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(54) **Title:** METHOD FOR DETECTING A PROTEIN-PROTEIN INTERACTION

(57) **Abstract:** Provided is a new method for detecting a protein-protein interaction, comprising fusing a TlucC(mut) domain having amino acid SEQ ID NO: 1 to the first protein; fusing a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4 to the second protein; allowing the fused first protein and the fused second protein to interact with each other; and detecting the light emitted from a complex of the fused first protein and the fused second protein.

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Description

METHOD FOR DETECTING A PROTEIN-PROTEIN INTERACTION

Technical Field

[0001] The present invention relates to methods for detecting a protein-protein interaction.

[Description of the Related Art]

[0002] Recently, taking advantage of the complementation of the split luciferase fragments, the system of detecting the interaction of two proteins of interest has been developed (Kim, S. B., Ozawa, T., Watanabe, S., Umezawa, Y., 2004. Proc. Natl. Acad. Sci. USA. 101, 11542-11547). Complementation strategies for detecting protein-protein interactions generally involve the fusion of split reporter protein fragments to the protein of interest in such a way that neither of the fragments retains significant activity by themselves. When the proteins of interest interact, the two inactive reporter protein fragment complement with each other such that the activity is regained, providing a readout signal for indirectly following the protein-protein interaction.

This complementation strategy has been used with a variety of reporter proteins, including dihydrofolate reductase, b-Lactamase, and green fluorescent protein. Several luciferases have been also used including Renilla luciferase, firefly luciferase, red click beetle luciferase, and green click beetle luciferase. The split fragments of these luciferases have their own specificity and a fragment can recover its luminescent ability only when it is placed close to another fragment derived from the same species of luciferase.

Disclosure of Invention

[0003] [Summary of the Invention]

[0004] The inventors have developed a mutant C-terminus fragment of the click beetle luciferase (TlucC(mut); SEQ ID NO: 1) which has the ability to complement the N-terminus fragment derived from other species of luciferase such as wild type firefly luciferase (FlucN; SEQ ID NO: 3) and green click beetle luciferase (GlucN; SEQ ID NO: 2), as well as that of the red click beetle luciferase (TlucN; SEQ ID NO: 4), using the same luciferin substrate.

[0005] One embodiment of the present invention is a protein comprising a TlucC(mut) domain. The TlucC(mut) domain has amino acid SEQ ID NO: 1.

[0006] Another embodiment is a complex comprising the protein comprising a TlucC(mut) domain and a protein comprising either a GlucN domain having amino acid SEQ ID

NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4. These proteins are capable of binding to each other.

[0007] Another embodiment is a method for detecting a protein comprising either a GlucN domain, a FlucN domain, or a TlucN domain. This method comprises allowing the protein to interact with the protein comprising a TlucC(mut) domain to form a complex. These domains in the complex complement to each other and become able to emit specific light. By detecting the emitted light, the protein comprising either a GlucN domain, a FlucN domain, or a TlucN domain can be detected.

[0008] Another embodiment is a method for detecting a protein comprising a TlucC(mut) domain. This method comprises allowing the protein to interact with a protein comprising either a GlucN domain, a FlucN domain, or a TlucN domain to form a complex. These domains in the complex complement to each other and become able to emit specific light. By detecting the emitted light, the protein comprising a TlucC(mut) domain can be detected.

[0009] Another embodiment is a method for detecting a complex comprising a first protein comprising a TlucC(mut) domain and a second protein comprising either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4. This method comprises detecting the light emitted from the complex.

[0010] Another embodiment is a method for detecting binding of a first protein comprising a TlucC(mut) domain and a second protein comprising either a GlucN domain, a FlucN domain, or a TlucN. This method comprises allowing the proteins to interact with each other to form a complex, and detecting the light emitted from the complex of the proteins.

[0011] Another embodiment is a method for detecting binding of a first protein and a second protein, the proteins being capable of binding to each other. The method comprises fusing a TlucC(mut) domain to the first protein, fusing either a GlucN domain, a FlucN domain, or a TlucN domain to the second protein, allowing the fused first protein and the fused second protein to interact with each other to form a complex, and detecting the light emitted from the complex.

[0012] Another embodiment is a method for selecting a binding protein of a first protein between a second protein and a third protein, the first protein being fused to a TlucC(mut) domain, each of the second protein and the third protein being fused to a different domain selected from a group consisting of a GlucN domain, a FlucN domain, and a TlucN domain, comprising allowing the first protein to interact with the second protein and the third protein, detecting the emitted light, and determining which complex of the proteins emits the light.

[0013] Another embodiment is a method for selecting a binding protein of a first protein

between a second protein and a third protein. This method comprises fusing a TlucC(mut) domain to the first protein, fusing each of two domains selected from a group consisting of a GlucN domain, a FlucN domain, a TlucN domain to the second protein and the third protein, allowing the first protein to interact with the second protein and the third protein, detecting the emitted light, and determining which complex of the proteins emits the light.

[0014] The protein containing a TlucC(mut) domain may have amino acid SEQ ID NO: 1 which contains one or a few of either substitution, deletion, or addition of an amino acid, as long as the protein is capable of binding to either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4, to form a complex.

Brief Description of the Drawings

[0015] [fig.1]Figure 1 shows a schematic of the principle of the bioluminescent probes based on the protein complementation.

[fig.2]Figure 2 shows the complementation of (a) FlucN and TlucC(mut), (b) GlucN and TlucC(mut), and (c) TlucC and TlucC(mut) in an example.

[fig.3]Figure 3 shows the constructs of luminescent probes used in an example.

[fig.4]Figure 4 shows expressions from the constructs of pBAD(wt)-TlucC(mut), pBAD(S112A)-TlucC(mut), pBAD(S136A)-TlucC(mut), pBAD(S155A)-TlucC(mut), pBAD(StrippleA)-TlucC(mut), pGlucN-14-3-3, and pGlucN-BCL-X_L in the COS-7 cells in an example.

[fig.5]Figure 5 show the interactions between BAD or BAD variants and 14-3-3 in an example.

[fig.6]Figure 6 shows that there was no phosphorylation of the 112nd, 136th, and 155th amino acids in BAD(S112A), BAD(S136A), BAD(S155A), respectively, and that there were phosphorylation of all of three amino acids in BAD(wt), in an example.

[fig.7]Figure 7 shows effects of various phosphorylation stimulators and inhibitors on BAD/14-3-3 interaction in an example.

[fig.8]Figure 8 shows the interactions between BAD and BCL-XL, and effects of BCL-XL inhibitors on the BAD/BCL-XL interaction in an example.

[fig.9]Fig. 9 shows the spectrum shift of lights emitted by the combinations of GlucN and Tluc(mut), FlucN and TlucC(mut), and TlucN and TlucC(mut) in an example.

[fig.10]Fig. 10 shows detection of luminescence in subcutaneous cells of nude mice in an example. "Em530nm" and "Em600nm" indicate wavelengths of detected lights. "OPEN" indicates the result obtained without narrowing wavelength for detection of light.

