mAG1, and phAG-MCLinker was used as a plasmid for fusing AG.

Specifically, in preparing phmAG1-MEK5(PB1) and phAG-MEK5(PB1), first, a DNA encoding a PB1 domain of MEK5 (region having the 16th to 109th amino acids of the MEK5 protein, the region had the amino acid sequence of SEQ ID NO: 6, also referred to as "MEK5(PB1)") (the DNA had the base sequence of SEQ ID NO: 5) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

MEK5(PB1) forward primer;

5'-CCGAATTCGGTGCTGGTAATTCGCATCAAGATCCCAA-3' (SEQ ID NO: 71), and

MEK5(PB1) reverse primer;

5'-TTCTCGAGTTAGCAGGCTCTTGGAATATCTGCAG-3' (SEQ ID NO: 72).

Then, the amplification product thus obtained was cleaved with EcoRI and XhoI, and inserted into

phmAG1-MCLinker and phAG-MCLinker having been treated with the same restriction enzymes; thus, phmAG1-MEK5(PB1) and phAG-MEK5(PB1) were prepared, respectively. Note that these plasmid DNAs encode a fusion protein composed of a
5 mAG1 protein and MEK5(PB1) (the fusion protein may also be referred to as "mAG1-MEK5(PB1)"), and a fusion protein composed of an AG protein and MEK5(PB1) (the fusion protein may also be referred to as "AG-MEK5(PB1)"), respectively.

Meanwhile, in preparing phmAG1-Nbr1(PB1) and
10 phAG-Nbr1(PB1), first, a DNA encoding a PB1 domain of Nbr1 (region having the 4th to 85th amino acids of the Nbr1 protein, the region had the amino acid sequence of SEQ ID NO: 8, also referred to as "Nbr1(PB1)") (the DNA had the base sequence of SEQ ID NO: 7) was amplified from the cDNA library
15 of HeLaS3 cells by PCR using the following primer set:

Nbr1(PB1) forward primer;
5'-AAGAATTCGGCAGGTTACTCTAAATGTGACTTTTAAA-3' (SEQ ID NO:
73), and

Nbr1(PB1) reverse primer;
20 5'-TTCTCGAGTTACCCTTCGTGGACTTGCATCTGCAGTT-3' (SEQ ID NO:
74).

Then, the amplification product thus obtained was cleaved with EcoRI and XhoI, and inserted into phmAG1-MCLinker and phAG-MCLinker having been treated with
25 the same restriction enzymes; thus, phmAG1-Nbr1(PB1) and phAG-Nbr1(PB1) were prepared, respectively. Note that

these plasmid DNAs encode a fusion protein composed of a mAG1 protein and Nbr1(PB1) (the fusion protein may also be referred to as "mAG1-Nbr1(PB1)"), and a fusion protein composed of an AG protein and Nbr1(PB1) (the fusion protein may also be referred to as "AG-Nbr1(PB1)"), respectively.

Further, in preparing phmAG1-PKCiota(PB1) and phAG-PKCiota(PB1), first, a DNA encoding a PB1 domain of PKCiota (region having the 16th to 99th amino acids of the PKCiota protein, the region had the amino acid sequence of SEQ ID NO: 10, also referred to as "PKCiota(PB1)") (the DNA had the base sequence of SEQ ID NO: 9) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

PKCiota(PB1) forward primer;
5'-AAGAATTCGCAGGTCCGGGTGAAAGCCTACTACCGCG-3' (SEQ ID NO: 75), and

PKCiota(PB1) reverse primer 18;
5'-TTCTCGAGTTAACAAGGGAACACATGAATCAAGAGTTCAG-3' (SEQ ID NO: 76).

Then, the amplification product thus obtained was cleaved with EcoRI and XhoI, and inserted into phmAG1-MCLinker and phAG-MCLinker having been treated with the same restriction enzymes; thus, phmAG1-PKCiota(PB1) and phAG-PKCiota(PB1) were prepared, respectively. Note that these plasmid DNAs encode a fusion protein composed of a mAG1 protein and PKCiota(PB1) (the fusion protein may

also be referred to as "mAG1-PKCiota(PB1)"), and a fusion protein composed of an AG protein and PKCiota(PB1) (the fusion protein may also be referred to as "AG-PKCiota(PB1)"), respectively.

5 Moreover, in preparing phmAG1-TFG(PB1) and phAG-TFG(PB1), first, a DNA encoding a PB1 domain of TFG (region having the 10th to 91st amino acids of the TFG protein, the region had the amino acid sequence of SEQ ID NO: 12, also referred to as "TFG(PB1)") (the DNA had the base
10 sequence of SEQ ID NO: 11) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

 TFG(PB1) forward primer 1;
5'-AACTGCAGCAAAGCTAATCATCAAAGCTCAACTTGGGGA-3' (SEQ ID NO: 77), and

15 TFG(PB1) reverse primer 1;
5'-TTAAGCTTTTAATTAACAAATAATGTCAGTTTCAGTAT-3' (SEQ ID NO: 78).

 Then, the amplification product thus obtained was cleaved with PstI and HindIII, and inserted into
20 phmAG1-MCLinker and phAG-MCLinker having been treated with the same restriction enzymes; thus, phmAG1-TFG(PB1) and phAG-TFG(PB1) were prepared, respectively. Note that these plasmid DNAs encode a fusion protein composed of a mAG1 protein and TFG(PB1) (the fusion protein may also be
25 referred to as "mAG1-TFG(PB1)"), and a fusion protein composed of an AG protein and TFG(PB1) (the fusion protein

may also be referred to as "AG-TFG(PB1)"), respectively.

Further, in preparing phmAG1-TEL(SAM) and phAG-TEL(SAM), first, a DNA encoding a SAM domain of TEL (region having the 38th to 124th amino acids of the TFG protein, the region had the amino acid sequence of SEQ ID NO: 14, also referred to as "TEL(SAM)") (the DNA had the base sequence of SEQ ID NO: 13) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

TEL(SAM) forward primer;
5'-AAAAGGATCCGCCACCATGCCTCGAGCGCTCAGGATGGAGGAA-3' (SEQ ID NO: 79), and

TEL(SAM) reverse primer;
5'-AAAAAAGCTTTTACCTCTGCTTCAGAATATGCTGAAGGAGTT-3' (SEQ ID NO: 80).

Then, the amplification product thus obtained was cleaved with BamHI and HindIII, and inserted into phmAG1-MCLinker and phAG-MCLinker having been treated with the same restriction enzymes; thus, phmAG1-TEL(SAM) and phAG-TEL(SAM) were prepared, respectively. Note that these plasmid DNAs encode a fusion protein composed of a mAG1 protein and TEL(SAM) (the fusion protein may also be referred to as "mAG1-TEL(SAM)"), and a fusion protein composed of an AG protein and TEL(SAM) (the fusion protein may also be referred to as "AG-TEL(SAM)"), respectively.

Additionally, in preparing phmAG1-EphB2(SAM) and

phAG-EphB2(SAM), first, a DNA encoding a SAM domain of EphB2 (region having the 905th to 981st amino acids of the EphB2 protein, the region had the amino acid sequence of SEQ ID NO: 16, also referred to as "EphB2(SAM)") (the DNA had the
5 base sequence of SEQ ID NO: 15) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

EphB2(SAM) forward primer;
5'-AAAAGGATCCGCCACCATGCTGGACCGCACGATCCCCGA-3' (SEQ ID
10 NO: 81), and

EphB2(SAM) reverse primer;
5'-AAAAAAGCTTTTAAATCTGGTTCATCTGCGCCCG-3' (SEQ ID NO: 82).

Then, the amplification product thus obtained was cleaved with BamHI and HindIII, and inserted into
15 phmAG1-MCLinker and phAG-MCLinker having been treated with the same restriction enzymes; thus, phmAG1-EphB2(SAM) and phAG-EphB2(SAM) were prepared, respectively. Note that these plasmid DNAs encode a fusion protein composed of a
20 mAG1 protein and EphB2(SAM) (the fusion protein may also be referred to as "mAG1-EphB2(SAM)"), and a fusion protein composed of an AG protein and EphB2(SAM) (the fusion protein may also be referred to as "AG-EphB2(SAM)"), respectively .

Furthermore, in preparing phmAG1-DGK delta(SAM) and phAG-DGK delta(SAM), first, a DNA encoding a SAM domain
25 of DGK delta (region having the 1097th to 1164th amino acids of the DGK delta protein, the region had the amino acid

sequence of SEQ ID NO: 18, also referred to as "DGK delta(SAM)") (the DNA had the base sequence of SEQ ID NO: 17) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

5 DGK delta(SAM) forward primer;
5'-AAAAGGTACCGCCACCATGCCGGTTCACCTCTGGGGGACA-3' (SEQ ID NO: 83), and

 DGK delta(SAM) reverse primer;
5'-AAAAAAGCTTTTAGCTGCGGCTCAGCTCCTTGAT-3' (SEQ ID NO: 84).

10 Then, the amplification product thus obtained was cleaved with KpnI and HindIII, and inserted into phmAG1-MCLinker and phAG-MCLinker having been treated with the same restriction enzymes; thus, phmAG1-DGK delta(SAM) and phAG-DGK delta(SAM) were prepared, respectively. Note
15 that these plasmid DNAs encode a fusion protein composed of a mAG1 protein and DGK delta(SAM) (the fusion protein may also be referred to as "mAG1-DGK delta(SAM)"), and a fusion protein composed of an AG protein and DGK delta(SAM) (the fusion protein may also be referred to as "AG-DGK
20 delta(SAM)"), respectively.

 In addition, in preparing phmAG1-Tankyrase(SAM) and phAG-Tankyrase(SAM), first, a DNA encoding a SAM domain of Tankyrase (region having the 952nd to 1078th amino acids of the Tankyrase protein, the region had the amino acid
25 sequence of SEQ ID NO: 20, also referred to as "Tankyrase(SAM)") (the DNA had the base sequence of SEQ

ID NO: 19) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

Tankyrase(SAM) forward primer;

5'-AAAAGGATCCGCCACCATGCTGATAGATGCCATGCCCCCAGA-3' (SEQ

ID NO: 85), and

Tankyrase(SAM) reverse primer;

5'-AAAAAAGCTTTTAAATTCGAATGACATTGTATCTGTTGAAGA-3' (SEQ

ID NO: 86).

Then, the amplification product thus obtained was
cleaved with BamHI and HindIII, and inserted into
phmAG1-MCLinker and phAG-MCLinker having been treated with
the same restriction enzymes; thus, phmAG1-Tankyrase(SAM)
and phAG-Tankyrase(SAM) were prepared, respectively.
Note that these plasmid DNAs encode a fusion protein
composed of a mAG1 protein and Tankyrase(SAM) (the fusion
protein may also be referred to as "mAG1-Tankyrase(SAM)"),
and a fusion protein composed of an AG protein and
Tankyrase(SAM) (the fusion protein may also be referred
to as "AG-Tankyrase(SAM)"), respectively.

(Transfection into Cultured Cells, and Observation
of Transfected Cells)

Each of the plasmid DNAs was introduced into HeLaS3
cells by the same method as that described in Example 1.
Moreover, the transfected cells were observed also by the
same method as that described in Example 1.

As apparent from the results shown in Figs. 13, 14,

and 15, PKC α (PB1), TFG(PB1), TEL(SAM), DGK δ (SAM), and Tankyrase(SAM) were dispersed when fused to mAG1, and formed fluorescent foci (assemblies) when fused to AG. Note that the cells expressing AG-PKC α (PB1) included both of cells forming fluorescent foci and cells not forming fluorescent foci. In contrast, in all the cells expressing the other fusion proteins comprising the AG protein, fluorescent foci were detected.

On the other hand, as apparent from the results shown in Figs. 12 and 15, when MEK5(PB1), Nbr1(PB1), and EphB2(SAM) were fused to mAG1 or when fused to AG, no fluorescent focus formation was observed.

Thus, it was revealed that TFG(PB1), TEL(SAM), DGK δ (SAM), and Tankyrase(SAM) were usable as the association-inducing protein according to the present invention.

(Example 5)

<Detection 3 of Protein-Protein Interaction>

In order to verify that the association-inducing proteins selected in Example 4 were suitably usable in the method for detecting a protein-protein interaction of the present invention, a test described below was conducted by the same method as that described in Example 2. Fig. 16 shows the obtained result.

(Preparation of Plasmid DNAs)

In preparing pFKBP12-TFG(PB1), first, a TFG(PB1) gene

was amplified from phAG-TFG(PB1) by PCR using the following primer set:

TFG(PB1) forward primer 2;
5'-AAACCGGTAAGCTAATCATCAAAGCTCAACTT-3' (SEQ ID NO: 87),

and

TFG(PB1) reverse primer 2;
5'-TTTCTAGATTAATTAACAAATAATGTCAGTTTCAGTAT-3' (SEQ ID NO: 88).

Then, the amplification product thus obtained was
cleaved with AgeI and XbaI, and inserted into
pFKBP12-p62(PB1) having been treated with the same
restriction enzymes to cut out a p62(PB1) region therefrom.
Thus, pFKBP12-TFG(PB1) was prepared. Note that the
pFKBP12-TFG(PB1) encodes a fusion protein composed of a
FKBP12 protein and TFG(PB1) (the fusion protein may also
be referred to as "FKBP12-TFG(PB1)").

Meanwhile, in preparing pFKBP12-TEL(SAM), first, a
TEL(SAM) gene was amplified from phAG-TEL(SAM) by PCR using
the following primer set:

TEL(SAM) forward primer 2;
5'-AAAAACCGGTCCTCGAGCGCTCAGGATGGAGGAA-3' (SEQ ID NO: 89),

and

TEL(SAM) reverse primer 2;
5'-AAAATCTAGATTACCTCTGCTTCAGAATATGCTGAAGGAGTT-3' (SEQ
ID NO: 90).

Then, the amplification product thus obtained was

cleaved with AgeI and XbaI, and inserted into pFKBP12-p62(PB1) having been treated with the same restriction enzymes to cut out a p62(PB1) region therefrom. Thus, pFKBP12-TEL(SAM) was prepared. Note that the
5 pFKBP12-TEL(SAM) encodes a fusion protein composed of a FKBP12 protein and TEL(SAM) (the fusion protein may also be referred to as "FKBP12-TEL(SAM)").

Further, in preparing pFKBP12-DGK delta(SAM), first, a DGK delta(SAM) gene was amplified from phAG-DGK
10 delta(SAM) by PCR using the following primer set:

DGK delta(SAM) forward primer 2;
5'-AAAAACCGGTCCGGTTCACCTCTGGGGGACAGA-3' (SEQ ID NO: 91),
and

DGK delta(SAM) reverse primer 2;
15 5'-AAAATCTAGATTAGCTGCGGCTCAGCTCCTTGAT-3' (SEQ ID NO: 92).

Then, the amplification product thus obtained was cleaved with AgeI and XbaI, and inserted into pFKBP12-p62(PB1) having been treated with the same restriction enzymes to cut out a p62(PB1) region therefrom.
20 Thus, pFKBP12-DGK delta(SAM) was prepared. Note that the pFKBP12-DGK delta(SAM) encodes a fusion protein composed of a FKBP12 protein and DGK delta(SAM) (the fusion protein may also be referred to as "FKBP12-DGKd(PB1)").

Moreover, in preparing pFKBP12-Tankyrase(SAM),
25 first, a Tankyrase(SAM) gene was amplified from phAG-Tankyrase(SAM) by PCR using the following primer set:

Tankyrase(SAM) forward primer 2;
5'-AAAAACCGGTCTGATAGATGCCATGCCCCAGA-3' (SEQ ID NO: 93),
and

5 Tankyrase(SAM) reverse primer 2;
5'-AAAATCTAGATTAAATTCGAATGACATTGTATCTGTTGAAGA-3' (SEQ
ID NO: 94).

10 Then, the amplification product thus obtained was
cleaved with AgeI and XbaI, and inserted into
pFKBP12-p62(PB1) having been treated with the same
restriction enzymes to cut out a p62(PB1) region therefrom.
Thus, pFKBP12-Tankyrase(SAM) was prepared. Note that
pFKBP12-Tankyrase(SAM) encodes a fusion protein composed
of a FKBP12 protein and Tankyrase(SAM) (the fusion protein
may also be referred to as "FKBP12-Tankyrase(SAM)").