Detailed Description of the Invention

[0016] The present invention provides a modified complementation strategy for detecting protein-protein interactions, using a mutant C-terminus fragment of the red click beetle luciferase (TlucC(mut); SEQ ID NO: 1).

[0017] The wild type C-terminus fragment of the red click beetle luciferase (wtTluc; SEQ ID NO: 1) can complement only the N-terminus fragment derived from the red click beetle luciferase (TlucN) but not those derived from other species of luciferase. As shown in the EXAMPLES, however, the TlucC(mut) has the ability to complement the N-terminus fragment derived from other species of luciferase such as wild type firefly luciferase (FlucN) and green click beetle luciferase (GlucN), as well as that of the red click beetle luciferase (TlucN), using the same luciferin substrate. These three domains, GlucN domain, FlucN domain, and TlucN domain, are referred to as anti-TlucC(mut) domains in the specification.

[0018] As shown in Example 4, depending on the combination of the TlucC(mut) domain with one of the anti-TlucC(mut) domains, the complex of proteins having each of the domains emits light with a specific wavelength i.e., the emitted light has peak near 600 nm in the case of a combination with TlucN, near 530 nm in the case of a combination with GlucN, and near 560 nm in the case of a combination with FlucN, respectively. Therefore, it can be determined which complex of the proteins emits the light, depending on the wavelength of the emitted light. For example, the complex can be identified as having a combination with TlucN, GlucN and FlucN, by detecting light with a wavelength of 600 nm, 530 nm and 560 nm, respectively. By taking advantage of this property, it can be further determined which complexes of the proteins emit the light even if more than one complexes emit light.

[0019] Emitted light from the complex can be detected as luminescence in cell lysates, cell extracts, and cultured cells, as well as luminescence in a living body as shown in Example 5. It is easier to detect luminescence in cells located near a body surface such as endodermic and subcutaneous cells.

[0020] Taking advantage of this property of the TlucC(mut), the following applications can be made as examples:

(1) detection of a protein having an anti-TlucC(mut) domain, using a protein having a TlucC(mut) domain, the proteins being capable of binding to each other;

(2) detection of a protein having a TlucC(mut) domain, using a protein having an anti-TlucC(mut) domain, the proteins being capable of binding to each other;

(3) detection of a complex of a protein having a TlucC(mut) domain and a protein having an anti-TlucC(mut) domain, the proteins being capable of binding to each other;

(4) detection of binding of a protein having a TlucC(mut) domain to a protein having an anti-TlucC(mut) domain; and

(5) selection of a binding protein of a protein having a TlucC(mut) domain between a second protein and a third protein, each of which has a different anti-TlucC(mut) domain.

[0021] In embodiment (1), existence of a protein having an anti-TlucC(mut) domain can be examined by allowing the protein to interact with a protein, which has a TlucC(mut) domain and is capable of binding with a protein having an anti-TlucC(mut) domain, and detecting the light emitted when the proteins are bound and the domains are placed in such a close position as to interact with each other.

[0022] In embodiment (2), existence of a protein having a TlucC(mut) domain can be examined by allowing the protein to interact with a protein, which has an anti-TlucC(mut) domain and is capable of binding with a protein having a TlucC(mut) domain, and detecting the light emitted when the proteins are bound and the domains are placed in such a close position as to interact with each other.

[0023] For example, if a gene encoding a protein of interest fused by an anti-TlucC(mut) domain is expressed in some cells, the expression can be detected by expressing another gene encoding a protein fused by a TlucC(mut) domain, which can bind to the protein of interest, in the cells together. In the cells, when both of the fusion proteins are bound and the domains are placed in such a close position as to interact with each other, the domains complement to each other and light specific to the combination of the domains are emitted. By detecting the emitted light, the existence of the protein of interest can be detected.

[0024] Such a system can be used for examining expression of a protein, the specificity of a promoter, etc. as applications.

[0025] In embodiment (3), since a complex of a protein having a TlucC(mut) domain and a protein having an anti-TlucC(mut) domain emit light with a specific wavelength, the existence of the complex can be detected by examining the emitted light in the assay system.

[0026] In embodiment (4), if a protein having a TlucC(mut) domain binds to a protein having an anti-TlucC(mut) domain, these proteins form a complex and thus the binding can be detected by examining the formed complex as described for embodiment (3).

[0027] In embodiment (5), a binding protein of a protein having a TlucC(mut) domain can be selected between two or three proteins, each of which has a different anti-TlucC(mut) domain. Depending on the combination of the domains derived from luciferases, the light with a different wavelength is emitted. Therefore, by detecting the emitted light and its wavelength, it will be known which complex is formed and a binding protein to the protein having a TlucC(mut) domain can be identified and selected.

[0028] This embodiment has its wide-range of applications. For example, if the property of

the protein having a TlucC(mut) domain is changed depending on the situation in a cell and the binding partner of the protein is changed depending on its property, then the situation in the cell can be detected by putting the protein having a TlucC(mut) domain and the other proteins having a anti-TlucC(mut) domain in the cell and examining the binding partner of the protein having a TlucC(mut) domain. If the complex is detected in real-time by fluorescence etc., the change of the property of the protein can be also detected in real-time.

[0029] In the above embodiments, the methods for producing the fusion protein are not limited but production by a chemical synthesis or a molecular biological synthesis is preferred and a molecular biological synthesis is most preferred. For example, a gene coding the fusion protein is inserted into an expression vector, the expression vector is introduced in a cell, and the fusion protein can be thus expressed in the cell.

[0030] The methods for detecting light emitted from the complex of the fusion proteins are not particularly limited and the most suitable method to the substrate and the emitted light can be used.

Examples

[0031] Example 1

Complementation

of TlucN, GlucN or FlucN with TlucC(mut)

[0032] To confirm that TlucN, GlucN or FlucN can complement to TlucC(mut), the interacting proteins, FKBP and FRB, which can be bound in the presence of rapamycin, were utilized.

[0033] To construct pTlucN-FKBP, pGlucN-FKBP, pFlucN-FKBP and pFRB-TlucC(mut), the cDNA of TlucN, GlucN, FlucN, FKBP, FRB, and TlucC(mut) were generated by standard polymerase chain reaction to add the Kozak sequence and restriction sites. The sequences of the primers used for generating these cDNAs are the followings:

(TlucN-1) 5'AAGCTTGCCATGGTAAAGCGTGAGAAAAATGTC3'(SEQ ID NO: 5)

(TlucN-2) 5'GGATCCTCCGCCTCCTCCGCCGTCGTCGATGGCCTC3' (SEQ ID NO: 6)

(GlucN-1) 5'AAGCTTGCCATGGAGAGAGAGAAGAAC3'(SEQ ID NO: 7)

(GlucN-2) 5'GGATCCTCCGCCTCCTCCTACCATAGGTCCCCAGAT3' (SEQ ID NO: 8)

(FlucN-1) 5'AAGCTTGCCATGGAAGACGCCAAAAACATAAAGAAAGGC3' (SEQ ID NO: 9)

(FlucN-2) 5'GGATCCTCCGCCTCCTCCATCCTTGTCAATCAAGGCGTTGGT3' (SEQ ID NO: 10)

(FKBP-1) 5'GGATCCATGGGCGTGCAGGTGGAG3' (SEQ ID NO: 11)

(FKBP-2) 5'CTCGAGCGTTCCAGTTTTAGAAGCTC3' (SEQ ID NO: 12)
(FRB-1) 5'GGATCCATGGTAGCCATCCTCTGG3' (SEQ ID NO: 13)
(FRB-2) 5'CTCGAGCGTGATATCCGTCTGAACAC3' (SEQ ID NO: 14)
(TlucC-1) 5'CTCGAGTGGAGGCGGCGGAAGCAAGGGTTATGTCAAT3'
(SEQ ID NO: 15)
(TlucC-2) 5'CCGCGGGCCCACACCGCCGGCCTTCACCAA3' (SEQ ID NO: 16)
(TlucC(mut)-1) 5'CTCGAGTGGAGGCGGCGGAAGCAAGGGTTATGTCAAT3'
(SEQ ID NO: 17)
(TlucC(mut)-2) 5'CCGCGGGCCCACACCGCCGGCCTTCACCAA3'
(SEQ ID NO: 18).

[0034] After confirming the sequence of these cDNAs, TlucN, GlucN and FlucN cDNA each was digested with HindIII and BamH1, and ligated with the pcDNA4/V5-His vector digested with the same enzymes to construct pTlucN, pGlucN, and pFlucN vector, respectively. Then, FKBP cDNA was digested with BamH1 and Xho1, and ligated with the pTlucN, pGlucN or pFlucN vector digested with the same enzymes to complete the construct of pTlucN-FKBP, pGlucN-FKBP and pFlucN-FKBP, respectively.

[0035] Similarly, TlucC and TlucC(mut) cDNA was digested with Xho1 and Apa1, and ligated with the pcDNA4/V5-His vector digested with the same enzymes to construct pTlucC and pTlucC(mut) vectors, respectively. Then, FRB cDNA was digested with BamH1 and Xho1 and ligated with the pTlucC and pTlucC(mut) vector digested with the same enzymes to complete the construct of pFRB-TlucC and pFRB-TlucC(mut), respectively.

[0036] pTK-Rluc(Promega), which expresses Renilla luciferase (Rluc), was used as internal control to normalize the transfection efficiency.

[0037] The COS-7 cells were plated on the 12-well plates and transiently transfected with pTlucN-FKBP, pFRB–TlucC(mut)and pTK-Rluc; pGlucN-FKBP, pFRB–TlucC(mut) and pTK-Rluc; and pFlucN-FKBP, pFRB–TlucC(mut)and pTK-Rluc, respectively using LipofectAMINE 2000 (GibcoBRL). As control experiments for each, pFRB–TlucC was used instead of pFRB–TlucC(mut). At 12-16 hours after transfection, the cells were stimulated by rapamycin (final conc. 100nM) for 30 min and then washed once with PBS. The cells as a negative control experiment were not stimulated by rapamycin but were subject to all other experimental procedures.

[0038] An 80 mL of specific substrate solution from the Dual-Luciferase assay kit (Promega) was added to each well of the plates. After 3 min incubation at 37 ^{o}C, the luminescence intensities from the cell lysates were recorded for 30 sec with the luminometer (Lumat LB9507). Wavelength for the measurement was set to 600 nm for

TlucN, 530 nm for GlucN, and 560 nm for FlucN (see results in Example 4 regarding these wavelengths). After measuring the activity (L_T) of the click beetle luciferase or the firefly luciferase, the specific substrate of Renilla luciferase was added and incubated for 3 min and its activity (L_R) was measured for 30 sec. The value of luciferase activity obtained by complementation of either TlucN, GlucN, or FlucN and TlucC(mut) was normalized by the value obtained by the Renilla luciferase was termed as "RLU ratio (Dual)"; i.e.(L_T)/ (L_R).

[0039] As shown in Fig.2, using the pTlucN-FKBP and pFRB–TlucC(mut) the signal obtained in the presence of rapamycin was 13-fold, compared with the control without rapamycin. By using the pGlucN-FKBP and pFRB–TlucC(mut) the signal obtained in the presence of rapamycin was ~20-fold, compared with the control without rapamycin. By using the pFlucN-FKBP and pFRB–TlucC(mut) the signal obtained in the presence of rapamycin was ~3800-fold, compared with the control without rapamycin. This example indicates that while TlucC(wt) scarcely binds to either GlucN or FlucN but TlucN, TlucC(mut) can serve as an excellent complementation partner of TlucN, GlucN or FlucN to evaluate protein-protein interactions.

[0040] Example 2

Interaction between BAD and 14-3-3

[0041] In this example, the interaction between the BAD protein or BAD derivatives and the 14-3-3 protein was examined using the complementation pair, GlucN and TlucC(mut) (Fig. 3).

[0042] To construct the pBAD-TlucC(mut) and pGlucN–14-3-3, the cDNAs of BAD and 14-3-3 were generated by standard polymerase chain reaction to add the Kozak sequence and restriction sites shown in Fig.3.

(BAD-1) 5'GGATCCGCCACCATGGGAACCCAAAG3' (SEQ ID NO: 19)

(BAD-2) 5'GAATCCCCCTGGGAGGGGGTGGAGCCTC3' (SEQ ID NO: 20)

(14-3-3-1) 5'GGATCCGCCACCATGGATAAAAATGAG3' (SEQ ID NO: 21)

(14-3-3-2) 5'GAATTCCCATTTTCCCCTCCTTCTCCTGC3' (SEQ ID NO: 22)

[0043] After confirming the sequence of these cDNAs, BAD cDNA was digested with BamH1 and EcoR1, and ligated with the pTlucC(mut) vector digested with the same enzymes to complete the BAD-TlucC(mut) construct. Similarly, 14-3-3 cDNA was digested with BamH1 and EcoR1, and ligated with the pGlucN digested with the same enzymes to complete the pGlucN–14-3-3 construct.

[0044] The COS-7 cells were plated on the 12-well plates and transiently transfected with p14-3-3–GlucN, pBAD–TlucC(mut), or pBCL-X_L–GlucN as described in Example 1. At 16-24 hours after transfection, the cells were lysed with the sample buffer (125 mM Tris pH6.8, 10% glycerol, 4% SDS, 0.006% Bromophenol blue, 1.8% beta-mercaptoethanol) and portions of the lysates were subject to Western

blotting using anti-V5 antibody [1 : 5000 in 1% skimmed milk in TBST (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20)] and alkaline phosphatase-labeled anti-mouse antibody (1 : 4000 in 1% skimmed milk in TBST). The protein expression was analyzed by an image analyzer (LAS-1000 plus, Fujifilm Co., Tokyo, Japan) using an ECL™ kit (Amersham Biosciences, UK) (Figure 4). The result confirmed expression from these constructs.