15 (Transfection into Cultured Cells, and Observation
of Transfected Cells)

Each of the following combinations of the plasmid
DNAs was mixed in equal amounts and introduced into HeLaS3
cells by the same method as that described in Example 1:

20 a combination of pmTOR(FRB domain)-hAG with
pFKBP12-TFG(PB1);

a combination of pmTOR(FRB domain)-hAG with
pFKBP12-TEL(SAM);

25 a combination of pmTOR(FRB domain)-hAG with
pFKBP12-DGK delta(SAM); and

a combination of pmTOR(FRB domain)-hAG with

pFKBP12-Tankyrase(SAM).

Moreover, the transfected cells were observed also by the same method as that described in Example 1. Then, it was confirmed that no fluorescent focus was detected in each of the cultured cells before 100 nM rapamycin (manufactured by Merck KGaA) was added, and fluorescence images were captured 300 seconds after the addition.

As apparent from the result shown in Fig. 16, even when any of the fusion proteins were expressed, mTOR(FRB domain)-AG was present in a dispersed manner before rapamycin was added (unillustrated); meanwhile, fluorescent foci were detected in all the cells after the addition. In other words, it was revealed that by the rapamycin-dependent interaction between mTOR(FRB domain) and the FKBP12 protein, mTOR(FRB domain)-AG was autonomously associated with FKBP12-TEL(SAM) and so forth, thereby forming fluorescent foci.

Thus, it was revealed that the use of not only p62(PB1) but also the other types of domains derived from the other proteins, such as TEL(SAM), as the association-inducing protein enabled detection of a protein-protein interaction. Moreover, this result verified that the model illustrated in Figs. 3 and 4 was practicable as the screening method for an association-inducing protein according to the present invention.

(Example 6)

<Detection 3 of Protein-Protein Interaction>

Whether or not fluorescent proteins other than the AG protein were applicable as the fluorescent protein having a multimerization ability in the method for detecting a protein interaction of the present invention was examined by a method described below. Fig. 17 shows the obtained result.

Note that the fluorescent proteins thus examined were Kusabira-Orange 1 (K01), dimeric Keima-Red (dKeima), and Kikume Green-Red (KikGR), which have been known to form a homodimer, a homodimer, and a homotetramer, respectively.

(Preparation of Plasmid DNAs)

In preparing pmTOR(FRB domain)-hK01, first, a hK01 gene was amplified from pHK01-MN1 (manufactured by limited company Amalgaam Co., Ltd.) by PCR using the following primer set:

hK01 forward primer;
5'-AAAAACCGGTATGGTGAGCGTGATCAAGCCCGAG-3' (SEQ ID NO: 95),
and

hK01 reverse primer;
5'-AAAATCTAGATTAGCAGTGGGCCACGGCGTCCTCC-3' (SEQ ID NO: 96).

Then, the amplification product thus obtained was cleaved with AgeI and XbaI, and inserted into pmTOR(FRB domain)-hAG having been treated with the same restriction enzymes to cut out a hAG region therefrom. Thus, pmTOR(FRB

domain)-hK01 was prepared.

Meanwhile, in preparing pmTOR(FRB domain)-hdKeima-Red, first, a hdKeima gene was prepared by cleaving phdKeima-Red-MNLinker (manufactured by limited company Amalgaam Co., Ltd.) with AgeI and XbaI. Then, the obtained hdKeima gene was inserted into pmTOR(FRB domain)-hAG having been treated with the same restriction enzymes to cut out a hAG region therefrom. Thus, pmTOR(FRB domain)-hdKeima-Red was prepared.

Further, in preparing pmTOR(FRB domain)-hKikGR1, first, a hKikGR1 gene was prepared by cleaving phKikGR1-MNLinker (manufactured by limited company Amalgaam Co., Ltd.) with AgeI and XbaI. Then, the obtained hKikGR1 gene was inserted into pmTOR(FRB domain)-hAG having been treated with the same restriction enzymes to cut out a hAG region therefrom. Thus, pmTOR(FRB domain)-phKikGR1 was prepared.

(Transfection into Cultured Cells, and Observation of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 1:

a combination of pmTOR(FRB domain)-hK01 with pp62(PB1)-FKBP12;

a combination of pmTOR(FRB domain)-hdKeima-Red with pp62(PB1)-FKBP12;

a combination of pmTOR(FRB domain)-hKikGR1 with pp62(PB1)-FKBP12; and

a combination of pmTOR(FRB domain)-hAG with pp62(PB1)-FKBP12.

5 Moreover, the transfected cells were observed also by the same method as that described in Example 1. Note that the K01 was observed using an excitation filter (BP520-540HQ, Olympus Corporation), a dichroic mirror (DM545HQ, manufactured by Olympus Corporation), and an
10 absorption filter (BA555-600HQ, manufactured by Olympus Corporation). The dKeima was observed using an excitation filter (440AF21, manufactured by OMEGA OPTICAL, INC.), a dichroic mirror (455DRLP, manufactured by OMEGA OPTICAL, INC.), and an absorption filter (610ALP, manufactured by
15 OMEGA OPTICAL, INC.). Then, fluorescence images were captured before 100 nM rapamycin (manufactured by Merck KGaA) was added and 300 seconds after the addition.

 As apparent from the result shown in Fig. 17, as in the case of using the AG protein, all of the examined
20 fluorescent proteins demonstrated that mTOR(FRB) fused to the fluorescent proteins was present in a dispersed manner before rapamycin was added; meanwhile, fluorescent foci (assemblies) were formed after the addition. Thus, it was verified that, in the present invention, the use of not
25 only the AG protein but also the other fluorescent proteins having a multimerization ability, such as the K01 protein,

enabled detection of a protein-protein interaction.

(Example 7)

<Screening 1 of Fluorescent Protein Having
Multimerization Ability>

5 Whether or not a fluorescent protein other than AG,
K01, dKeima, and KikGR was applicable as the fluorescent
protein having a multimerization ability in the method for
detecting a protein interaction of the present invention
was examined by a method described below. Specifically,
10 examined was whether or not an assembly (fluorescent focus)
as shown in Fig. 3 was formed when monomeric Kusabira-Orange
2 (mK02) was fused with p62(PB1) serving as the
association-inducing protein and expressed in cells. Fig.
18 shows the obtained result.

15 First, as in the case of "phmAG1-p62(PB1) and
phAG-p62(PB1)" described in Example 1, the DNA encoding
p62(PB1) was amplified by PCR. The amplification product
thus obtained was cleaved with EcoRI and NotI, and inserted
into phmK02-MCLinker (manufactured by limited company
20 Amalgaam Co., Ltd.) having been treated with the same
restriction enzymes. Thus, phmK02-p62(PB1) was prepared.
Then, the phmK02-p62(PB1) was introduced into HeLaS3 cells
by the same method as that described in Example 1. Moreover,
the transfected cells were observed also by the same method
25 as that described in Example 6. Fig. 18 shows the obtained
result.

As apparent from the result shown in Fig. 18, mK02-p62(PB1) proteins encoded by phmK02-p62(PB1) were associated with each other, thereby forming fluorescent foci. Thus, it was revealed that not only the above-described fluorescent proteins capable of forming a homomultimer in cells, such as AG, K01, dKeima, and KikGR, but also mK02 generally believed to be a monomeric fluorescent protein were usable as the fluorescent protein having a multimerization ability in the method of the present invention.

(Example 8)

<Screening 2 of Fluorescent Proteins Having Multimerization Ability>

It was confirmed by the following method that other than AG, K01, dKeima, and KikGR described above, MiCy1, KCy1, dAG (AB), and dAG (AC) (fluorescent protein capable of homodimerization) as well as TGuv, Momiji, COR3.01, COR5, and DsRed2 (fluorescent protein capable of homotetramerization), which had been known as fluorescent proteins capable of forming a homomultimer in cells, were also usable in the method for detecting a protein interaction of the present invention.

Moreover, in order to find out fluorescent proteins generally believed to be monomeric fluorescent proteins such as mK02 but usable in the method of the present invention, screening was carried out by the following method.

(Method for Detecting Protein-Protein Interaction)

By the same method as that described in Examples 5 and 6, p62(PB1)-FKBP12, FKBP12-DGKd(SAM), FKBP12-TEL(SAM), or FKBP12-Tankyrase(SAM), and mTOR(FRB) fused to
5 corresponding one of fluorescent proteins shown in Table 1 were expressed in HeLaS3 cells to evaluate a degree of fluorescent focus (assembly) formation after rapamycin was added. Note that plasmid DNAs encoding fusion proteins composed of mTOR(FRB) and the corresponding fluorescent
10 proteins were prepared as appropriate by the same method as that described in Example 2. Moreover, regarding the combinations of FKBP12-Tankyrase(SAM) with mK02, mKeima, mMiCy1, mK01, MiCy1, and TGuv, the test was conducted by introducing the genes into 293T cells. Specifically, the
15 293T cells were cultured in DMEM High glucose (manufactured by SIGMA ALDRICH CO.) containing 10% FBS (manufactured by Equitech-Bio Inc.). Moreover, the 293T cells were seeded onto an 8-well chamber (manufactured by Nunc A/S) 6 hours before the plasmid DNAs were introduced. Further, at the
20 time of the transfection, 200 ng of the plasmid DNA encoding FKBP12-Tankyrase(SAM) and 200 ng of one of the plasmid DNAs encoding fusion proteins composed of the fluorescent proteins and mTOR(FRB) were diluted with 30 μ l of OptiMEM (manufactured by Life Technologies Corporation), and 1.2
25 μ l of TurboFect Transfection Reagent (manufactured by Thermo Fisher Scientific Inc.) was added thereto and

stirred. Then, the resultant was further mixed with 300
µl of the culture solution, subsequently added to the 293T
cells, and observed 48 hours later. Table 1 shows the
obtained result. In Table 1, "+++" indicates a combination
5 from which fluorescent foci were observed in 50% or more
of the HeLaS3 cells; "++" indicates a combination from which
fluorescent foci were observed in 50% or less of the HeLaS3
cells; and "+" indicates a combination from which
fluorescent foci were observed in 293T cells expressing
10 a larger amount of proteins than that in the HeLaS3 cells.

[Table 1]

		P62 (PB1)	DGKd (SAM)	TEL (SAM)	Tankyrase (SAM)
mKO2	monomer	+++	+++	++	+
mKeima	monomer	+++	+++	++	+
mMiCy1	monomer	+++	+++	+++	+
mKO1	monomer	+++	+++	+++	+
mKikGR1	monomer	+++	+++	+++	+++
MiCy1	dimer	+++	+++	+++	+
KCy1	dimer	+++	+++	+++	++
KO1	dimer	+++	+++	+++	++
dKeima	dimer	+++	+++	+++	++
dAG (AB)	dimer	+++	+++	+++	++
dAG (AC)	dimer	+++	+++	+++	++
TGuv	tetramer	+++	+++	+++	+
Momiji	tetramer	+++	+++	+++	+++
KikGR	tetramer	+++	+++	+++	+++
AG	tetramer	+++	+++	+++	+++
COR3.01	tetramer	+++	+++	+++	+++
COR5	tetramer	+++	+++	+++	+++
DsRed2	tetramer	+++	+++	+++	+++

As apparent from the result shown in Table 1, it was confirmed that all of the fluorescent proteins capable of forming a homomultimer in cells were usable in the method of the present invention. Moreover, the results of TEL(SAM) and Tankyrase(SAM) seem to suggest a tendency that the higher the multimerization ability of a fluorescent protein, the more likely that fluorescent foci (assemblies)

are formed by the protein-protein interaction. Further, as shown in Table 1, it was revealed that mKeima, mMiCy1, mK01, and mKikGR1 generally believed to be monomeric fluorescent proteins, other than mK02, were also usable in the method of the present invention.

(Example 9)

<Detection 5 of Protein-Protein Interaction>

It was confirmed by a method described below that, even in combination with TFG(PB1) serving as the association-inducing protein, the use of the fluorescent proteins having a multimerization ability and confirmed in Examples 6 to 8 to be usable in the method of the present invention enabled detection of a protein-protein interaction. Table 2 shows the obtained result.

(Method for Detecting Protein-Protein Interaction)

By the same method as that described in Examples 5 and 6, TFG(PB1) and mTOR(FRB) fused to mKikGR1, dAG(AC), Momiji, KikGR, AG, COR3.01, COR5, or DsRed2 were expressed in HeLaS3 cells to evaluate a degree of fluorescent focus (assembly) formation after rapamycin was added. Moreover, by the same method as that described in Example 8, TFG(PB1) and mTOR(FRB) fused to K01 or dAG (AB) were expressed in 293T cells to evaluate a degree of fluorescent focus (assembly) formation after rapamycin was added. Table 2 shows the obtained result. In Table 2, "++" indicates a combination from which fluorescent foci were observed in

50% or less of the HeLaS3 cells, and "+" indicates a combination from which fluorescent foci were observed in the 293T cells expressing a larger amount of proteins than that in the HeLaS3 cells.

[Table 2]

		TFG(PB1)
mKikGR1	monomer	++
KO1	dimer	+
dAG(AB)	dimer	+
dAG(AC)	dimer	++
Momiji	tetramer	++
KikGR	tetramer	++
AG	tetramer	++
COR3.01	tetramer	++
COR5	tetramer	++
DsRed2	tetramer	++

As apparent from the result shown in Table 2, it was confirmed that the use of the fluorescent proteins having a multimerization ability in combination with the other association-inducing protein than the proteins described in Example 8 also enabled detection of a protein-protein interaction.

(Example 10)

<Detection 6 of Protein-Protein Interaction>

The fluorescent focus (assembly) according to the present invention is, as described above, attributable to a protein-protein interaction. Hence, a fluorescence intensity of a fluorescent focus presumably reflects a strength of the protein-protein interaction. Moreover, in the method for detecting a protein-protein interaction, quantification and comparison, if possible, of the strength of the interaction are useful in evaluating a substance (inhibitor) suppressing a protein-protein interaction,

and evaluating a factor modulating a protein-protein interaction.

For this reason, the concentration of a compound inducing a protein-protein interaction was changed to test whether or not the fluorescence intensity of fluorescent foci (assemblies) was changed in a manner dependent on the concentration of the compound by a method described below.

(Transfection into Cells, and Observation and Analysis of Transfected Cells)

pmTOR(FRB domain)-hAG and pFKBP12-p62(PB1) were mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 1. The cells were collected 24 hours after the introduction, and seeded onto 96 MicroWell Optical Bottom Plate (manufactured by Nunc A/S) at 20000 cells/well. Then, 24 hours after the seeding, a solution of Hoechst 33342 (manufactured by Dojindo Laboratories) diluted with an observation buffer to 5.6 µg/ml was added to the plate, and allowed for further culturing for 30 minutes. Thereafter, the plate was washed twice with D-PBS(-) (manufactured by Wako Pure Chemical Industries, Ltd.). Then, the medium was replaced with rapamycin having been diluted with an observation buffer to predetermined concentrations, and 15 minutes later the resultant was fixed with 4% Paraformaldehyde Phosphate Buffer Solution (manufactured by Wako Pure Chemical Industries, Ltd.). Note that the concentrations of

rapamycin used were 0.1 nM, 0.2 nM, 0.5 nM, 1.4 nM, 4.1 nM, 12.3 nM, 37.0 nM, 111.1 nM, 333.3 nM, and 1000 nM. Then, the prepared samples were observed using IN Cell Analyzer 1000 (manufactured by GE Healthcare). Fig. 19 shows part of the obtained result.