[0045] Next, the constructs having cDNAs of BAD(S112A), BAD(S136A), and BAD(S155A) were generated, in which a serine of the phosphorylation sites, S112, S136, or S155, was mutated to alanine (Fig. 3).

[0046] The cDNAs of BAD(S112A), BAD(S136A), BAD(S155A), and BAD(StrippleA) were generated by standard polymerase chain reaction to add the Kozak sequence and restriction sites. After confirming the sequence of these cDNAs, BAD(S112A), BAD(S136A), BAD(S155A) and BAD(StrippleA) were digested with BamH1 and EcoR1, and ligated with the pTlucC(mut) vector digested with the same enzymes to complete the pBAD(S112A)-TlucC(mut), pBAD(S136A)-TlucC(mut), pBAD(S155A)-TlucC(mut), and pBAD(StrippleA)-TlucC(mut) constructs, respectively.

[0047] The sequences of the oligonucleotide pairs used in PCR for generating BAD(S112A), BAD(S136A), and BAD(S155A) are the followings:

(BAD(S112A)-1) 5'GAGACT CGGAGTCGCCACAGTGCGTAC-CCAGCGGGGACCGAG3' (SEQ ID NO: 23)

(BAD(S112A)-2) 5'CTCGGTCCCCGCTGGGTACGCACTGTGGCGACTCCGAGTCTC3' (SEQ ID NO: 24)

(BAD(S136A)-1) 5'CGAGGACGCTCGCGTGCGGCTCCCCCCAATCTCTGGGCAGCG3' (SEQ ID NO: 25)

(BAD(S136A)-2) 5'CGCTGCCCAGAGATTGGGGGGAGCCGCACGCGAGCGTCCTCG3' (SEQ ID NO: 26)

(BAD(S155A)-1) 5'GAGCTCCGAAGGATGGCCGATGAGTTTGAGGGTTCCTTC3' (SEQ ID NO: 27)

(BAD(S155A)-2) 5'GAAGGAACCCTCAAACTCATCGGCCATCCTTCGGAGCTC3' (SEQ ID NO: 28)

[0048] The expression vectors, p14-3-3–GlucN and either one of pBAD–TlucC(mut), pBAD(S112A)–TlucC(mut),

pBAD(S136A)–TlucC(mut), pBAD(S155A)-TlucC(mut) or pBAD(StrippleA)-TlucC(mut) were cotransfected in COS-7 cells as described above. At 12-16 hours after transfection, cells were lysed and luminescence was monitored by luminometer. The result is shown in Fig. 5.

[0049] Using a wild-type pair of p14-3-3–GlucN and pBAD–TlucC(mut), a very strong luminescence intensity was observed, reflecting the fact that 14-3-3 and BAD proteins are in strong interaction endogenously. However, the luminescence intensity for a pair of 14-3-3 and BAD(S112A) or BAD(S136A)BAD was half of that for a wild-type pair. In the case of BAD(S155A), the intensity was reduced to one-fifth, and in the case of BAD(StrippleA) in which S136, S112, and S155 all were mutated to alanine, the interaction between BAD(StrippleA) and 14-3-3 was negligible.

[0050] Next, we confirmed that there was no phosphorylation of the 112nd, 136th, and 155th amino acids in BAD(S112A), BAD(S136A), BAD(S155A), respectively, while there were phophorylation of all of three amino acids in BAD(wt), in the COS-7 cells by Western blotting using antibodies against phospho-BAD(Ser112), phospho-BAD(Ser136) or phospho-BAD(Ser155). As shown in Figure 6, a signal was observed for BAD(wt)-TlucC(mut), while no signal appeared for BAD(S112A)-TlucC(mut), BAD(S136A)-TlucC(mut), and BAD(S155A)-TlucC(mut) despite the expression levels comparable to BAD (wt)-TlucC(mut). Identical results were obtained using another cell line, HeLa cells (data not shown).

[0051] Further, we examined the effects of the various compounds on BAD/14-3-3 interactions (Figure 7). Forskolin and IBMX are known to be phosphorylation stimulators, and DATS and oleic acid are known to be dephosphorylation agents. Experimentally, COS-7 cells were cultured and pBAD-TlucC(mut) and pGlucN-14-3-3 were introduced into the cells, as described above. The cells were then incubated in the media with Forskolin, IBMX, DATS or oleic acid (100 mM each) for 60 minutes before luminescence assay. As shown in Fig. 7, Forskolin and IBMX enhanced the luminescence signal, while DATS and oleic acid reduced it. This result indicates that the luminescence signal reflects the binding strength of the proteins.

[0052] Example 3

Interaction between BAD and BCL-X_L

[0053] In this example, BAD and BCL-X_L were used as the interacting proteins.

[0054] To construct the pGlucN–BCL-X_L, the cDNAs of BCL-X_L were generated by standard polymerase chain reaction to add the Kozak sequence and restriction sites. After confirming the sequence of the cDNA, BCL-X_L cDNA was digested with EcoR1 and Xho1, and ligated with the pGlucN vector to complete pGlucN-BCL-X_L construct.

[0055] The expression vectors, pBAD-TlucC(mut) and pGlucN–BCL-XL, were co-

transfected in COS-7 cells and their luminescence signals were measured as described in Example 1. A strong luminescence signal was observed in the absence of any ligand (Figure 8).

[0056] Next, we evaluated the abilities of the BCL-XL inhibitors, antimycin and HA 14-1. Experimentally, the cells containing BAD-TlucC(mut) and GlucN-BCL-X_L were incubated with antimycin or HA 14-1 (100 mM each) for 30-60 minutes before luminescence assay. As shown in Figure 8, both compounds significantly reduced the luminescence signal. It was thus shown again that luminescence signal by BAD-TlucC(mut) and GlucN-BCL-XL reflects their binding strength.

[0057] Example 4

Spectrum of the emitted light

[0058] In this example, the spectrum of the light emitted by combination of GlucN and Tluc(mut) is shown.

[0059] The cultured COS-7 cells were transfected with the pGlucN-FKBP and pFRB-TlucC(mut) and incubated at 37 ^{o}C for 24 hours. Cells were stimulated with rapamycin (1.0 mM) and again incubated for another 10-12 hours at 37 ^{o}C. The cultured medium was then removed and luciferin substrate solution was added. After 3-5 minutes, cells were lysed and the spectrum was obtained using fluorescence spectrophotometer. As shown in Fig. 9, the spectrum had a peak (lmax) near 530 nm. Similarly, the spectrum of the detected light by the combination of pFlucN-FKBP and pFRB-TlucC(mut) had a peak (lmax) near 560 nm, and the spectrum of the detected light by the combination of pTlucN-FKBP and pFRB-TlucC(mut) had a peak (lmax) near 600 nm.

[0060] Example 5

Detection of luminescence in subcutaneously located murine cells

[0061] In this example, the spectrum of the light emitted by combination of GlucN and Tluc(mut) is shown.

[0062] The expression vectors, p14-3-3–GlucN and either one of pBAD–TlucC(mut) or pBAD(StrippleA)–TlucC(mut) were introduced into mice as follows.

[0063] The constructs of p14-3-3-GlucN, pBAD-TlucC(mut), and pBAD(StrippleA)-TlucC(mut) were the same as those used in Example 2. pGluc(wt) was constructed as follows.

[0064] First, the cDNA of Gluc(wt) was generated by standard polymerase chain reaction to add restriction sites. After confirming the sequence of the cDNA, Gluc(wt) was digested with HindIII and Xho1 and inserted in the vector digested with the same enzymes to construct pGluc(wt). The sequences of the oligonucleotide pairs used in PCR for generating Gluc(wt)are the followings:

(Gluc(wt)-1) 5'AAGCTTATGGAGAGAGAGAAGAACGTGTACGGC 3'

(SEQ ID NO: 29)

(Gluc(wt)-2) 5'CTCGAGCGCAGCTTAGAAGCCTTCTCCATCAG 3'

(SEQ ID NO: 30)

[0065] COS-7 cells were cultured in four 10 cm dishes. Each dish was: (1) transfected with Gluc(wt)alone; (2) cotransfected with pBAD-TlucC(mut) and pGlucN-14-3-3-TlucN; (3) cotransfected with pBAD-TlucC(mut), pGlucN-14-3-3-TlucN and Gluc(wt); and (4) cotransfected with pBAD(StrippleA)-TlucC(mut), pGlucN-14-3-3-TlucN and Gluc(wt). The cells were harvested after incubated at 37 ^{o}C for 18-24 hours. The cells were suspended in phosphate buffer saline, and an aliquot of $1x10^{6}$ cells was implanted in anesthetized BALB/c nude mice (female, 4 weeks old, ~15g body weight) at positions indicated in Fig.1. Fifteen minutes after cells implantation, luciferin, 3mg dissolved in 100 mL of PBS, was injected i.p. Ten minutes after the injection, mice were imaged by using a CCD camera (Versarray: 1300B, Prinston Instruments).

[0066] As shown in Fig. 10, light with the wavelength of 530 nm was detected in cases (1), (2) and (3). In case (4), no light was detected since BAD(StrippleA) protein and 14-3-3 protein did not bind to each other and thus TlucC(mut) and TlucN did not bind to each other.

[0067] As shown above, when complexes of a protein having TlucC(mut) domain and proteins having each of anti-TlucC(mut) domains emit light in a living body, not only the lights can be detected from outside of the body but also each of the complexes can be distinguished by detecting the lights with wavelength specific to each of the complexes.

Industrial Applicability

[0068] The present invention provides a modified complementation strategy for detecting protein-protein interactions.

Claims

[1] A protein comprising a TlucC(mut) domain having amino acid SEQ ID NO: 1.

[2] A protein comprising a TlucC(mut) domain having amino acid SEQ ID NO: 1 which comprises one or a few of either substitution, deletion, or addition of an amino acid, the protein being capable of binding to either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4, to form a complex.

[3] A complex comprising the protein of either claim 1 or 2 and a protein comprising either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4.

[4] A method for detecting a protein comprising either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4, comprising:
allowing the protein to interact with the protein of either claim 1 or 2, the proteins being capable of binding to each other; and
detecting light emitted from the complex of the proteins.

[5] A method for detecting a protein of claim 4, comprising:
allowing a protein comprising either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4 to interact with the protein of either claim 1 or 2 to form a complex, the proteins being capable of binding to each other; and
detecting light emitted from the complex of the proteins.

[6] A method for detecting a complex of claim 5, comprising:
detecting the light emitted from the complex.

[7] A method for detecting binding of a first protein of either claim 1 or 2 and a second protein comprising either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4, comprising steps of:
allowing the first protein and the second protein to interact with each other to form a complex; and
detecting the light emitted from the complex.

[8] A method for detecting binding of a first protein and a second protein, the proteins being capable of binding to each other, comprising steps of:
fusing a TlucC(mut) domain having amino acid SEQ ID NO: 1 to the first protein;
fusing either a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain

having amino acid SEQ ID NO: 3, or a TlucN domain having amino acid SEQ ID NO: 4 to the second protein;

allowing the fused first protein and the fused second protein to interact with each other to form a complex; and

detecting the light emitted from the complex.

[9] A method for selecting a binding protein of a first protein between a second protein and a third protein, the first protein being fused to a TlucC(mut) domain having amino acid SEQ ID NO: 1, each of the second protein and the third protein being fused to a different domain selected from a group consisting of a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, and a TlucN domain having amino acid SEQ ID NO: 4, comprising:

allowing the first protein to interact with the second protein and the third protein; and

detecting the emitted light and determining which complex of the proteins emits the light.

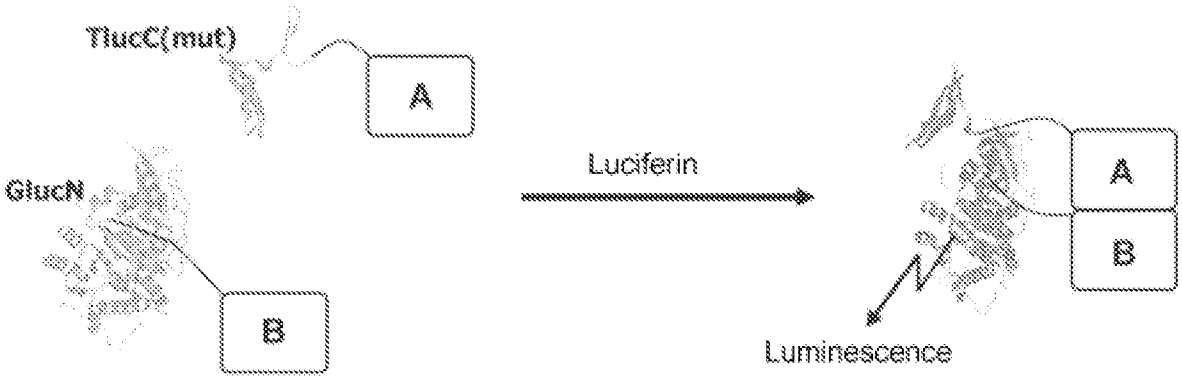
[10] The method of claim 8, further comprising:

fusing a TlucC(mut) domain having amino acid SEQ ID NO: 1 to the first protein; and