Further, fluorescence images were analyzed in multiple fields of view. A total luminance (total fluorescence intensity) of fluorescent foci (assemblies) per cell in an image of wells to which rapamycin was added at predetermined concentrations was calculated to analyze a correlation with the rapamycin concentration. Fig. 20 shows part of the obtained result. Note that, in Fig. 20, the X axis represents the concentration of rapamycin added to each well; the Y axis represents the total luminance (total fluorescence intensity) of fluorescent foci (assemblies) per cell; and dots represent the measurement values. Moreover, using Igor(R) (manufactured by WaveMetrics, Inc.), a fitting curve was drawn through the dots, which represents a function fitted to an equation: $y = \text{base} + (\text{max} - \text{base}) / [1 + (\text{xhalf} / x)^{\text{rate}}]$, where $[\text{Dot Intensity} / \text{Cells}] = y$, and $[\text{Canc. (nM)}] = x$. (base=0.0028731, max=0.1823, rate=1.4516, xhalf=46.99).

As apparent from the results shown in Figs. 19 and 20, it was revealed that when the compound rapamycin inducing an interaction between mTOR(FRB domain) and a FKBP12 protein was added to the cells expressing mTOR(FRB

domain)-AG and FKBP12-p62(PB1), the fluorescence intensity of fluorescent foci was increased in a manner dependent on the concentration of rapamycin added. It was also demonstrated that the assemblies between mTOR(FRB domain)-AG and FKBP12-p62(PB1) were formed in a manner dependent on the concentration.

(Example 11)

<Detection 7 of Protein-Protein Interaction>

Whether or not the method of the present invention was utilizable in determining the 50% effective concentration (EC50) and the 50% inhibitory concentration (IC50) of a drug against a protein-protein interaction was evaluated by a method described below.

(Preparation of Plasmid DNA)

Using pmTOR(FRB domain)-hAG described in Example 2 and pFucci-S/G2/M Green-Hyg (manufactured by limited company Amalgaam Co., Ltd.), pmTOR(FRB domain)-hAG_Hyg was prepared according to a conventional method, so that the drug resistance gene was converted to a hygromycin B resistance gene.

(Preparation of Stably-Expressing Cell Line)

By the same method as that described in Example 1, the pmTOR(FRB domain)-hAG_Hyg and pp62(PB1)-FKBP12 described in Example 2 were introduced in a HeLaS3 cell line, and cultured.

Moreover, 24 hours after the plasmid DNAs were

introduced in the HeLaS3 cell line, the medium was replaced with a medium containing 600 µg/mL of G418 (manufactured by Wako Pure Chemical Industries, Ltd.) and 150 µg/mL of hygromycin (manufactured by Nacalai Tesque, Inc.). Then, cells survived after culturing for one week with this medium were cloned by a colony pick-up method.

(Observation and Analysis of Transfected Cells)

The cloned cell lines were seeded onto 96-well plates. Then, after washing twice with PBS on the next day, an observation buffer containing Hoechst 33342 (manufactured by Dojindo Laboratories) was added to the cell line seeded onto each well, followed by incubation at 37°C for 15 minutes for nuclear staining.

Further, these cell lines were washed twice with an observation buffer. Then, an observation buffer containing rapamycin, or rapamycin and FK506 at certain concentrations was added to each well, and incubated for 20 minutes.

Note that the concentration of rapamycin added was 0.39 nM, 0.78 nM, 1.56 nM, 3.13 nM, 6.25 nM, 12.50 nM, 25.00 nM, 50.00 nM, 100.00 nM, 200.00 nM, 400.00 nM, or 800.00 nM. Moreover, FK506 is known as a substance competitively inhibiting an interaction between FKBP12 and rapamycin. In this Example, FK506 was added to each well after diluted with a buffer containing 20 nM rapamycin in such a manner that the FK506 concentration was 0.008 µM, 0.016 µM, 0.031

μM , 0.063 μM , 0.125 μM , 0.250 μM , 0.500 μM , 1.000 μM , 2.000 μM , 4.000 μM , 8.000 μM , or 16.000 μM .

After each drug was added and incubated, 4% Paraformaldehyde Phosphate Buffer Solution (manufactured by Wako Pure Chemical Industries, Ltd.) was added to each well, followed by incubation at room temperature for 15 minutes. Thereby, these cell lines were fixed. Then, these cell lines were washed three times with an observation buffer. Subsequently, an image of three fields of view was obtained for each well using a fluorescence microscope. Thereafter, each fluorescence image was analyzed using iCY (see de Chaumont F et al., Nature Methods, June 28, vol. 9, no. 7, pp. 690 to 696), and a total luminance (total fluorescence intensity) of fluorescent foci (assemblies) per cell was calculated to analyze a correlation with the concentration of the drugs added. Fig. 21 shows the result obtained by adding only rapamycin, and Fig. 22 shows the result obtained by adding rapamycin and FK506.

Note that, in Figs. 21 and 22, the X axis represents the concentration of the drug added to the cell line; the Y axis represents the total luminance (total fluorescence intensity) of fluorescent foci (assemblies) per cell; and dots represent measurement values. Moreover, a fitting curve drawn through the dots shows the analysis result using Igor(R) (manufactured by WaveMetrics, Inc.).

As apparent from the result shown in Fig. 21, it was

revealed that when the compound rapamycin inducing an interaction between mTOR(FRB domain) and a FKBP12 protein was added to the cells stably expressing mTOR(FRB domain)-AG and p62(PB1)-FKBP12, the fluorescence intensity of fluorescent foci was increased in a manner dependent on the concentration of rapamycin added. It was demonstrated that assemblies between mTOR(FRB domain)-AG and p62(PB1)-FKBP12 were formed in a manner dependent on the concentration. Further, the EC50 of rapamycin for the protein-protein interaction between mTOR(FRB domain) and FKBP12 was 3.36 nM by the calculation according to $f(x)=\max+(\min-\max)/(1+(x/EC50)^{\text{hill}})$ based on the fitting curve shown in Fig. 21.

On the other hand, as apparent from the result shown in Fig. 22, it was revealed that when FK506 was added in the presence of rapamycin, the fluorescence intensity of fluorescent foci was decreased in a manner dependent on the concentration of FK506 added. It was demonstrated that the assembly formation between mTOR(FRB domain)-AG and p62(PB1)-FKBP12 was inhibited in a manner dependent on the concentration. Further, the IC50 of FK506 for the interaction between rapamycin and FKBP12 and eventually for the protein-protein interaction between mTOR(FRB domain) and FKBP12 was 0.68 μM by the calculation according to $f(x)=\min+(\max-\min)/(1+(x/IC50)^{\text{hill}})$ based on the fitting curve shown in Fig. 22.

(Example 12)

<Detection 8 of Protein-Protein Interaction>

For the same purposes as those in Examples 10 and 11, whether or not fusion proteins constituting assemblies (fluorescent foci) were dispersed by an inhibitor specific to a protein-protein interaction in the method of the present invention, and whether or not the fluorescence intensity of fluorescent foci was changed in a manner dependent on the inhibitor concentration by changing the inhibitor concentration were tested by a method described below.

Note that the detection target in this test was an interaction between a p53 protein and an MDM2 protein, and that Nutlin-3 known as an inhibitor against the interaction was used in Example 12 (see Vassilev LT et al., Science, February 6, 2004, vol. 303, no. 5659, pp. 844 to 848).

(Preparation of Plasmid DNAs)

In preparing pp62(PB1)-p53, first, a DNA encoding a portion of p53 (region having the 1st to 70th amino acids of the p53 protein, the region had the amino acid sequence of SEQ ID NO: 26) (the DNA had the base sequence of SEQ ID NO: 25) was amplified from a cDNA library of U2OS cells by PCR using the following primer set:

p53	forward	primer;
5'-AAGGATCCATGGAGGAGCCGCGAGTCAGATCCTAGCGTCG-3'	(SEQ ID NO: 97),	and

p53 reverse primer48;
5'-TTGCGGCCGCTTAAGCAGCCTCTGGCATTCTGGGAGCTTCATC-3' (SEQ
ID NO: 98)

Then, the amplification product thus obtained was
5 cleaved with BamHI and NotI, and inserted into
pp62(PB1)-MCLinker having been treated with the same
restriction enzymes. Thus, pp62(PB1)-p53 was prepared.

Moreover, in preparing phAG-MDM2, first, a DNA
encoding a portion of MDM2 (region having the 7th to 125th
10 amino acids of the MDM2 protein, the region had the amino
acid sequence of SEQ ID NO: 28) (the DNA had the base sequence
of SEQ ID NO: 27) was amplified from the cDNA library of
U2OS cells by PCR using the following primer set:

MDM2 forward primer;
15 5'-AAGGATCCATGTGCAATACCAACATGTCTGTACCTACTGATGGTGC-3'
(SEQ ID NO: 99), and

MDM2 reverse primer;
5'-TTCTCGAGTTAACCTGAGTCCGATGATTCCTGCTGATTG-3' (SEQ ID
NO: 100).

20 Then, the amplification product thus obtained was
cleaved with BamHI and XhoI, and inserted into
phAG-MCLinker having been treated with the same restriction
enzymes. Thus, phAG-MDM2 was prepared.

(Transfection into Cells, and Observation and
25 Analysis of Transfected Cells)

pp62(PB1)-p53 and phAG-MDM2 were mixed in equal

amounts and introduced into HeLaS3 cells by the same method as that described in Example 1. The culture solution was discarded 24 hours after the introduction, and 1.5 ml of an observation buffer containing 0.19 μ M Nutlin-3 was added to the resultant. Fluorescence images were captured 15 minutes thereafter. Subsequently, the observation buffer was discarded, and 1.5 ml of an observation buffer containing 0.77 μ M Nutlin-3 was added to the resultant. Fluorescence images were again captured 15 minutes thereafter. The same procedure was carried out using 4.8 μ M and 12 μ M Nutlin-3, as well. Fig. 23 shows the obtained result. Moreover, a total luminance of fluorescent foci (assemblies) in a fluorescence image of cells to which Nutlin-3 was added at predetermined concentrations was calculated to create a graph for illustrating a correlation with the Nutlin-3 concentration. Fig. 24 shows the obtained result. Note that, in Fig. 24, the X axis represents the concentration of Nutlin-3 added to the cells, and the Y axis represents the total luminance (total fluorescence intensity) of fluorescent foci per fluorescence image (one field of view).

As apparent from the result shown in Fig. 23, when p62(PB1)-p53 and AG-MDM2 were expressed in the cells, fluorescent foci were detected. Moreover, as a result of the stepwise increase in the concentration of the inhibitor (Nutlin-3) added against the interaction between p53 and

MDM2, fluorescent foci (assemblies) were observed to be gradually extinguished within the same field of view, confirming that the fusion proteins constituting the assemblies were being dispersed. Further, as apparent
5 from the result shown in Fig. 24, the fluorescence luminance of the fluorescent foci in the field of view was decreased in a manner dependent on the inhibitor concentration.

(Example 13)

<Detection 9 of Protein-Protein Interaction>

10 As in Example 11, whether or not the method of the present invention was utilizable in determining the IC₅₀ of an inhibitor specific to a protein-protein interaction was evaluated by a method described below.

(Preparation of Plasmid DNA)

15 First, using phAG-MDM2 described in Example 12 and pFucci-S/G2/M Green-Hyg (manufactured by limited company Amalgaam Co., Ltd.), phAG-MDM2_Hyg was prepared according to a conventional method, so that the drug resistance gene was converted from a G418 resistance gene to a hygromycin
20 B resistance gene.

(Preparation of Stably-Expressing Cell Line)

Next, the phAG-MDM2_Hyg and pp62(PB1)-p53 described in Example 12 were introduced into a CHO-K1 cell line. Note that CHO-K1 cells were cultured in NUTRIENT MIXTURE F-12
25 HAM (manufactured by SIGMA ALDRICH CO.) containing 10% FBS (manufactured by Equitech-Bio Inc.).

Then, 24 hours after the plasmid DNAs were introduced in the CHO-K1 cell line, the medium was replaced with one containing 100 µg/ml of G418 (manufactured by Wako Pure Chemical Industries, Ltd.) and 200 µg/ml of hygromycin (manufactured by Nacalai Tesque, Inc.). Further, cells survived after culturing for one week with this medium were monocloned by limiting dilution.

(Observation and Analysis of Transfected Cells)

After the nuclear staining on the monocloned cell line by the same method as that described in Example 11, an observation buffer containing Nutlin-3 (manufactured by CALBIOCHEM) at certain concentrations was added to each well, and incubated for 20 minutes. Note that Nutlin-3 was prepared and added in such a manner that the final concentration was 0.2 µM, 0.3 µM, 0.6 µM, 0.9 µM, 1.6 µM, 2.6 µM, 4.3 µM, 7.2 µM, 12.0 µM, or 20.0 µM.

Subsequently, the cell line was fixed by the same method as that described in Example 11. An image of each well was obtained with a fluorescence microscope, and each fluorescence image was analyzed using iCY. A total luminance of fluorescent foci per cell was calculated to analyze a correlation with the concentration of Nutlin-3 added. Fig. 25 shows the obtained result. Note that, in Fig. 25, the X axis represents the concentration of the drug added to the cell line; the Y axis represents the total luminance (total fluorescence intensity) of fluorescent

foci (assemblies) per cell; and dots represent measurement values. Moreover, a fitting curve drawn through the dots shows the analysis result using Igor(R).

Although unillustrated, in CHO-K1 cells also stably
5 expressing p62(PB1)-p53 and AG-MDM2, fluorescent foci
(assemblies) attributable to a protein-protein
interaction between p53 and MDM2 were observed as in Example
12. Moreover, as shown in Fig. 25, the fluorescence
luminance of these assemblies was decreased in a manner
10 dependent on the concentration of the inhibitor Nutlin-3.
Further, the IC₅₀ of Nutlin-3 for the protein-protein
interaction between p53 and MDM2 was 8.9 μ M by the
calculation according to
$$f(x) = \min + (\max - \min) / (1 + (x / IC_{50})^{\text{hill}})$$

15 based on the fitting
curve shown in Fig. 25.

The results described in Examples 10 to 13 above
verified that the fluorescence luminance of the fluorescent
focus according to the present invention reflected the
strength of the protein-protein interaction, making
20 quantification of the protein-protein interaction
possible. Furthermore, it was also revealed that the
assembly formation was reversible. It was demonstrated
that the quantification was practicable by using fixed
cells (see Fig. 20), and also using a live imaging technique
25 with living cells (see Fig. 24). In addition, it was
revealed as described in Examples 11 and 13 that the present

invention enabled detection of a promoting reaction and an inhibiting reaction both dependent on the concentration of a drug for a protein-protein interaction, and further enabled calculations of the EC50 and the IC50 of the drug.

Thus, it was demonstrated that the method for detecting a protein-protein interaction of the present invention was applicable to evaluation of and screening for a substance modulating a protein-protein interaction.

(Example 14)

<Detection of Protein-Protein Interaction>

It has been known that p50 and p65 form a heterodimer, constituting NF κ B. Further, NF κ B functions in the nucleus as a transcription factor playing a role in modulating inflammatory cytokine expression. However, it has been known that the interaction with I κ B α retains NF κ B in the cytoplasm, suppressing the transcription function (see Marc D. Jacobs et al., Cell, December 11, 1998, vol. 95, pp. 749 to 758). Thus, an overexpression of p50 and p65 disturbs the stoichiometric balance with endogenous I κ B α , so that the heterodimer is localized mainly in the nucleus. On the other hand, if I κ B α is overexpressed, the heterotrimer further including I κ B α is retained in the cytoplasm.

For this reason, in this Example, whether or not the method of the present invention enabled detection of a change in intracellular localization of a complex

containing p50 and p65 in accordance with the presence or absence of I κ B α was tested by a method described below.

First, each of pp62(PB1)-p50 and phAG-p65 was prepared by the same method as that described in Example 2. DNAs (SEQ ID NOs: 155 and 157, respectively) encoding regions having the amino acid sequences of SEQ ID NOs: 156 and 158 had been inserted in the pp62(PB1)-p50 and the phAG-p65, respectively.