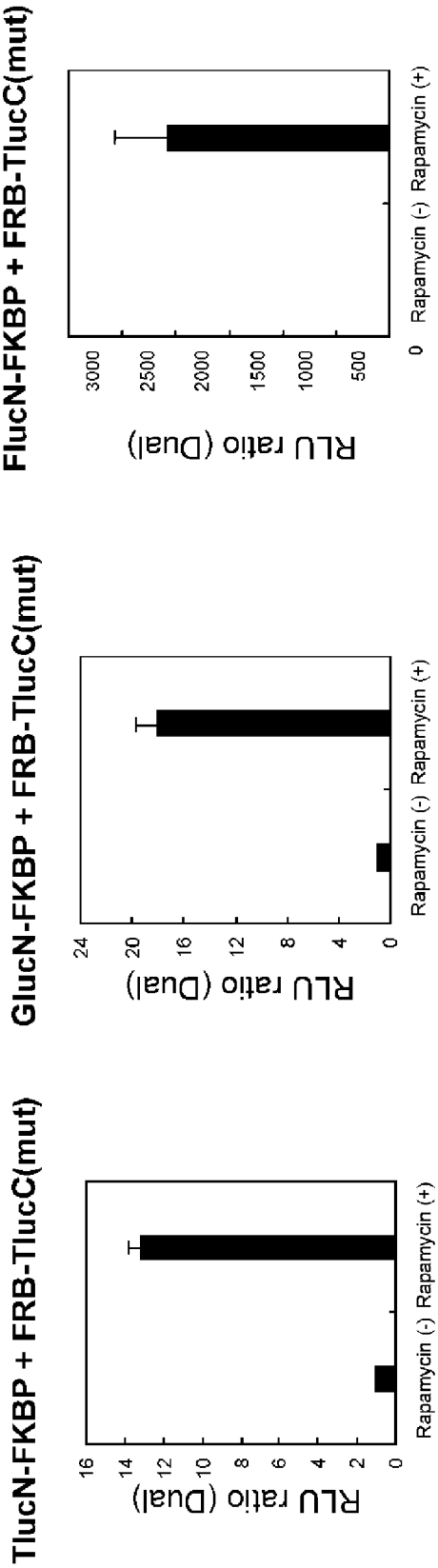
fusing each of two domains selected from a group consisting of a GlucN domain having amino acid SEQ ID NO: 2, a FlucN domain having amino acid SEQ ID NO: 3, a TlucN domain having amino acid SEQ ID NO: 4 to the second protein and the third protein.

[11] A vector comprising a sequence encoding a TlucC(mut) domain having amino acid SEQ ID NO: 1.

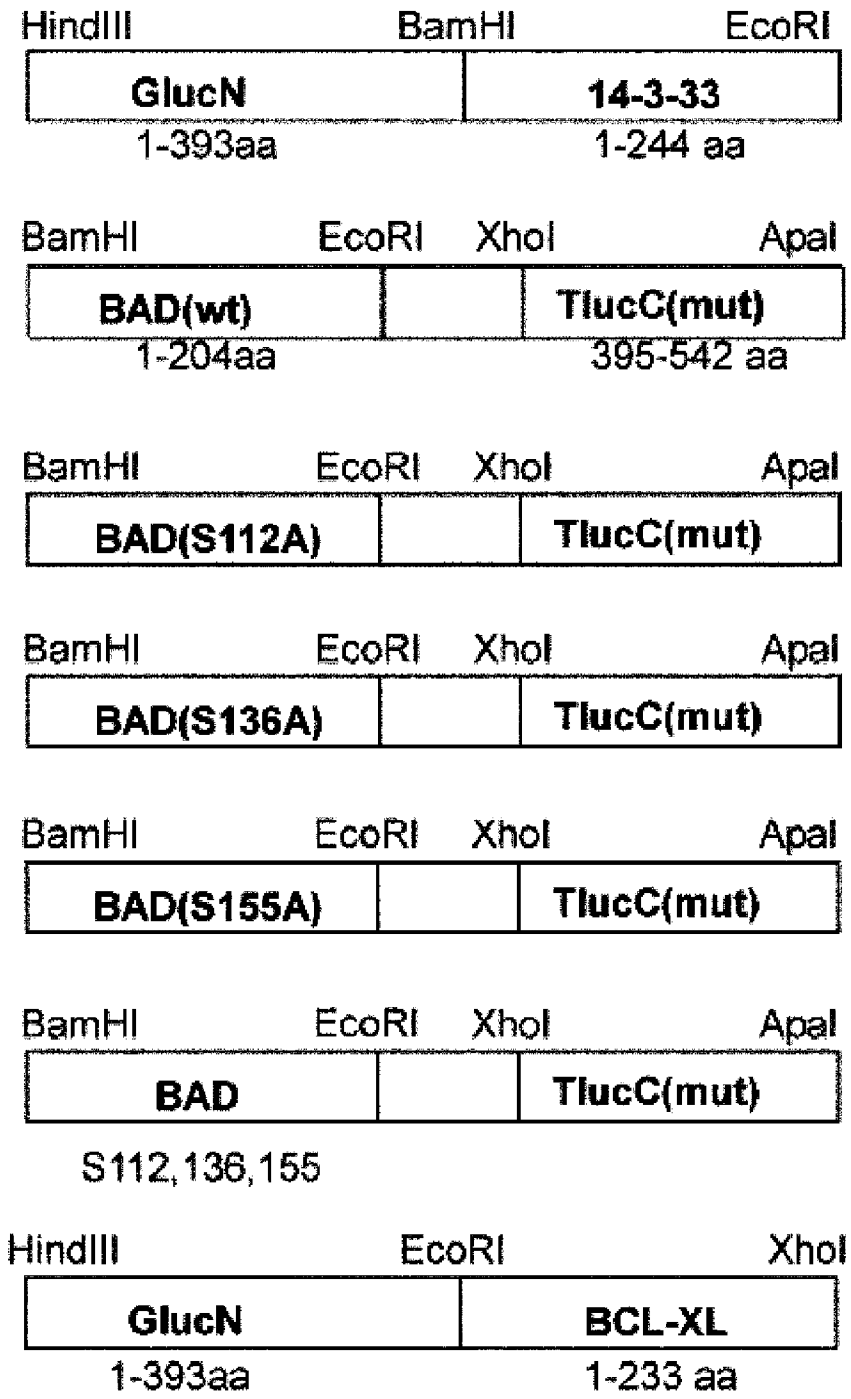
[Fig. 1]



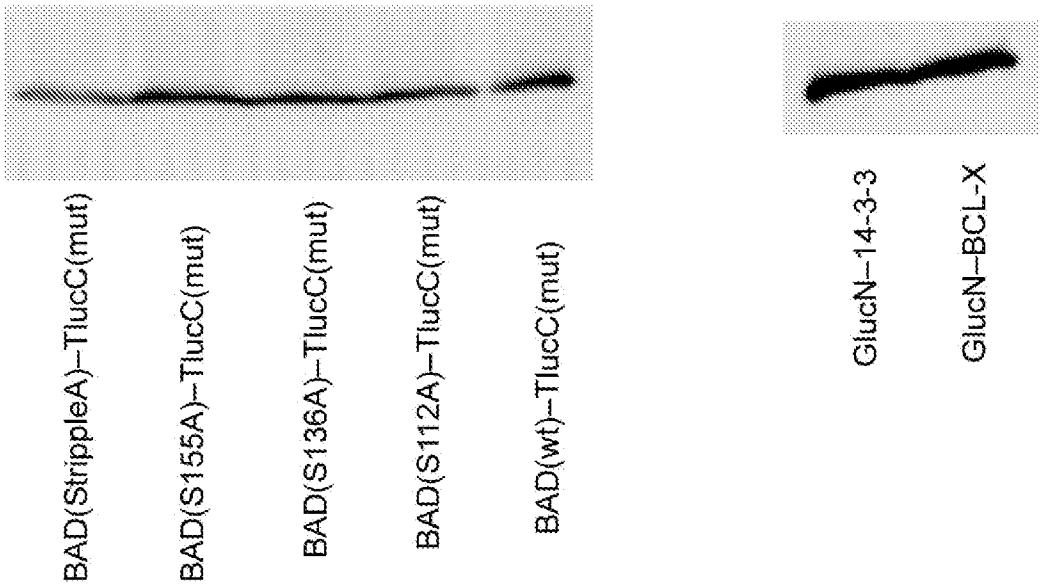
[Fig. 2]


TlucN-FKBP + FRB-TlucC(mut)
GlucN-FKBP + FRB-TlucC(mut)
FlucN-FKBP + FRB-TlucC(mut)
RLU ratio (Dual)
0
2
4
6
8
10
12
14
16
20
24
500
1000
1500
2000
2500
3000
Rapamycin (-)
Rapamycin (+)

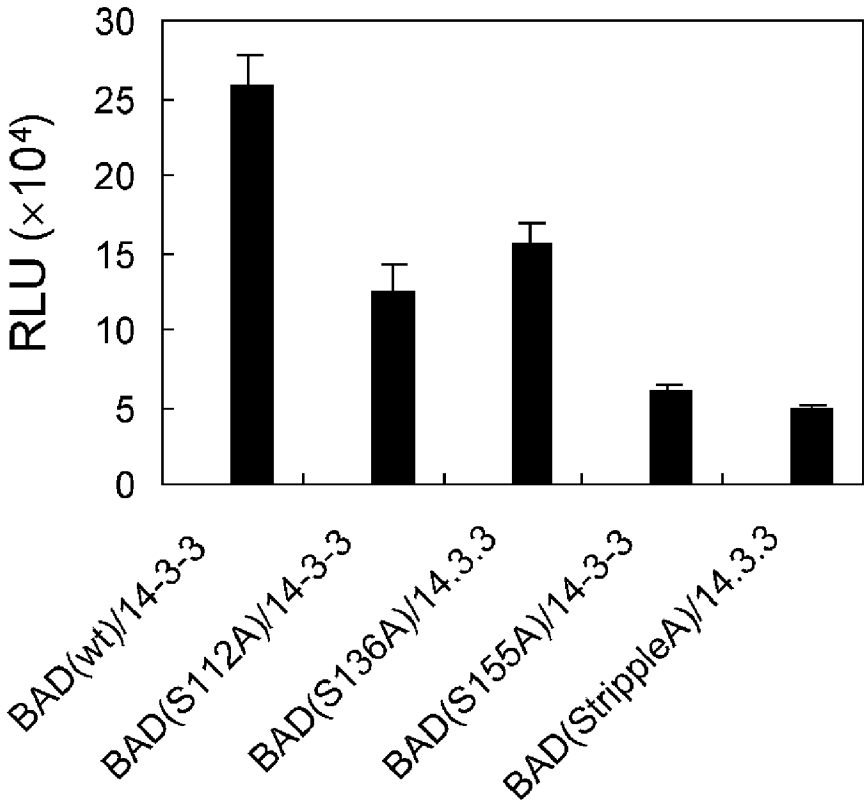
[Fig. 3]


HindIII
BamHI
EcoRI
GlucN
14-3-33
1-393aa
1-244 aa
BamHI
EcoRI
XhoI
ApaI
BAD(wt)
TlucC(mut)
1-204aa
395-542 aa
BamHI
EcoRI
XhoI
ApaI
BAD(S112A)
TlucC(mut)
BamHI
EcoRI
XhoI
ApaI
BAD(S136A)
TlucC(mut)
BamHI
EcoRI
XhoI
ApaI
BAD(S155A)
TlucC(mut)
BamHI
EcoRI
XhoI
ApaI
BAD
TlucC(mut)
S112,136,155
HindIII
EcoRI
XhoI
GlucN
BCL-XL
1-393aa
1-233 aa

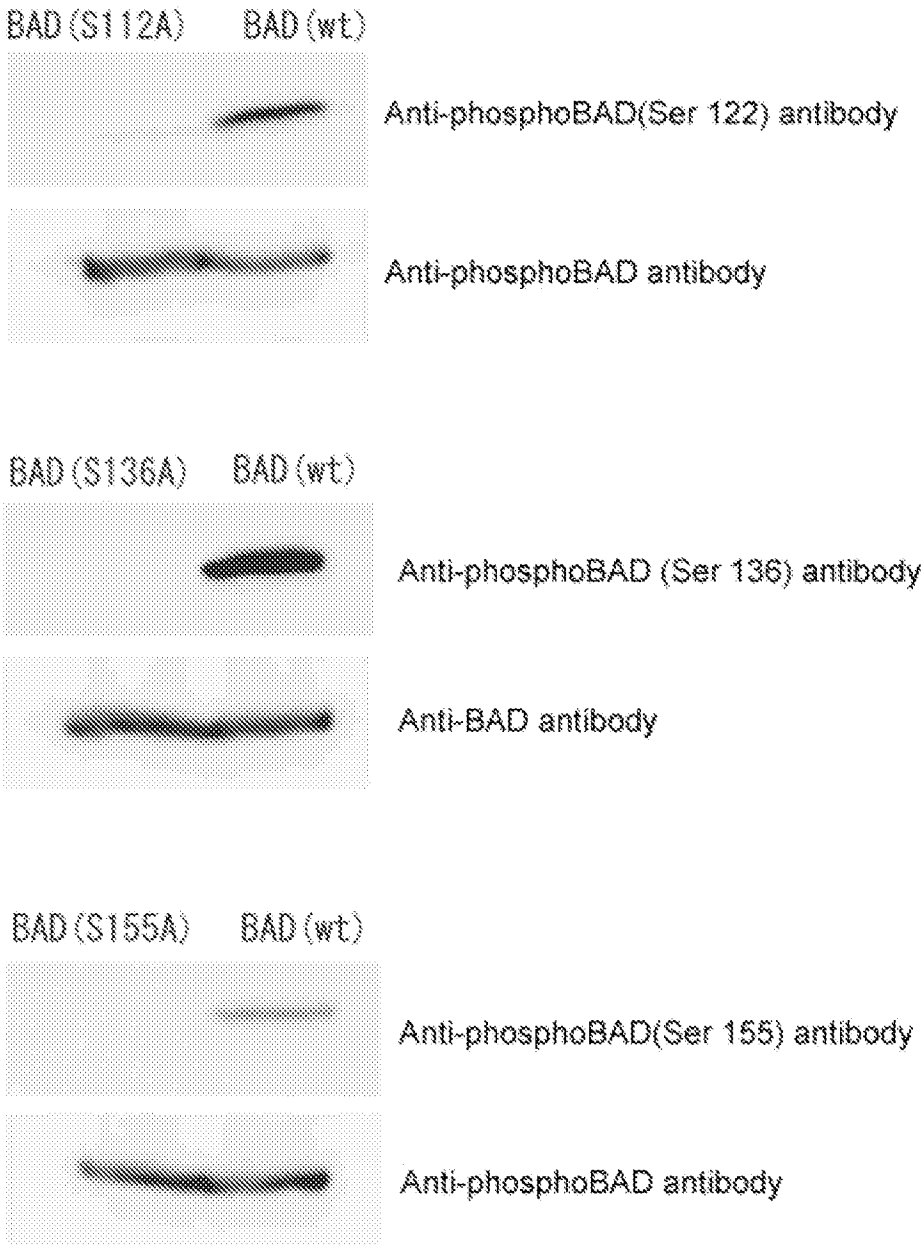
[Fig. 4]