Meanwhile, pI κ B α was prepared by the same method as that described in Example 1 using a DNA sequence encoding the amino acid sequence specified under Genbank ACCESSION No: NP_065390.1.

Then, HeLaS3 cells were seeded onto 4 wells in an 8-well chamber (manufactured by Nunc A/S). On the next day, the plasmid DNAs were introduced into these cells. In the transfection, 100 ng of each of pp62(PB1)-p50 and phAG-p65 was added to OptiMEM (manufactured by Life Technologies Corporation), and pI κ B α was further added thereto in different amounts for the use. Note that the amounts of the pI κ B α added were 0 ng (not added) or 100 ng. Further, to make total amounts of the plasmid DNAs added all equal, 300 ng or 0 ng of pmKeima-Red-S1 (manufactured by limited company Amalgaam Co., Ltd.) was added. Then, 1.5 μ l of PolyFect(R) Transfection Reagent was added to each OptiMEM and stirred. Furthermore, after mixed with 200 μ l of the culture solution, the resultant

was added to the HeLaS3 cells. Subsequently, 22 hours after this transfection, the cells were fixed with 4% Paraformaldehyde Phosphate Buffer Solution (manufactured by Wako Pure Chemical Industries, Ltd.) at room temperature for 15 minutes. After the cell membranes were solubilized with 0.2% TritonX-100/PBS for 5 minutes, immunostaining was carried out using an anti-I κ B α antibody (manufactured by Cell Signaling Technology, Inc.). Furthermore, the nuclei were stained using Hoechst 33342. Then, the immunostained cells were observed by the same method as that described in Example 1. Fig. 26 shows the obtained result. Note that, in the figure, "merging" shows the result of merging images of AG-derived fluorescence (two panels on the left in the figure), images showing the result of the immunostaining with the anti-I κ B α antibody (two panels in the middles of the figure), and images showing the result of the nuclear staining with Hoechst 33342.

As apparent from the result shown in Fig. 26, it was confirmed that, in the cells into which I κ B α was not introduced, heterodimers formed from p50 and p65 were formed in the nuclei. On the other hand, when I κ B α was introduced, I κ B α -derived signals were detected (see the lower panel in the middle of the figure) at the same locations as the fluorescent foci detected in the image of the AG-derived fluorescence (see the lower left panel in the figure), confirming that the complex containing p50 and

p65 included I κ B α . Further, the method of the present invention also confirmed that in the presence of I κ B α , the localization of the complex containing p50 and p65 was changed from the inside of the nuclei to the inside of the cytoplasms.

(Example 15)

<Detection 11 of Protein-Protein Interaction>

In this Example, utilizing the complex containing p50 and p65 detected also in Example 14, whether or not the method of the present invention enabled quantitative detection of a change in intracellular localization of the complex in accordance with the quantitative balance with I κ B α was tested by a method described below.

First, HeLaS3 cells were seeded onto 4 wells in an 8-well chamber (manufactured by Nunc A/S). Then, on the next day, pp62(PB1)-p50, phAG-p65, and pI κ B α , which were described in Example 13, were introduced into these cells. In the transfection, 100 ng of each of pp62(PB1)-p50 and phAG-p65 added to OptiMEM (manufactured by Life Technologies Corporation), and the pI κ B α was further added thereto in different amounts for the use. Note that the amounts of the pI κ B α added were (0) not added, (1) 33 ng, (2) 100ng, and (3) 300 ng. Further, to make total amounts of the plasmid DNAs added all equal, pmKeima-Red-S1 (manufactured by limited company Amalgaam Co., Ltd.) was added in amounts of: 300 ng in the case of (0); 267 ng,

(1); 200 ng, (2); and 0 ng, (3). Then, 1.5 μ l of PolyFect(R) Transfection Reagent was added to each OptiMEM and stirred. Furthermore, after mixed with 200 μ l of the culture solution, the resultant was added to the HeLaS3 cells, and observed 22 hours thereafter by the same method as that described in Example 1.

After 150 or more cells in which the plasmid DNAs were introduced under the (0) to (3) conditions were photographed, the cells were classified into three groups (A) to (C) according to the fluorescent focus localization. Specifically, cells are classified as (A) if fluorescent foci were detected only in the cytoplasms; (B), if detected in the cytoplasms and the nuclei; and (C), if detected only in the nuclei. Then, a percentage of the cell count in each group was calculated, and a graph was created. Fig. 27 shows the obtained result.

As apparent from the result shown in Fig. 27, the percentage of fluorescent foci detected in the cytoplasms was increased in a manner dependent on the I κ B α amount. Thus, the present invention can provide a method for grouping cells according to fluorescent focus localization, and comparing the number of cells in each group. Further, it was revealed as described in Example 14 also that by utilizing localization of an interaction between direct detection targets, a first protein and a second protein (for example, p50 and p65), the method of the present

invention enabled quantification of an influence of a third protein (for example, I κ B α) on the interaction as well as the amount of the third protein expressed.

(Example 16)

5 <Detection 12 of Protein-Protein Interaction>

It has been known that, in the nucleus of a cell, p21 recognizes and interacts with a complex composed of CDK4 and Cyclin D1 (see LaBaer J et al., Genes Dev., April 1, 1997, vol. 11, no. 7, pp. 847 to 862). Moreover, it
10 has also been revealed that such heterotrimer formation inhibits cell-cycle progression (transition from a G1 phase to an S phase) otherwise promoted by a complex composed of CDK4 and Cyclin D1.

For this reason, in this Example, as in Examples 14
15 and 15, whether or not the present invention enabled detection of formation of a complex composed of three types of different proteins was tested by a method described below.

(Preparation of Plasmid DNA)

20 pp62(PB1)-CDK4, phAG-p21, and pCyclin D1 were prepared by the same method as that described in Example 1 on the basis of DNA sequences encoding the amino acid sequences specified under Genbank ACCESSION Nos: NP_000066.1, NP_000380.1, and NP_444284.1, respectively.

25 (Transfection into Cultured Cells, Cell Immunostaining, and Observation of Cells)

HeLaS3 cells were used as cultured cells into which the plasmid DNAs were introduced. Moreover, the HeLaS3 cells were cultured by the same method as that described in Example 1. Further, in the transfection, the HeLaS3
5 cells were seeded onto 2 wells of an 8-well chamber (manufactured by Nunc A/S). On the next day, by the same method as that described in Example 1, 130 ng of each plasmid DNA in the following combinations was introduced into the HeLaS3 cells using 1 µl of Transfection Reagent:

10 a combination of pp62(PB1)-CDK4 and phAG-p21 with pCyclin D1; and

a combination of pp62(PB1)-CDK4 and phAG-p21 with phmKGC-MN (manufactured by limited company Amalgaam Co., Ltd.) (note that the phmKGC-MN was added to makes total
15 amounts of the plasmids all equal).

Then, 24 hours after the transfection, the cells were fixed with 4% Paraformaldehyde Phosphate Buffer Solution (manufactured by Wako Pure Chemical Industries, Ltd.) at room temperature for 15 minutes. After the cell membranes
20 were solubilized with 0.2% TritonX-100/PBS for 5 minutes, immunostaining was carried out using 4 µg/ml of an anti-Cyclin D1 antibody (manufactured by MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.). The immunostained cells were observed by the same method as that described
25 in Example 1. Fig. 28 shows the obtained result.

As apparent from the result shown in Fig. 28, no clear

assembly (fluorescent focus) was observed in many cells only by expressing p62(PB1)-CDK4 and AG-p21. Nevertheless, when Cyclin D1 was forcibly expressed together with p62(PB1)-CDK4 and AG-p21, this made possible stoichiometrically uniform expression of the elements necessary for the heterotrimer formation, and clear fluorescent foci were observed in almost all the cells. Further, the immunostaining images of Cyclin D1 confirmed that Cyclin D1 was localized in the observed fluorescent foci.

Thus, similarly to the results described in Examples 14 and 15, it was confirmed that the present invention enabled detection of a protein-protein interaction in formation of a complex of a trimer or a higher multimer.

Moreover, suppose a case where two types of proteins (p50 and p65 in Examples 14 and 15, CDK4 and p21 in Example 16) are expected to form a complex including a "certain molecule" (I κ B α in Examples 14 and 15, Cyclin D1 in Example 16) as described above. In this case, if the two types of proteins are expressed in cells as a "first fusion protein comprising an association-inducing protein" and a "second fusion protein comprising a fluorescent protein having a multimerization ability" respectively, and a protein encoded by a cDNA expression library is further expressed in the cells, the present invention makes it possible to search for the "certain molecule" (constitutional factor

of the complex) on the basis of a fluorescent focus (for example, formation or extinction a fluorescent focus, a change in fluorescent focus localization).

Further, as described in Examples 14 to 16, the present invention makes it possible to analyze an expressed amount of a constitutional factor (I κ B α in Examples 14 and 15, Cyclin D1 in Example 16) of a complex on the basis of a fluorescent focus, and eventually, by utilizing the expressed amount, to analyze states of cells, such as a cell cycle controlled by the complex and a stress to which the complex is to respond.

(Example 17)

<Detection 13 of Protein Interaction>

As described in Examples 10 to 13, it was revealed that the fluorescence intensity of the fluorescent focus according to the present invention reflected a strength of a protein-protein interaction. Thus, the method for detecting a protein-protein interaction of the present invention presumably is capable of identifying an amino acid important for the interaction on the basis of the fluorescence intensity of the fluorescent focus according to the present invention. For this reason, a test was conducted by a method described below, utilizing an amino acid mutation known to be involved in reduction or enhancement of a protein-protein interaction.

Note that the detection target in this test was an

interaction between a Sec5 protein and a RalB protein. A Sec5 protein has been known to interact with a RalB protein in a GTP-activated form (see Moskalenko S et al., Nat Cell Biol., January 2002, vol. 4, no. 1, pp. 66 to 72). It has been known that the interaction is reduced with an inactive mutant RalB(S28N) of RalB, but enhanced with an active mutant RalB(Q72L) of RalB (see Shipitsin M et al., Mol Cell Biol., July 2004, vol. 24, iss. 13, pp. 5746 to 5756). Further, a RalB protein, which is a membrane-anchored protein, has been revealed to be localized at the cell membranes by palmitoylation of the C-terminus thereof.

(Preparation of Plasmid DNA)

In preparing pp62(PB1)-Sec5, first, a DNA encoding a portion of Sec5 (region having the 1st to 99th amino acids of the Sec5 protein, the region had the amino acid sequence of SEQ ID NO: 30) (the DNA had the base sequence of SEQ ID NO: 29) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

Sec5 forward primer;
5'-CCCGGATCCATGTCTCGATCACGACAACCC-3' (SEQ ID NO: 101),
and
Sec5 reverse primer;
5'-GGGAAGCTTTTATTAGCCTATTTTCTCAGGTTTGAGTA-3' (SEQ ID NO: 102).

Then, the amplification product thus obtained was cleaved with BamHI and HindIII, and inserted into

pp62(PB1)-MCLinker having been treated with the same restriction enzymes. Thus, pp62(PB1)-Sec5 was prepared. Note that the pp62(PB1)-Sec5 encodes a fusion protein composed of p62(PB1) and a partial Sec5 protein (the fusion protein may also be referred to as "p62(PB1)-Sec5").

Meanwhile, in preparing phAG-RalB(WT), first, a DNA encoding RalB (protein having the amino acid sequence of SEQ ID NO: 32) (the DNA had the base sequence of SEQ ID NO: 31) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

RalB forward primer;
5'-CCCGGATCCATGGCTGCCAACAAGAGTAAG-3' (SEQ ID NO: 103),
and

RalB reverse primer;
5'-GGGAAGCTTTTATCATAGTAAGCAACATCTTTC-3' (SEQ ID NO: 104).

Then, the amplification product thus obtained was cleaved with BamHI and HindIII, and inserted into phAG-MCLinker having been treated with the same restriction enzymes. Thus, phAG-RalB(WT) was prepared. Note that the phAG-RalB(WT) encodes a fusion protein composed of an AG protein and a RalB protein (the fusion protein may also be referred to as "AG-RalB(WT)").

Further, phAG-RalB(Q72L) was prepared using phAG-RalB(WT) as a template, and AMAP(TM) Multi Site-directed Mutagenesis Kit (manufactured by limited company Amalgaam Co., Ltd.), and according to the attached

instruction, a mutation was introduced using the following primer:

RalB(Q72L) primer;
5'-CTGGACACCGCTGGGCTAGAGGACTACGCAGCCA-3' (SEQ ID NO:
5 105).

Note that the amino acid sequence of a RalB(Q72L) protein is shown in SEQ ID NO: 34. Moreover, the base sequence of a DNA encoding the protein is shown in SEQ ID NO: 33. Further, phAG-RalB(Q72L) encodes a fusion protein
10 composed of an AG protein and a RalB(Q72L) protein (the fusion protein may also be referred to as "AG-RalB(Q72L)").

In addition, phAG-RalB(S28N) was prepared using phAG-RalB(WT) as a template, and AMAP(TM) Multi Site-directed Mutagenesis Kit, and according to the
15 attached instruction, a mutation was introduced using the following primer:

RalB(S28N) primer;
5'-CAGCGGAGGCGTTGGCAAGAACGCCCTGACGCTTCAGTTCA-3' (SEQ ID
NO: 106).

Note that the amino acid sequence of a RalB(S28N) protein is shown in SEQ ID NO: 36. Moreover, the base sequence of a DNA encoding the protein is shown in SEQ ID NO: 35. phAG-RalB(S28N) encodes a fusion protein composed
20 of an AG protein and a RalB(S28N) protein (the fusion protein may also be referred to as "AG-RalB(S28N)").
25

(Transfection into Cultured Cells, and Observation

of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 1:

5 a combination of pp62(PB1)-Sec5 with phAG-RalB(WT);

a combination of pp62(PB1)-Sec5 with phAG-RalB(Q72L);

a combination of pp62(PB1)-Sec5 with phAG-RalB(S28N); and

10 a combination of pp62(PB1) with phAG-RalB(WT).

Moreover, the transfected cells were observed also by the same method as that described in Example 1. Fig. 29 shows the obtained result. Further, images were obtained using a total internal reflection fluorescence microscopy system with arc lamp source (manufactured by Olympus Corporation, IX71-ARCEVA) capable of exciting only the vicinity of the cell membrane. Fig. 30 shows the obtained result.

As apparent from the result shown in Fig. 29, since the RalB protein having the C-terminus palmitoylated was localized at the cell membranes, when the wildtype RalB protein (RalB(WT)) was expressed, fluorescent foci derived from the interaction with the Sec5 protein were detected in the vicinity of the cell membranes. On the other hand, no fluorescent focus was detected in the cells co-expressing p62(PB1)-Sec5 and AG-RalB(S28N). It was

confirmed as described above that the inactive mutant RalB(S28N) of RalB reduced the interaction with the Sec5 protein. Moreover, in the cells co-expressing p62(PB1)-Sec5 and AG-RalB(Q72L), fluorescent foci were
5 detected, which had a higher fluorescence intensity (luminance) than that in the cells co-expressing p62(PB1)-Sec5 and AG-RalB(WT). It was also confirmed as described above that the active mutant RalB(Q72L) of RalB enhanced the interaction with the Sec5 protein.

10 Further, as apparent from the result shown in Fig. 30, in the cells co-expressing p62(PB1)-Sec5 and AG-RalB(WT), assemblies were observed. The result shown in Fig. 30 was a result of using the observation system capable of exciting only the vicinity of the cell membrane.
15 Thus, similarly to the result shown in Fig. 29, it was verified that the method of the present invention enabled detection of the interaction between the wildtype RalB protein and the Sec5 protein in the vicinity of the cell membranes.

20 Furthermore, as apparent from the result shown in Fig. 30, in the cells co-expressing p62(PB1)-Sec5 and AG-RalB(Q72L), more significant assembly formation was observed. On the other hand, in the cells co-expressing p62(PB1)-Sec5 and AG-RalB(S28N), no assembly was observed.
25 Thus, similarly to the result shown in Fig. 29, it was verified that the method of the present invention enabled

detection of reduction of the interaction with the Sec5 protein by RalB(S28N), and enhancement of the interaction by RalB(Q72L).

(Example 18)

5 <Detection 14 of Protein Interaction>

As in Example 17, a test was conducted by a method described below, using an amino acid mutation known to be involved in reduction of a protein-protein interaction.

(Preparation of Plasmid DNA)

10 pp62(PB1)-p53_W23L was prepared by introducing a mutation into pp62(PB1)-p53 described in Example 12 by the same method as that described in Example 17 using a primer having the base sequence of SEQ ID NO: 159 (5'-ACATTTTCAGACCTATTGAACTACTTCCTGAAAACAACGT-3').

15 Note that the amino acid at position 23 of p53 is located at an interaction interface site between p53 and MDM2. Further, a "W23L" mutation of p53 has been known as a mutation resulting in reduction of the interaction (see literature Zondlo SC, Biochemistry., October 3, 2006, 20 vol. 45, no. 39, pp. 11945 to 11957).

(Transfection into Cultured Cells, and Observation of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into cells 25 by the same method as that described in Example 1, and the cells were observed. Fig. 31 shows the obtained result.

A combination of pp62(PB1)-p53 with phAG-MDM2.

A combination of pp62(PB1)-p53_W23L with phAG-MDM2.

As apparent from the result shown in Fig. 31, in the cells co-expressing p62(PB1)-p53 and AG-MDM2, fluorescent foci (assembly formation) were significantly observed (the left panel in the figure). On the other hand, in the cells co-expressing p62(PB1)-p53_W23L and AG-MDM2, no assembly was observed (the right panel in the figure).

Thus, as described in Examples 17 and 18, it was revealed that the method for detecting a protein-protein interaction of the present invention was also capable of identifying the amino acid important for the interaction on the basis of the fluorescence intensity of the fluorescent focus according to the present invention. Particularly, the method of the present invention is capable of very easily specifying an amino acid involved in a protein-protein interaction by introducing a mutation into an interface of the interaction and detecting the presence or absence of the interaction. In other words, combining a method for introducing a mutation into a protein such as alanine scanning with the method of the present invention makes it possible to search for a site (hot spot) important for a protein interaction very easily.

(Example 19)

<Detection 15 of Protein-Protein Interaction>

As described above, it was demonstrated that the

method for detecting a protein-protein interaction of the present invention enabled quantitative measurement of when a protein-protein interaction took place and ended in real time. Hence, a test was conducted regarding whether or not the use of the method of the present invention enabled detection of how an endogenous signal transduction in cells changed over time, according to a protein-protein interaction that took place in response to the signal.

Specifically, the protein-protein interaction as the detection target in this test was an interaction between calmodulin and a partial sequence (M13 peptide) of myosin light chain kinase 2. It has been revealed that the interaction takes place in response to a transient increase in intracellular calcium ion concentration (second messenger) that occurs when a G protein-coupled receptor (GPCR) receives a ligand (see Miyawaki A et al., Nature, August 28, 1997, vol. 388, no. 6645, pp. 882 to 887). Hence, whether or not it was possible to detect a change in intracellular calcium ion concentration over time according to the interaction was tested by a method described below. Fig. 32 shows the obtained result.

(Preparation of Plasmid DNAs)

In preparing pCalmodulin-hAG, first, a DNA encoding calmodulin (protein having the amino acid sequence of SEQ ID NO: 38) (the DNA had the base sequence of SEQ ID NO: 37) was amplified from the cDNA library of HeLaS3 cells

by PCR using the following primer set:

calmodulin forward primer;

5'-TTGGATCCGCCACCATGGACCAACTGACAGAAGAGCAGATTGC-3' (SEQ ID NO: 107), and

5 calmodulin reverse primer;

5'-AAGAATTCCCCTTTGCTGTCATCATTTGTACAAACTCTTC-3' (SEQ ID NO: 108)

10 Then, the amplification product thus obtained was cleaved with BamHI and EcoRI, and inserted into phAG-MNLinker having been treated with the same restriction enzymes. Thus, pCalmodulin-hAG was prepared. Note that the pCalmodulin-hAG encodes a fusion protein composed of a calmodulin protein and an AG protein (the fusion protein may also be referred to as "Calmodulin-AG").

15 Meanwhile, in preparing pM13peptide-p62(PB1), first, a DNA encoding a portion of myosin light chain kinase 2 (region having the 566th to 591st amino acids of the myosin light chain kinase 2 protein, the region had the amino acid sequence of SEQ ID NO: 40) (the DNA had the base sequence of SEQ ID NO: 39) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

M13 peptide forward primer;

5'-TTGGATCCGCCACCATGAAGAGGCGCTGGAAGAAAACTTCATTGC-3' (SEQ ID NO: 109), and

25 M13 peptide reverse primer;

5'-CCGAATTCCCCAGTGCCCCGGAGCTGGAGATCTTCTTG-3' (SEQ ID NO:

110).

Then, the amplification product thus obtained was cleaved with BamHI and EcoRI, and inserted into pp62(PB1)-MNLinker having been treated with the same restriction enzymes. Thus, pM13peptide-p62(PB1) was prepared. Note that the pM13peptide-p62(PB1) encodes a fusion protein composed of an M13 peptide and p62(PB1) (the fusion protein may also be referred to as "M13peptide-p62(PB1)").

(Transfection into Cultured Cells, and Observation of Transfected Cells)

pCalmodulin-hAG and pM13peptide-p62(PB1) were mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 1. Then, 200 μ M histamine (manufactured by Wako Pure Chemical Industries, Ltd.) was added thereto, and fluorescence images were captured over time. Note that it has been revealed that histamine functions as a ligand of an H1 receptor, one of GPCRs, which is expressed also in HeLaS3 cells.

As apparent from the result shown in Fig. 32, before the ligand (histamine) was added, Calmodulin-AG was present in a dispersed manner in the cells. Meanwhile, 90 seconds after the ligand was added, fluorescent focus formation was detected, confirming the assembly formation with M13peptide-p62(PB1). Nevertheless, 490 seconds after the cytoplasm calcium ion concentration was decreased, the

fluorescent foci (assemblies) were extinguished.

Thus, it was revealed that the use of the method for detecting a protein-protein interaction of the present invention enabled real-time measurement of the calcium ion concentration transiently increased by the signal transduction from the H1 receptor.

Moreover, the result of this Example 19 also demonstrated that the present invention enabled detection of the transient protein-protein interaction, further that the present invention was applicable to detection of and screening for: an endogenous factor such as a second messenger causing a protein-protein interaction; a signal transduction to which the second messenger or the like contributed; and a stimulus from the outside such as an extracellular ligand eliciting the signal transduction.

(Example 20)

<Detection 16 of Protein-Protein Interaction>

In conventional methods for detecting a protein-protein interaction represented by W02000/017221 A and W02006/099486 A, one of proteins constituting a complex formed by a protein-protein interaction is forcibly (artificially) confined in a particular region in a cell. Accordingly, the detection was impossible in an intracellular environment unique to the interaction. In contrast, in the method for detecting a protein-protein interaction of the present invention, fluorescent foci

(assemblies) are autonomously formed only when an interaction takes place. Hence, it is expected that the problems in the conventional method can be solved. For this reason, whether or not the present invention enabled
5 detection of an interaction in any region in a cell was tested by a method described below.

(Preparation of Plasmid DNA)

In preparing pmTOR(FRB domain)-AGNLS, first, an AGNLS gene was amplified from pNP-AG (manufactured by limited
10 company Amalgaam Co., Ltd.) by PCR using the following primer set:

AGNLS forward primer;
5'-AAACCGGTATGGTGAGTGTGATTAAACCAGAG-3' (SEQ ID NO: 111),
and

15 AGNLS reverse primer;
5'-AATCTAGATTATTTATCCTTTTCCTTTTACTCTTCTTAGCTACTTC-
3' (SEQ ID NO: 112).

Then, the amplification product thus obtained was cleaved with AgeI and XbaI, and inserted into pmTOR(FRB
20 domain)-hAG having been treated with the same restriction enzymes to cut out a hAG region therefrom. Thus, pmTOR(FRB domain)-AGNLS was prepared. Note that the pmTOR(FRB domain)-AGNLS encodes a fusion protein composed of mTOR(FRB domain), an AG protein, and a nuclear localization
25 signal (NLS) (the fusion protein may also be referred to as "mTOR(FRB domain)-AGNLS"). Moreover, mTOR(FRB

domain)-AGNLS is to be localized in the nucleus of a cell because the nuclear localization signal is fused to the C-terminus of the mTOR(FRB domain)-AG.

Meanwhile, in preparing pp62(PB1)-HRas, first, a DNA
5 encoding an HRas protein was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set. The amplification product thus obtained was cleaved with EcoRI and XhoI.

HRas forward primer;
10 5'-AAGAATTCGATGACGGAATATAAGCTGGTGGTGGTGGGCGCCGTCGGTGTG
GGCAAGAGTGC-3' (SEQ ID NO: 113), and

HRas reverse primer;
5'-TTCTCGAGACCTCCGGAGACGTTTCAGCTTCCGCAGCTTGTGCTGCCGGATC
TCACGCACCAAC-3' (SEQ ID NO: 114).

15 Further, a prenylated sequence derived from a KRas protein was amplified by PCR using the following primer set. The amplification product thus obtained was cleaved with XhoI and NotI.

KRas forward primer;
20 5'-AACTCGAGAAGATGAGCAAAGATGGTAAAAAGAAGAAAAAGAAGTCAAAGA
CAAAGTGTG-3' (SEQ ID NO: 115), and

KRas reverse primer;
5'-TTGCGGCCGCTTACATAATTACACACTTTGTCTTTGACTTCTTTTCTTCT
TTTTACCAT-3' (SEQ ID NO: 116).

25 Then, the two DNA fragments prepared in this manner were inserted into pp62(PB1)-MCLinker having been treated

with EcoRI and NotI. Thus, pp62(PB1)-HRas(WT) was prepared.

Furthermore, pp62(PB1)-HRas encoding a fusion protein composed of a constitutively-active mutant HRas and p62(PB1) was prepared by introducing a mutation using pp62(PB1)-HRas(WT) as a template, and AMAP(TM) Multi Site-directed Mutagenesis Kit (manufactured by limited company Amalgaam Co., Ltd.) according to the attached instruction, with the following primer:

HRas mutant primer;
5'-GCTGGTGGTGGTGGGCGCCGTCGGTGTGGGCAAGAGTGCGC-3' (SEQ ID NO: 117).

Note that the pp62(PB1)-HRas encodes a protein obtained by fusing p62(PB1) with a DNA encoding HRas having the C-terminus to which the KRas protein-derived prenylated sequence is added (the protein had the amino acid sequence of SEQ ID NO: 42) (the DNA had the base sequence of SEQ ID NO: 41). Moreover, since having the prenylated sequence, this fusion protein is subjected to post-translation lipid modification in cells, and localized at the cell membranes.

In preparing phAG-cRaf, first, a DNA encoding a portion of cRaf (region having the 51st to 131st amino acids of the cRaf protein, the region had the amino acid sequence of SEQ ID NO: 44) (the DNA had the base sequence of SEQ ID NO: 43) was amplified by PCR using the cDNA library of HeLaS3 cells as a template and the following primer set:

cRaf forward primer;
5'-AAGGTACCCCTTCTAAGACAAGCAACACTATCCGTGTTTTCTTGCCGAACA
AGCAAAGAA-3' (SEQ ID NO: 118), and

cRaf reverse primer 71;
5'-TTAAGCTTTTACAGGAAATCTACTTGAAGTTCTTCTCCAATCAAAGACGCA
G-3' (SEQ ID NO: 119).

Then, the amplification product thus obtained was
cleaved with KpnI and HindIII, and inserted into
phAG-MCLinker having been treated with the same restriction
enzymes. Thus, phAG-cRaf was prepared. Note that the
phAG-cRaf is a fusion protein composed of an AG protein
and a portion of a cRaf protein (the fusion protein may
also be referred to as "AG-cRaf"). Moreover, the portion
of of a cRaf protein has been known to interact with an
HRas protein (see Mochizuki N et al., Nature, June 28, 2001,
vol. 411, no. 6841, pp. 1065 to 1068).

Further, in preparing pSmac-p62(PB1), first, a DNA
encoding a fusion protein composed of a portion of Smac
(region having the 1st to 10th amino acids of the Smac protein,
the region had the amino acid sequence of SEQ ID NO: 46)
and p62(PB1) was amplified from pp62(PB1)-MNL by PCR using
the following primer set:

Smac forward primer;
5'-AGGATCCGCCACCATGGCCGTGCCCATCGCCCAGAAATCAGAGAATTCGG-
3' (SEQ ID NO: 120), and

p62(PB1) reverse primer 2;

5'-ACCTCTAGATTATTTCTCTTTAATGTAGATTCGGAAGATG-3' (SEQ ID NO: 64)

Then, the amplification product thus obtained was cleaved with BamHI and XbaI, and inserted into pp62(PB1)-MNL having been treated with the same restriction enzymes to cut out the linker and p62(PB1) therefrom. Thus, pSmac-p62(PB1) was prepared. Note that the pSmac-p62(PB1) encodes a fusion protein composed of a portion of Smac and p62(PB1) (the fusion protein may also be referred to as "Smac-p62(PB1)").

In addition, in preparing pXIAP-hAG, first, a DNA encoding a portion of XIAP (region having the 243rd to 356th amino acids of the XIAP protein, the region had the amino acid sequence of SEQ ID NO: 48) (the DNA had the base sequence of SEQ ID NO: 47) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

XIAP forward primer;
5'-TTGGATCCGCCACCATGGCTGTGAGTTCTGATAGGAATTTCCCAAATTC-3'
(SEQ ID NO: 121), and

XIAP reverse primer;
5'-TTGAATTCTCAGTAGTTCTTACCAGACACTCCTCAAGTGAATGAG-3'
(SEQ ID NO: 122).

Then, the amplification product thus obtained was cleaved with BamHI and EcoRI, and inserted into phAG-MNL linker having been treated with the same restriction enzymes. Thus, pXIAP-hAG was prepared. Note that the

pXIAP-hAG encodes a fusion protein composed of a portion of XIAP and an AG protein (the fusion protein may also be referred to as "XIAP-AG"). Moreover, it has been known that the portion of Smac and the portion of XIAP interact with each other in the cytoplasm (see Liu Z et al., Nature, December 21-28, 2000, vol. 408, no. 6815, pp. 1004 to 1008).

Further, in preparing pp62(PB1)-BclX(L), first, a DNA encoding a portion of BclX(L) (region having the 1st to 209th amino acids of the BclX(L) protein, the region had the amino acid sequence of SEQ ID NO: 50) (the DNA had the base sequence of SEQ ID NO: 49) was amplified from the cDNA library of HeLaS3 cells by PCR using the following primer set:

BclX(L) forward primer;
5'-TTCTCGAGGATGTCTCAGAGCAACCGGGAGCTGGTGGTTGAC-3' (SEQ ID NO: 123), and
BclX(L) reverse primer;
5'-CTAAGCGGCCGCTTAGCGTTCCTGGCCCTTTCGGCTCTCGGCTG-3' (SEQ ID NO: 124).

Then, the amplification product thus obtained was cleaved with XhoI and NotI, and inserted into pp62(PB1)-MCLinker having been treated with the same restriction enzymes. Thus, pp62(PB1)-BclX(L) was prepared. Note that the pp62(PB1)-BclX(L) encodes a fusion protein composed of p62(PB1) and a portion of BclX(L) (the fusion protein may also be referred to as

"p62(PB1)-BclX(L)").

In addition, in preparing phAG-BAD, first, a DNA encoding a portion of BAD (region having the 103rd to 127th amino acids of the BAD protein, the region had the amino acid sequence of SEQ ID NO: 52) (the DNA had the base sequence of SEQ ID NO: 51) was amplified by PCR using the following primer set:

BAD forward primer 1;
5'-GCAGCACAGCGCTATGGCCGCGAGCTCCGGAGGATGAGTGACGAGTTTGT-

3' (SEQ ID NO: 125),

BAD forward primer 2;
5'-TTGGATCCAACCTCTGGGCAGCACAGCGCTATGGCCGCGAGCTCCGGAGG-

3' (SEQ ID NO: 126), and

BAD reverse primer;
5'-TTGAATTCTTACTTCTTAAAGGAGTCCACAACTCGTCACTCATCCTCCG-

3' (SEQ ID NO: 127).

Then, the amplification product thus obtained was cleaved with BamHI and EcoRI, and inserted into phAG-MCLinker having been treated with the same restriction enzymes. Thus, phAG-BAD was prepared. Note that the phAG-BAD encodes a fusion protein composed of an AG protein and a portion of BAD (the fusion protein may also be referred to as "AG-BAD"). Moreover, it has been known that the portion of BclX(L) and the portion of BAD interact with each other in the cytoplasm (see Sattler M et al., Science, February 14, 1997, vol. 275, no. 5302, pp. 983 to 986).

(Transfection into Cultured Cells, and Observation of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into HeLaS3 cells by the same method as that described in Example 1:

a combination of pFKBP12-p62(PB1) with pmTOR(FRB domain)-AGNLS;

a combination of pp62(PB1)-HRas with phAG-cRaf;

a combination of pSmac-p62(PB1) with pXIAP-hAG; and

a combination of pp62(PB1)-BclX(L) with phAG-BAD.

Moreover, the transfected cells were observed also by the same method as that described in Example 1. Nevertheless, regarding the cells in which the pFKBP12-p62(PB1) and the pmTOR(FRB domain)-AGNLS were introduced, fluorescence images and phase contrast images were captured 300 seconds after 100 nM rapamycin was added. Figs. 33 to 36 show the obtained results.

As apparent from the result shown in Fig. 33, when mTOR(FRB domain)-AGNLS and FKBP12-p62(PB1) were expressed in the cells, mTOR(FRB domain)-AGNLS was localized in a dispersed state because the mTOR(FRB domain)-AGNLS had the nuclear localization signal sequence at the C-terminus thereof (see the left panel in Fig. 33). Moreover, by the addition of rapamycin, fluorescent foci formed by an association between FKBP12-p62(PB1) and mTOR(FRB domain)-AGNLS were detected only in the nuclei (see two

panels on the right in Fig. 33).

Further, as apparent from the result shown in Fig. 34, when p62(PB1)-HRas having the prenylated sequence at the C-terminus thereof and AG-cRaf were expressed in the cells, by the prenylated sequence, fluorescent foci formed by an association between the p62(PB1)-HRas and the AG-cRaf were detected from the cell membranes.

Furthermore, as apparent from the result shown in Fig. 35, when Smac-p62(PB1) and XIAP-AG were expressed in the cells, fluorescent foci formed by an association between Smac-p62(PB1) and XIAP-AG were also detected in the cytoplasms, reflecting the interaction between the Smac protein and the XIAP protein, which had been known to take place in the cytoplasm.

Additionally, as apparent from the result shown in Fig. 36, when p62(PB1)-BclX(L) and AG-BAD were expressed in the cells, fluorescent foci formed by an association between p62(PB1)-BclX(L) and AG-BAD were detected also in the cytoplasms, reflecting the interaction between the BclX(L) protein and the BAD protein, which had been known to take place in the cytoplasm.

(Example 21)

<Detection 17 of Protein-Protein Interaction>

As in the case of Example 20, whether or not the use of the method of the present invention enabled detection of a protein-protein interaction in an intracellular

environment unique to a target protein was tested by a method described below.

Note that, in this Example 21, the detection target was a protein-protein interaction between a Rac1 protein and PBD (p21 binding domain). The Rac1 protein is a low-molecular-weight G protein, and guanine nucleotide exchange factors (GEFs) such as Tiam1, Trio, and VAV1 convert the Rac1 protein from an inactive GDP-bound form to an active GTP-bound form. Moreover, it has been known that an active Rac1 protein and PBD of a Cdc42/Rac effector protein (p21-activated kinase 1: PAK1) interact with each other. Further, GEFs are localized differently depending on the type, and are localized inside the nucleus, at the border (near the cell membrane), and so on, of a cell. For this reason, while a Rac1 protein is present everywhere in the cell, an activation of the Rac1 protein and an interaction between an active Rac1 protein and PBD take place in intracellular regions in accordance with GEFs localized differently depending on the type (see Benard V et al., J Biol Chem., May 7, 1999, vol. 274, no. 19, pp. 13198 to 13204).

(Preparation of Plasmid DNA)

In preparing phAG-Rac1, first, a DNA encoding a Rac1 protein (protein having the amino acid sequence of SEQ ID NO: 54) (the DNA had the base sequence of SEQ ID NO: 53) was amplified from the cDNA library of HeLaS3 cells by PCR

using the following primer set:

Rac1 forward primer;

5'-GAGAATTCGATGCAGGCCATCAAGTGTGTGGTGG-3' (SEQ ID NO: 128),

and

5 Rac1 reverse primer;

5'-GGCTCGAGTTACAACAGCAGGCATTTTCTCTTCC-3' (SEQ ID NO: 129).

Then, the amplification product thus obtained was
cleaved with EcoRI and XhoI, and inserted into
10 phAG-MNLinker having been treated with the same restriction
enzymes. Thus, phAG-Rac1 was prepared.

Meanwhile, in preparing pp62(PB1)-PBD, first, a DNA
encoding PBD(region having the 67th to 150th amino acids
of the PAK1 protein, the region had the amino acid sequence
15 of SEQ ID NO: 56) (the DNA had the base sequence of SEQ
ID NO: 55) was amplified from the cDNA library of HeLaS3
cells by PCR using the following primer set:

PBD forward primer;

5'-TTGGATCCAAGAAAGAGAAAGAGCGGCCAGAGATTTCTCTCCC-3' (SEQ
20 ID NO: 130), and

PBD reverse primer;

5'-CCGAATTCTTACGCTGACTTATCTGTAAAGCTCATGTATTCTGGC-3'
(SEQ ID NO: 131).

Then, the amplification product thus obtained was
25 cleaved with BamHI and EcoRI, and inserted into
pp62(PB1)-MCLinker having been treated with the same

restriction enzymes. Thus, pp62(PB1)-PBD was prepared.

(Transfection into Cultured Cells, and Observation of Transfected Cells)

Each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into U2OS cells by the same method as that described in Example 1:

a combination of phAG-Rac1 with pp62(PB1)-PBD; and

a combination of phAG-Rac1 with pp62(PB1)-MNLinker.

Figs. 37 to 39 show the obtained results.

As apparent from the results shown in Figs. 37 and 38, when AG-Rac1 and p62(PB1)-PBD were expressed in the cells, fluorescent foci formed by an association between AG-Rac1 and p62(PB1)-PBD were detected in the nuclei (Fig. 37) and at the borders of the cells (Fig. 38).

On the other hand, as apparent from the result shown in Fig. 39, when AG-Rac1 and p62(PB1) not fused to PBD were expressed in the cells, no fluorescent focus was detected.

The above results demonstrated that it was possible to detect the same protein-protein interaction in multiple regions in the cell. Thus, it was revealed that without forcibly (artificially) confining in a particular region in a cell a protein constituting a complex formed by a protein-protein interaction, the present invention enabled detection of the protein-protein interaction in a unique intracellular environment in accordance with the localization of the protein.

Moreover, it was also demonstrated that by detecting the protein-protein interaction, the present invention enabled detection of the active (GTP-bound form) Rac1 protein. Further, it was verified that it was also possible to detect the localization and activity of the intracellular enzyme GEF by detecting the conversion to the active GTP-bound form, thus revealing that the method of the present invention enabled detection of an activity of an endogenous factor according to a protein-protein interaction.

(Example 22)

<Detection 18 of Protein-Protein Interaction>

As described in Example 21, it has been revealed that an active Rac1 protein and PBD of a Cdc42/Rac effector protein (p21-activated kinase 1: PAK1) interact with each other.

Moreover, it has also been known that the localization of a Rac1 protein is changed by geranylgeranyl group modification (prenylation) on the C-terminus of the Rac1 protein. Further, it has also been known that a Rac1 protein interacts with RhoGDI via a geranylgeranyl group of the Rac1 protein.

Hence, it was confirmed by a method described below that the method of the present invention enabled detection of a change in localization of these protein-protein interactions.

First, pRhoGDI-p62(PB1) was prepared by the same method as that described in Example 2 on the basis of a DNA sequence encoding the amino acid sequence specified under Genbank ACCESSION No: NP_004300. Moreover,
5 phAG-Rac1 and pp62(PB1)-PBD were as described in Example 21.

Then, each of the following combinations of the plasmid DNAs was mixed in equal amounts and introduced into cells by the same method as in Example 2:

- 10 a combination of phAG-Rac1 with pp62(PB1)-PBD; and
 a combination of phAG-Rac1 with pRhoGDI-p62(PB1).

Then, after culturing for 6 hours after the transfection, an inhibitor mevastatin (Enzo Life Sciences, Inc.) against geranylgeranyl group modification was added
15 to the final concentration of 10 μ M, allowed for further culturing for 15 hours, and observed. Fig. 40 shows the result of the cells co-expressing AG-Rac1 and p62(PB1)-PBD. Fig. 41 shows the result of the cells co-expressing AG-Rac1 and RhoGDI-p62(PB1). Note that the same cells are shown
20 in each of Figs. 40 and 41: (A) is photographed by the same method as that described in Example 1 using a normal inverted epifluorescence microscope; and (B) is photographed by the same method as that described in Example 17 using a total internal reflection fluorescence microscopy system with
25 arc lamp source.

As apparent from the result shown in the two upper

panels of Fig. 40, the method of the present invention confirmed that, in a normal state, the protein-protein interaction between Rac1 and PBD took place both inside and outside the nuclei; in other words, Rac1 was present in an activated state.

It has been known that if geranylgeranyl group modification is inhibited with a drug such as mevastatin, Rac1 is localized in the nucleus. Regarding this knowledge, the method of the present invention also confirmed as apparent from the result shown in the two lower panels of Fig. 40 that the interaction between Rac1 and PBD was changed by the mevastatin treatment, so that the interaction took place only in the nuclei. Further, by detecting the interaction, the method of the present invention also confirmed that Rac1 was present in an activated state even without the geranylgeranyl group modification.

Meanwhile, regarding the interaction between Rac1 and RhoGDI, the method of the present invention confirmed as apparent from the result shown in the two upper panels of Fig. 41 that, in a normal state, the protein-protein interaction between Rac1 and RhoGDI took place outside the nuclei.

On the other hand, as apparent from the result shown in the two lower panels of Fig. 41, the method of the present invention confirmed that Rac1 was localized only in the nuclei by the mevastatin treatment as described above, and

further that since the geranylgeranyl group modification of Rac1 was suppressed, the interaction between Rac1 and RhoGDI was reduced.

Thus, the present invention confirmed that it was possible to detect how the protein-protein interaction in multiple regions in the cell was changed by a stimulus from the outside. Furthermore, it was confirmed that it was also possible to detect the presence or absence of a modification of a protein influencing a protein-protein interaction (for example, geranylgeranyl group modification of Rac1 in relation to a Rac1-RhoGDI interaction).

(Example 23)

An interaction between KRas and cRaf is one of important signal transductions for cell proliferation, differentiation, and so forth. Moreover, it has been revealed that this protein-protein interaction takes place by an activation of KRas as a result of signaling via Grb2-SOS from an EGF receptor activated by an epidermal growth factor (EGF). Further, it has also been known that this protein-protein interaction changes the localization of cRaf from the cytoplasm to the cell membrane.

As described above, since the interaction between KRas and cRaf is dependent on EGF, the interaction does not take place in the absence of EGF. However, among KRas mutants, there are also constitutively-active mutants (for

example, KRasG12D) capable of interacting with cRaf in the absence of EGF. Furthermore, such mutants have been detected in various cancers. Thus, in the development of effective cancer treatments, and so forth, it is important to detect positional information and temporal information on a protein-protein interaction such as assembly formation between KRas or mutants thereof and cRaf, and localization of the assembly.

For this reason, whether or not the present invention enabled detection of a difference in protein-protein interaction between a protein having a disease-associated mutation and a wildtype protein thereof was tested by a method described below.

(Preparation of Plasmid DNAs)

pp62(PB1)-KRas(WT) was prepared by the same method as that described in Example 2 on the basis of a DNA sequence encoding the amino acid sequence specified under Genbank ACCESSION No: NP_004976.

Regarding pp62(PB1)-KRas(G12D), a mutation was introduced into pp62(PB1)-KRas(WT) by the same method as that described in Example 17 using a primer having the DNA sequence of SEQ ID NO: 160 (5'-CTTGTGGTAGTTGGAGCTGACGGCGTAGGCAAGAGTGCCTTG-3').

phAG-cRaf(R59A) was prepared by the same method as that described in Example 2 on the basis of a DNA sequence (SEQ ID NO: 161) encoding a protein having the amino acid sequence

of SEQ ID NO: 162. Note that, as cRaf, a mutant of the protein (cRaf(R59A)) was used in this Example with reference to the method described in Harvey CD et al., Science., July 4, 2008, vol. 321, no. 5885, pp. 136-140.

5 (Transfection into Cultured Cells, and Observation of Transfected Cells)

The plasmid DNAs were mixed in equal amounts in the following combinations, and were introduced into Cos-7 cells by the same method as that described in Example 2:

10 a combination of pp62(PB1)-KRas(WT) with phAG-cRaf(R59A); and

a combination of pp62(PB1)-KRas(G12D) with phAG-cRaf(R59A).

15 The cells were observed using a total internal reflection fluorescence microscopy system with arc lamp source (manufactured by Olympus Corporation, IX71-ARCEVA) capable of exciting only the vicinity of the cell membrane. Additionally, regarding the combination of pp62(PB1)-KRas(WT) with the phAG-cRaf(R59A), an image was
20 obtained when no EGF was added. After that, EGF (manufactured by SIGMA CO.) was added to the cells to the final concentration of 50 ng/ml and left standing at 37°C for 30 minutes. Then, the resultant was observed again. Fig. 42 shows the obtained result.

25 As apparent from the result shown in Fig. 42, in the cells co-expressing p62(PB1)-KRas(WT) and AG-cRaf(R59A),

no fluorescent focus (assembly formation) was observed when no stimulus was applied from the outside (no EGF addition). Meanwhile, as EGF was added, the assembly formation was observed at the cell membranes. On the other hand, in the combination of pp62(PB1)-KRas(G12D) with phAG-cRaf(R59A), the assembly formation was observed at the cell membranes even without a stimulus from the outside.

Thus, the present invention makes it possible to clearly understand a difference (such as dependency on external stimulus) of a protein-protein interaction caused by a mutation associated with a disease. Therefore, the method of the present invention is effective in analyzing the intracellular dynamics and the function of a protein and the like associated with the disease.

(Example 24)

<Detection of Protein-Protein Interaction>

As described in Example 19 also, it was confirmed by a method described below that the present invention enabled detection of a change in protein-protein interaction over time.

Note that, in Example 24, the targeted protein-protein interactions were: an interaction between BclX(L) and Bak, and an interaction between BclX(L) and Bax. It has been revealed that both Bak and Bax interact with BclX(L) via BH3 domains thereof. It has been revealed that the dissociation constant between BclX(L) and Bak BH3

domain is 340 nM, and the dissociation constant between BclX(L) and Bax BH3 domain is 13 μ M (see Sattler M et al., Science., February 14, 1997, vol. 275, no. 5302, pp. 983 to 986). Additionally, it has also been known that these
5 protein-protein interactions are competitively inhibited by ABT-737 (BH3 mimetic).

(Preparation of Plasmid DNAs)

pBak-hAG was prepared by the same method as that described in Example 2 using a DNA (SEQ ID NO: 163) encoding
10 a region having the amino acid sequence of SEQ ID NO: 164. phAG-Bax was prepared by the same method as that described in Example 2 using a DNA (SEQ ID NO: 165) encoding a region having the amino acid sequence of SEQ ID NO: 166. Moreover, pp62(PB1)-BclX(L) was as described in Example 20.

(Transfection into Cultured Cells, and Observation of Transfected Cells)

The plasmid DNAs were mixed in equal amounts in the following combinations, and the genes were introduced into 293 cells by the same method as that described in Example
20 2:

a combination of pp62(PB1)-BclX(L) with pBak-hAG;
and

a combination of pp62(PB1)-BclX(L) with phAG-Bax.

Then, to the 293 cells into these plasmid DNAs were
25 introduced, ABT-737 (manufactured by Santa Cruz Biotechnology, Inc.) was added to the final concentration

of 15 μ M, and images were obtained by the same method as that described in Example 2 every 30 minutes for the cells co-expressing p62(PB1)-BclX(L) and Bak-AG, and every 5 minutes for the cells co-expressing p62(PB1)-BclX(L) and AG-Bax. On the basis of the obtained images, analysis was performed by the same method as that described in Example 11, and a graph was prepared by plotting a total fluorescence intensity of fluorescent foci (assemblies) against time. Note that, in the graph, the x axis represents time (minutes), provided that time when the drug was added is 0, and the y axis represents the total fluorescence intensity of fluorescent foci (assemblies). Fig. 43 shows the result of the cells co-expressing p62(PB1)-BclX(L) and Bak-AG. Fig. 44 shows the result of the cells co-expressing p62(PB1)-BclX(L) and AG-Bax.

As apparent from the results shown in Figs. 43 and 44, in both of the interaction between BclX(L) and Bak and the interaction between BclX(L) and Bax, the total fluorescence luminances of assemblies formed in a manner dependent on these protein-protein interactions were decreased over time by the addition of ABT-737. Thus, the present invention verified that by detecting the fluorescent foci, it was possible to detect a period until the protein-protein interactions ended.

Moreover, the time when the total fluorescence luminance reached a half value was approximately 80 minutes

in the case of BclX(L) and Bak, and approximately 35 minutes in the case of BclX(L) and Bax. Since the rate of decreasing assemblies was slower in BclX(L) and Bak with a smaller dissociation constant (dissociation constant: 340 nM) than in BclX(L) and Bax (dissociation constant: 13 μ M), it was confirmed as described above that the present invention enabled evaluation of a strength of the protein-protein interaction.

(Example 25)

<Detection 21 of Protein-Protein Interaction>

As described in Example 24 also, it was confirmed by a method described below that the present invention enabled detection of a change over time in when a protein-protein interaction took place.

First, CHO-K1 cells stably expressing p62(PB1)-p53 and AG-MDM2 described in Example 13 were seeded onto a 35-mm glass base dish (manufactured by Asahi Glass Co., Ltd.). Then, on the next day, Nutlin-3 (manufactured by CALBIOCHEM) was added thereto to the final concentration of 10 μ M, and observed by the same method as that described in Example 1. Images were obtained 2 minutes before the addition and thereafter every 15 seconds. The obtained images were used to analyze a total fluorescence luminance of assemblies by employing the same method as that described in Example 11, and a graph was prepared by plotting the total fluorescence luminance against time. Fig. 45 shows

the obtained result.

As apparent from the result shown in Fig. 45, the total fluorescence luminance of assemblies was decreased as soon as Nutlin-3 (manufactured by CALBIOCHEM) was added. The time when the half value was reached was approximately 3 minutes. Thus, the present invention confirmed that, by detecting the fluorescent foci, it was possible to detect a period until the protein-protein interaction ended and the process.

(Example 26)

<Detection 22 of Protein-Protein Interaction>

As described above, it was confirmed by a method described below that the present invention enabled detection of a change over time in when a protein-protein interaction took place.

First, to HeLaS3 cells stably expressing mTOR(FRB domain)-AG and p62(PB1)-FKBP12 described in Example 11, rapamycin was added to the final concentration of 20 nM, and analyzed by the same method as that described in Example 11. Fig. 46 shows the obtained result.

As apparent from the result shown in Fig. 46, after rapamycin was added, assembly formation was induced as time elapsed. The time when the half value was reached was approximately 3 minutes. Thus, it was confirmed that the present invention enabled detection of time when the protein-protein interaction took place and the process,

by detecting the fluorescent foci.

(Example 27)

<Detection 23 of Protein-Protein Interaction>

It has been revealed that when ERK in cells is activated
5 by an EGF stimulus, the ERK substrate (ERK_substrate) is
phosphorylated; as a result, the ERK_substrate and a ww
domain of a Pin1 protein (Pin1(ww)) interact with each other.
Further, it has also been known that if a MEK inhibitor
U0126 is added, the ERK activity is decreased; as a result,
10 the ERK substrate is dephosphorylated, terminating the
interaction between the ERK substrate and Pin1(ww).

In this Example, it was confirmed by a method described
below that: the present invention enabled detection of an
interaction between an ERK substrate and Pin1(ww) induced
15 indirectly by EGF through ERK activation; the present
invention enabled detection of an interaction between an
ERK substrate and Pin1(ww) suppressed indirectly by U0126
through ERK inactivation; and the present invention enabled
detection of an EGF stimulus-dependent signal transduction
20 over time.

(Preparation of Plasmid DNA)

In this Example, in order to detect an EGF
stimulus-dependent interaction between an ERK substrate
and Pin1(ww),
25 pp62(PB1)-ERK_substrate-P2A-hAG-Pin1(ww)-NES was
prepared with reference to a system for detecting the

interaction by utilizing FRET (see Christopher D. Harvey et al., Proc Natl Acad Sci U S A., December 9, 2008, vol. 105, no. 49, pp. 19264 to 19269). Specifically, pp62(PB1)-ERK_substrate-P2A-hAG-Pin1(ww)-NES was prepared by the same method as that described in Example 2 using a chemically synthesized DNA (SEQ ID NO: 167) encoding a region having the amino acid sequence of SEQ ID NO: 168.

Note that, in the amino acid sequence of SEQ ID NO: 168, the amino acid sequence from positions 1 to 102 shows the amino acid sequence of p62(PB1). The amino acid sequence from positions 103 to 128 shows a linker sequence. The amino acid sequence from positions 129 to 138 shows the amino acid sequence from positions 43 to 52 of human Cdc25C that is the ERK substrate, and the amino acid sequence from positions 139 to 142 shows the amino acid sequence of an ERK docking site. The amino acid sequence from positions 146 to 164 shows the amino acid sequence of a P2A peptide. The amino acid sequence from positions 165 to 390 shows the amino acid sequence of AG. The amino acid sequence from positions 391 to 416 shows a linker sequence. The amino acid sequence from positions 417 to 470 shows the amino acid sequence of Pin1(ww). The amino acid sequence from positions 471 to 482 shows the amino acid sequence of a nuclear export signal (NES) of MEK.

Moreover, the P2A peptide inserted between

p62(PB1)-ERK_substrate and AG-Pin1(ww)-NES is a CHYSEL (cis-acting hydrolase element) sequence derived from *porcine teschovirus*. It has been known that when the protein is translated, ribosome skipping occurs, generating cleavage in front of proline at the end of the amino acid sequence (ATNFSLLKQAGDVEENPGP) (see Donnelly ML et al., J Gen Virol., may 2001, vol. 82, no. 5, pp. 1013 to 1025). Thus, if the pp62(PB1)-ERK_substrate-P2A-hAG-Pin1(ww)-NES is introduced into cells, this is consequently cleaved into: a product having a portion of the P2A peptide fused to the C-terminus of p62(PB1)-ERK_substrate; and a product having a portion of the P2A peptide fused to the N-terminus of AG-Pin1(ww)-NES. The two are expressed in the cells.

(Transfection into Cultured Cells, and Observation of Transfected Cells)

pp62(PB1)-ERK_substrate-P2A-hAG-Pin1(ww)-NES was transfected into 293 cells by the same method as that described in Example 1. On the next day, EGF (manufactured by SIGMA CO.) was added to the cells to the final concentration of 50 ng/ml. Further, 14 minutes thereafter, U0126 was added to the cells to the final concentration of 10 μ M. Meanwhile, the cell observation was started 2 minutes before the EGF addition, and the observed images were obtained every 15 seconds. The obtained images were used to analyze a total fluorescence luminance of

fluorescent foci (assemblies) by the same method as that described in Example 11, and a graph was prepared by plotting the total fluorescence luminance against time. Fig. 47 shows the obtained result.

5 As apparent from the result shown in Fig. 47, in the cells expressing p62(PB1)-ERK_substrate and AG-Pin1(ww)-NES, after EGF was added, fluorescent foci (assemblies) were significantly observed as time elapsed. However, after U0126 was added, the fluorescent foci were
10 slowly decreased, reflecting the promotion of ERK substrate dephosphorylation in response to endogenous ERK inactivation due to the addition.

 The change over time in the total fluorescence luminance of fluorescent foci (assemblies) representing
15 the interaction between the ERK substrate and Pin1(ww) was substantially the same as the result obtained by measuring an ERK substrate-Pin1(ww) interaction using FRET described in Christopher D. Harvey et al., Proc Natl Acad Sci U S A., December 9, 2008, vol. 105, no. 49, pp. 19264 to 19269.

20 In this manner, the present invention makes it possible to detect a protein-protein interaction induced or inhibited indirectly by a particular stimulus, by detecting fluorescent foci. Moreover, the present invention also makes it possible to detect a signal
25 transduction over time.

(Example 28)

<Detection 24 of Protein-Protein Interaction>

As described above, based on the interaction between HRas and cRaf described in Example 20, it was confirmed that the present invention enabled detection of a change over time in when a protein-protein interaction took place.

Specifically, pp62(PB1)-HRas(WT) described in Example 20 and phAG-cRaf(R59A) described in Example 23 were introduced into cells by the same method as that described in Example 23. EGF (manufactured by SIGMA CO.) was added thereto to the final concentration of 50 ng/ml. A measurement apparatus capable of detecting a fluorescent signal only in the vicinity of the cell membrane was used for the observation. Note that the observation was started 5 minutes before the EGF addition, and continued at 5-minute intervals for 30 minutes after the addition. The observation was made another 30 minutes thereafter. On the basis of the obtained image data, analysis was performed by the same method as that described in Example 11, and a graph was prepared by plotting a total fluorescence intensity of fluorescent foci (assemblies) against time. Note that, in the graph, the x axis represents time (minutes), provided that time when EGF was added is 0, and the y axis represents the total fluorescence intensity of fluorescent foci (assemblies). Fig. 48 shows the obtained result.

As apparent from the result shown in Fig. 48, the interaction between HRas and cRaf that took place by adding

EGF was successfully detected over time.

Moreover, as described above, it has been known that cRaf interacts with HRas activated as a result of signaling via a Grb2-SOS complex from an EGF receptor of cells to which EGF has been added. Thus, the result shown in Fig. 48 confirmed that the present invention enabled tracing over time of a process in which an intracellular signal transduction pathway (such as a signal transduction pathway via a Grb2-SOS complex) was activated in response to a stimulus (such as EGF) applied to cells from the outside.

(Example 29)

<Detection 25 of Protein-Protein Interaction>

As described above, it has been revealed that rapamycin binds to a FKBP12 protein, and this complex further binds to a FRB domain of a mTOR protein (mTOR(FRB)). A mTOR protein is a serine/threonine kinase having a function of activating signal transductions involved in protein synthesis and cell proliferation. It has also been revealed that the function is inhibited by such complex formation between a FKBP12 protein and rapamycin.

Furthermore, a FKBP12 protein has been known to interact with a protein phosphatase (calcineurin) composed of a catalytic subunit A (calcineurin A) and a regulatory subunit B (calcineurin B) via FK506. Calcineurin is an enzyme having a very important function in signal transductions in T cells and the like. It has also been

revealed that the function is inhibited by such complex formation between a FKBP12 protein and FK506.

Hence, in this Example, a test was conducted by a method described below regarding whether or not the present invention enabled detection and distinguishment of: a complex of FKBP12 and mTOR(FRB) formed in a manner dependent on rapamycin in a single cell in which FKBP12, mTOR(FRB), calcineurin A and calcineurin B were co-expressed; a complex of FKBP12 with calcineurin A and calcineurin B formed in a manner dependent on FK506; and eventually an inhibition of signal transductions in which these complexes were involved.

Note that as the "calcineurin A and calcineurin B" expressed in the cell, a mCAB protein was used, which was composed of a portion of calcineurin A fused to a portion of calcineurin B (see Clemons PA et al., Chem Biol., January 2002, vol. 9, iss. 1, pp. 49 to 61).

First, phAG-mCAB, pp62(PB1)-FKBP12, and pmTOR(FRB)-hK01 were introduced into HeLaS3 cells. The phAG-mCAB was prepared by the same method as that described in Example 2 using an artificially synthesized DNA (SEQ ID NO: 169) encoding a region having the amino acid sequence of SEQ ID NO; 170. The pp62(PB1)-FKBP12 was as described in Example 2. The pmTOR(FRB)-hK01 (pmTOR(FRB domain)-hK01) was as described in Example 6.

Moreover, the HeLaS3 cells were cultured by the same

method as that described in Example 1. Further, in the transfection, the HeLaS3 cells were seeded onto 2 wells of an 8-well chamber (manufactured by Nunc A/S). On the next day, by the same method as that described in Example 1, 130 ng of each of the plasmid DNAs was introduced into the HeLaS3 cells using 1 µl of Transfection Reagent.

Then, 24 hours thereafter, the transfected cells were observed by the same method as that described in Example 1. To the cells, rapamycin or FK506 was added to the final concentration of 500 nM, and observed another 15 minutes thereafter. Fig. 49 shows the obtained result.

As apparent from the result shown in Fig. 49, by adding rapamycin to the cells expressing AG-mCAB, p62(PB1)-FKBP12, and mTOR(FRB)-K01, the interaction between mTOR(FRB) and FKBP12 was observed in the form of fluorescent foci emitting a K01-derived fluorescent signal. On the other hand, by adding FK506, the interaction between mCAB and FKBP12 was observed in the form of fluorescent foci emitting an AG-derived fluorescent signal.

Thus, it was confirmed that the present invention enabled detection of multiple types of protein-protein interactions in a single cell, particularly, various protein-protein interactions dependent on different stimuli in a single cell.

Moreover, the present invention can provide a method for detecting and distinguishing multiple types of signal

transductions in a single cell by detecting various protein-protein interactions involved in signal transductions in a single cell.

Furthermore, as described in this Example also, unlike FRET that is another method for detecting a protein-protein interaction in living cells, it is not necessary to select a fluorescent protein meeting the conditions of an acceptor and a donor; in addition, it is not necessary to take into consideration cross excitation by which an acceptor fluorescent protein is excited, and bleed-through in which fluorescence of a donor fluorescent protein bleeds through a filter (absorption filter) set for detecting fluorescence of an acceptor fluorescent protein. Thus, in the present invention, combinations of various fluorescent proteins having different wavelength characteristics can be easily selected and utilized.

(Example 30)

<Detection 26 of Protein-Protein Interaction>

Whether or not the method of the present invention enabled detection of known protein-protein interactions shown in Table 3 by using p62(PB1) as the association-inducing protein and an AG protein as the fluorescent protein having a multimerization ability was tested by the method described in Example 2.

[Table 3]

Protein-protein interaction	
Cdk5	P25
Plk	Wee1
calcineurin A α	VIVIT peptide
JNK	JIP
CREB	CBP
ERK2	MEK
MEK	cRaf
β -catenin	TCF

As a result, although unillustrated, it was verified that it was possible to detect the protein-protein interactions in all the combinations in the form of fluorescent foci. It was demonstrated that the present invention was a generally-adoptable method for detecting a protein-protein interaction.

[Industrial Applicability]

As has been described above, the present invention makes it possible to detect a protein-protein interaction in an intracellular environment unique thereto, and to detect positional information and temporal information on the protein-protein interaction. Moreover, in the present invention, a strength of a protein-protein interaction correlates with the fluorescence intensity of a fluorescent focus. Accordingly, the method is utilizable in identifying an amino acid residue involved in a protein-protein interaction, and also in screening for a substance modulating a protein-protein interaction, on the

basis of the fluorescence intensity.

Thus, the method for detecting a protein-protein interaction and so forth of the present invention and a kit for use in these methods are useful in the development of pharmaceutical products and so on through elucidations of various signal transductions *in vivo*, various biological reaction controls, and the like, and eventually through elucidations of disease mechanisms.

[Sequence Listing Free Text]

10 SEQ ID NOs: 1 and 2

<223> humanized-codon Azami Green (AG)

SEQ ID NOs: 3 and 4

<223> PB1 domain of p62

SEQ ID NOs: 5 and 6

15 <223> PB1 domain of MEK5

SEQ ID NOs: 7 and 8

<223> PB1 domain of Nbr1

SEQ ID NOs: 9 and 10

<223> PB1 domain of PKCιota

20 SEQ ID NOs: 11 and 12

<223> PB1 domain of TFG

SEQ ID NOs: 13 and 14

<223> SAM domain of TEL

SEQ ID NOs: 15 and 16

25 <223> SAM domain of EphB2

SEQ ID NOs: 17 and 18

<223> SAM domain of DGK delta
 SEQ ID NOs: 19 and 20
 <223> SAM domain of Tankyrase-1
 SEQ ID NOs: 21 and 22
 5 <223> FRB domain of mTOR
 SEQ ID NOs: 23 and 24
 <223> FKBP12
 SEQ ID NOs: 25 and 26
 <223> p53
 10 SEQ ID NOs: 27 and 28
 <223> MDM2
 SEQ ID NOs: 29 and 30
 <223> Sec5
 SEQ ID NOs: 31 and 32
 15 <223> RalB
 SEQ ID NOs: 33 and 34
 <223> RalB protein Q72L mutant
 SEQ ID NOs: 35 and 36
 <223> RalB protein S28N mutant
 20 SEQ ID NOs: 37 and 38
 <223> calmodulin
 SEQ ID NOs: 39 and 40
 <223> M13 peptide
 SEQ ID NOs: 41 and 42
 25 <223> HRas
 SEQ ID NOs: 43 and 44

<223> cRaf
 SEQ ID NOs: 45 and 46
 <223> Smac
 SEQ ID NOs: 47 and 48
 5 <223> XIAP
 SEQ ID NOs: 49 and 50
 <223> BclX(L)
 SEQ ID NOs: 51 and 52
 <223> BAD
 10 SEQ ID NOs: 53 and 54
 <223> Rac1
 SEQ ID NOs: 55 and 56
 <223> PBD
 SEQ ID NO: 57
 15 <223> artificially synthesized hAG forward primer 1
 sequence
 SEQ ID NO: 58
 <223> artificially synthesized hAG reverse primer 1
 sequence
 20 SEQ ID NO: 59
 <223> artificially synthesized p62(PB1) forward primer 1
 sequence
 SEQ ID NO: 60
 <223> artificially synthesized p62(PB1) reverse primer 1
 25 sequence
 SEQ ID NO: 61

<223> artificially synthesized hAG forward primer 2
sequence

SEQ ID NO: 62

<223> artificially synthesized hAG reverse primer 2
sequence

SEQ ID NO: 63

<223> artificially synthesized p62(PB1) forward primer 2
sequence

SEQ ID NO: 64

<223> artificially synthesized p62(PB1) reverse primer 2
sequence

SEQ ID NO: 65

<223> artificially synthesized p62(PB1) forward primer 3
sequence

SEQ ID NO: 66

<223> artificially synthesized p62(PB1) reverse primer 3
sequence

SEQ ID NO: 67

<223> artificially synthesized mTOR(FRB) forward primer
sequence

SEQ ID NO: 68

<223> artificially synthesized mTOR(FRB) reverse primer
sequence

SEQ ID NO: 69

<223> artificially synthesized FKBP12 forward primer
sequence

SEQ ID NO: 70

<223> artificially synthesized FKBP12 reverse primer
sequence

SEQ ID NO: 71

5 <223> artificially synthesized MEK(PB1) forward primer
sequence

SEQ ID NO: 72

<223> artificially synthesized MEK(PB1) reverse primer
sequence

10 SEQ ID NO: 73

<223> artificially synthesized Nbr1(PB1) forward primer
sequence

SEQ ID NO: 74

<223> artificially synthesized Nbr1(PB1) reverse primer
15 sequence

SEQ ID NO: 75

<223> artificially synthesized PKCιota(PB1) forward
primer sequence

SEQ ID NO: 76

20 <223> artificially synthesized PKCιota(PB1) reverse
primer sequence

SEQ ID NO: 77

<223> artificially synthesized TFG(PB1) forward primer
sequence

25 SEQ ID NO: 78

<223> artificially synthesized TFG(PB1) reverse primer

sequence

SEQ ID NO: 79

<223> artificially synthesized TEL(SAM) forward primer
sequence

5 SEQ ID NO: 80

<223> artificially synthesized TEL(SAM) reverse primer
sequence

SEQ ID NO: 81

10 <223> artificially synthesized EphB2(SAM) forward primer
sequence

SEQ ID NO: 82

<223> artificially synthesized EphB2(SAM) reverse primer
sequence

SEQ ID NO: 83

15 <223> artificially synthesized DGK delta(SAM) forward
primer sequence

SEQ ID NO: 84

<223> artificially synthesized DGK delta(SAM) reverse
primer sequence

20 SEQ ID NO: 85

<223> artificially synthesized Tankyrase(SAM) forward
primer sequence

SEQ ID NO: 86

25 <223> artificially synthesized Tankyrase(SAM) reverse
primer sequence

SEQ ID NO: 87

<223> artificially synthesized TFG(PB1) forward primer 2
sequence

SEQ ID NO: 88

<223> artificially synthesized TFG(PB1) reverse primer 2
sequence

SEQ ID NO: 89

<223> artificially synthesized TEL(SAM) forward primer 2
sequence

SEQ ID NO: 90

<223> artificially synthesized TEL(SAM) reverse primer 2
sequence

SEQ ID NO: 91

<223> artificially synthesized DGK delta(SAM) forward
primer 2 sequence

SEQ ID NO: 92

<223> artificially synthesized DGK delta(SAM) reverse
primer 2 sequence

SEQ ID NO: 93

<223> artificially synthesized Tankyrase(SAM) forward
primer 2 sequence

SEQ ID NO: 94

<223> artificially synthesized Tankyrase(SAM) reverse
primer 2 sequence

SEQ ID NO: 95

<223> artificially synthesized hK01 forward primer
sequence

SEQ ID NO: 96

<223> artificially synthesized hK01 reverse primer
sequence

SEQ ID NO: 97

5 <223> artificially synthesized p53 forward primer sequence

SEQ ID NO: 98

<223> artificially synthesized p53 reverse primer sequence

SEQ ID NO: 99

10 <223> artificially synthesized MDM2 forward primer
sequence

SEQ ID NO: 100

<223> artificially synthesized MDM2 reverse primer
sequence

SEQ ID NO: 101

15 <223> artificially synthesized Sec5 forward primer
sequence

SEQ ID NO: 102

<223> artificially synthesized Sec5 reverse primer
sequence

20 SEQ ID NO: 103

<223> artificially synthesized RalB forward primer
sequence

SEQ ID NO: 104

25 <223> artificially synthesized RalB reverse primer
sequence

SEQ ID NO: 105

<223> artificially synthesized RalB(Q72L) mutation primer
sequence

SEQ ID NO: 106

<223> artificially synthesized RalB(S28N) mutation primer
sequence

SEQ ID NO: 107

<223> artificially synthesized calmodulin forward primer
sequence

SEQ ID NO: 108

<223> artificially synthesized calmodulin reverse primer
sequence

SEQ ID NO: 109

<223> artificially synthesized M13 peptide forward primer
sequence

SEQ ID NO: 110

<223> artificially synthesized M13 peptide reverse primer
sequence

SEQ ID NO: 111

<223> artificially synthesized AGNLS forward primer
sequence

SEQ ID NO: 112

<223> artificially synthesized AGNLS reverse primer
sequence

SEQ ID NO: 113

<223> artificially synthesized HRas forward primer
sequence

SEQ ID NO: 114

<223> artificially synthesized HRas reverse primer
sequence

SEQ ID NO: 115

5 <223> artificially synthesized KRas forward primer
sequence

SEQ ID NO: 116

<223> artificially synthesized KRas reverse primer
sequence

10 SEQ ID NO: 117

<223> artificially synthesized HRas mutant primer sequence

SEQ ID NO: 118

<223> artificially synthesized cRaf forward primer
sequence

15 SEQ ID NO: 119

<223> artificially synthesized cRaf reverse primer
sequence

SEQ ID NO: 120

20 <223> artificially synthesized Smac forward primer
sequence

SEQ ID NO: 121

<223> artificially synthesized XIAP forward primer
sequence

SEQ ID NO: 122

25 <223> artificially synthesized XIAP reverse primer
sequence

SEQ ID NO: 123

<223> artificially synthesized BclX(L) forward primer sequence

SEQ ID NO: 124

5 <223> artificially synthesized BclX(L) reverse primer sequence

SEQ ID NO: 125

<223> artificially synthesized BAD forward primer 1 sequence

10 SEQ ID NO: 126

<223> artificially synthesized BAD forward primer 2 sequence

SEQ ID NO: 127

<223> artificially synthesized BAD reverse primer sequence

15 SEQ ID NO: 128

<223> artificially synthesized Rac1 forward primer sequence

SEQ ID NO: 129

20 <223> artificially synthesized Rac1 reverse primer sequence

SEQ ID NO: 130

<223> artificially synthesized PBD forward primer sequence

SEQ ID NO: 131

<223> artificially synthesized PBD reverse primer sequence

25 SEQ ID NO: 132

<223> base sequence of monomeric K0 (Kusabira-Orange)

SEQ ID NO: 133

<223> amino acid sequence of monomeric KO (Kusabira-Orange)

SEQ ID NOs: 134 and 135

<223> humanized-codon mAG1

5 SEQ ID NOs: 136 and 137

<223> mMiCy1

SEQ ID NOs: 138 and 139

<223> mKikGR1

SEQ ID NOs: 140 and 141

10 <223> KCy1

SEQ ID NOs: 142 and 143

<223> dAG (AB)

SEQ ID NOs: 144 and 145

<223> dAG (AC)

15 SEQ ID NOs: 146 and 147

<223> TGuv

SEQ ID NOs: 148 and 149

<223> Momiji

SEQ ID NOs: 150 and 151

20 <223> COR3.01

SEQ ID NOs: 152 and 153

<223> DsRed2

SEQ ID NOs: 154, 159, and 160

<223> artificially synthesized primer sequences

25 SEQ ID NOs: 155, 157, 161, 163, 165, 167, and 169

<223> artificially synthesized polynucleotide sequences

SEQ ID NOs: 156, 158, 162, 164, 166, 168, and 170

<223> artificially synthesized polypeptide sequences

SEQ ID NOs: 171 and 172

<223> COR5

[CLAIMS]

[Claim 1]

A method for detecting an interaction between a first protein and a second protein, the method comprising the steps of:

expressing in a cell a first fusion protein comprising the first protein and an association-inducing protein, and a second fusion protein comprising the second protein and a fluorescent protein capable of forming a homomultimer;

detecting a fluorescent focus formed by an association between the first fusion protein and the second fusion protein in the cell; and

determining an interaction between the first protein and the second protein according to the detection of the fluorescent focus,

wherein the association-inducing protein is at least one protein selected from the group consisting of a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota, a SAM domain of TEL, a SAM domain of DGK delta, and a SAM domain of Tankyrase-1.

[Claim 2]

The method according to claim 1, wherein the fluorescent focus is detected to detect the interaction taking place or ending, a period until the interaction takes place or ends, or a duration of the interaction.

[Claim 3]

The method according to claim 1, wherein the fluorescent focus is detected to detect the interaction taking place or ending in response to a particular stimulus, a period until the interaction takes place or ends, or a duration of the interaction.

[Claim 4]

The method according to any one of claims 1 to 3 for screening for a protein interacting with a particular protein, wherein

one of the first protein and the second protein is the particular protein, while the other is a test protein, and

a protein interacting with the particular protein is selected according to the detection of the fluorescent focus.

[Claim 5]

The method according to any one of claims 1 to 3 for identifying any one of an amino acid residue in the first protein and an amino acid residue in the second protein, which are involved in the interaction, wherein

in a case where a protein in which a mutation is introduced is used as any one of the first protein and the second protein, if an intensity of the fluorescent focus is reduced in comparison with a case of using a protein in which no mutation is introduced, the amino acid residue in which the mutation is introduced is determined to be

involved in the interaction.

[Claim 6]

A method for screening for a substance modulating
an interaction between a first protein and a second protein,
5 the method comprising the steps of:

expressing in a cell a first fusion protein comprising
the first protein and an association-inducing protein, and
a second fusion protein comprising the second protein and
a fluorescent protein capable of forming a homomultimer,
10 in presence of a test compound;

detecting a fluorescent focus formed by an
association between the first fusion protein and the second
fusion protein in the cell; and

selecting the test compound as a substance inducing
15 the interaction if a fluorescence intensity of the
fluorescent focus is higher than a fluorescence intensity
of a fluorescent focus formed in absence of the test compound,
or selecting the test compound as a substance suppressing
the interaction if the fluorescence intensity of the
20 fluorescent focus is lower than the fluorescence intensity
of the fluorescent focus formed in the absence of the test
compound,

wherein the association-inducing protein is at least
one protein selected from the group consisting of a PB1
25 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota,
a SAM domain of TEL, a SAM domain of DGK delta, and a SAM

domain of Tankyrase-1.

[Claim 7]

A method for screening for an association-inducing protein, the method comprising the steps of:

5 (a) expressing in a cell a fusion protein comprising a test protein and mAG1;

(b) expressing in a cell a fusion protein comprising the test protein and a fluorescent protein capable of forming a homomultimer; and

10 (c) selecting the test protein as an association-inducing protein if a fluorescent focus is not detected in step (a) but a fluorescent focus is detected in step (b).

[Claim 8]

15 The method according to any one of claims 1 to 7, wherein the fluorescent protein capable of forming a homomultimer is at least one fluorescent protein selected from the group consisting of Azami-Green, Kusabira-Orange 1, dimeric Keima-Red, Kikume Green-Red, Midoriishi-Cyan1, 20 Kusabira-Cyan1, dimeric Azami-Green (AB), dimeric Azami-Green (AC), TGuv, Momiji, COR3.01, COR5, and DsRed2.

[Claim 9]

A kit for use in the method according to any one of claims 1 to 8, the kit comprising an instruction and at 25 least one substance selected from the group consisting of the following (a) to (j):

(a) a vector comprising a DNA encoding the association-inducing protein and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the association-inducing protein when expressed;

(b) a vector comprising a DNA encoding the fluorescent protein capable of forming a homomultimer and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the fluorescent protein when expressed;

(c) a vector comprising a DNA encoding mAg1 and a cloning site allowing an insertion of a DNA encoding a certain protein in such a manner that the certain protein is fused to the fluorescent protein when expressed;

(d) a vector encoding the first fusion protein;

(e) a vector encoding the second fusion protein;

(f) a vector set comprising the vector according to any one of (a) and (d) and the vector according to any one of (b) and (e);

(g) a vector set comprising the vector according to (b) and the vector according to (c);

(h) a transformed cell comprising a vector encoding the first fusion protein;

(i) a transformed cell comprising a vector encoding the second fusion protein; and

(j) a transformed cell comprising a vector encoding

the first fusion protein and a vector encoding the second fusion protein,

wherein the association-inducing protein is at least one protein selected from the group consisting of a PB1 domain of p62, a PB1 domain of TFG, a PB1 domain of PKCιota, 5 a SAM domain of TEL, a SAM domain of DGK delta, and a SAM domain of Tankyrase-1.