BAD(StrippleA)–TlucC(mut)
BAD(S155A)–TlucC(mut)
BAD(S136A)–TlucC(mut)
BAD(S112A)–TlucC(mut)
BAD(wt)–TlucC(mut)
GlucN–14-3-3
GlucN–BCL-X

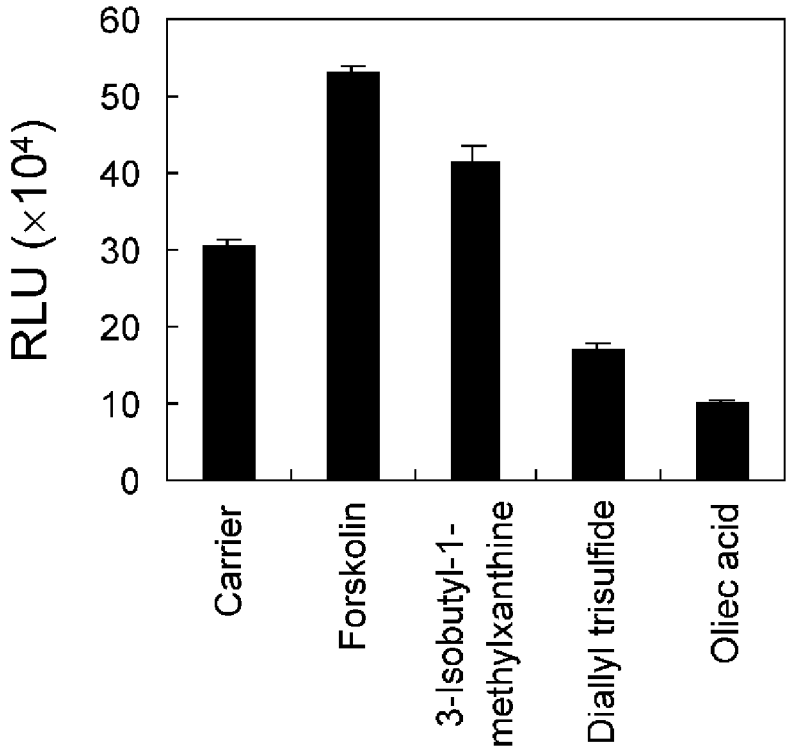
[Fig. 5]


RLU (×10⁴)
30
25
20
15
10
5
0
BAD(wt)/14-3-3
BAD(S112A)/14-3-3
BAD(S136A)/14.3.3
BAD(S155A)/14-3-3
BAD(StrippleA)/14.3.3

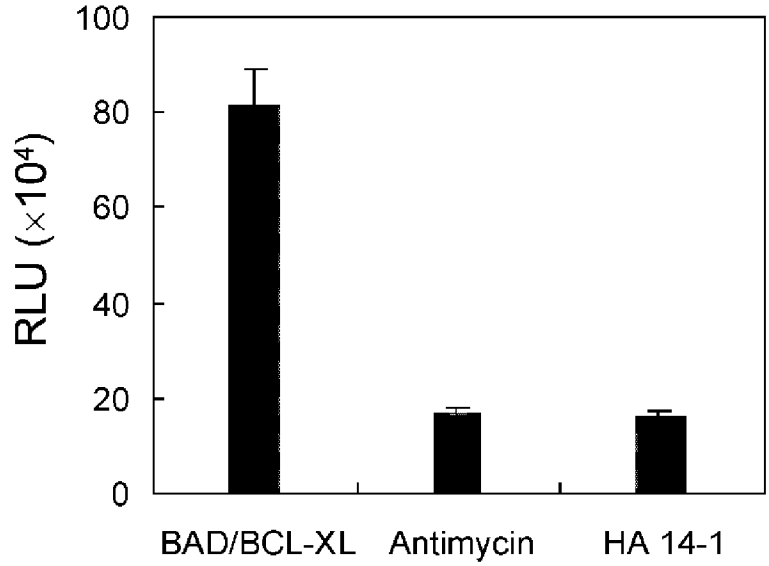
[Fig. 6]


BAD (S112A)
BAD (wt)
Anti-phosphoBAD(Ser 122) antibody
Anti-phosphoBAD antibody
BAD (S136A)
BAD (wt)
Anti-phosphoBAD (Ser 136) antibody
Anti-BAD antibody
BAD (S155A)
BAD (wt)
Anti-phosphoBAD(Ser 155) antibody
Anti-phosphoBAD antibody

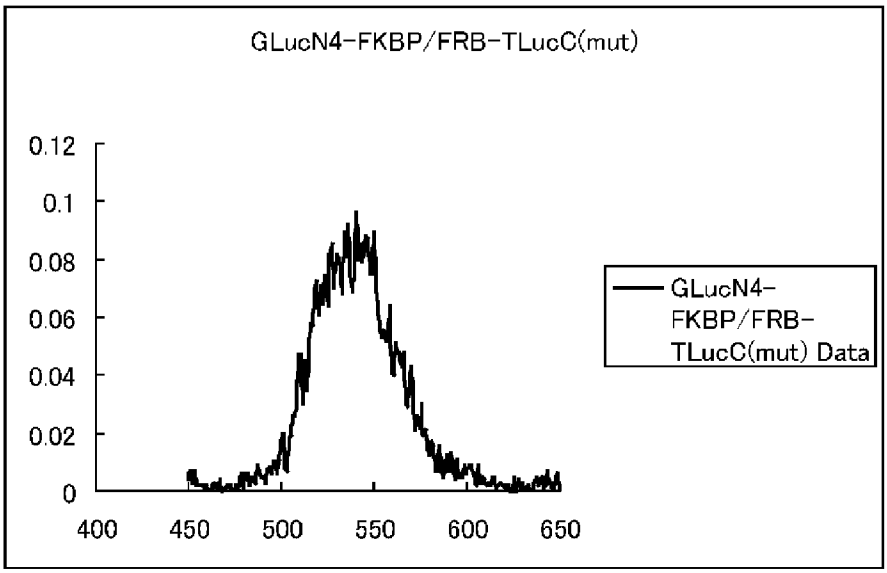
[Fig. 7]

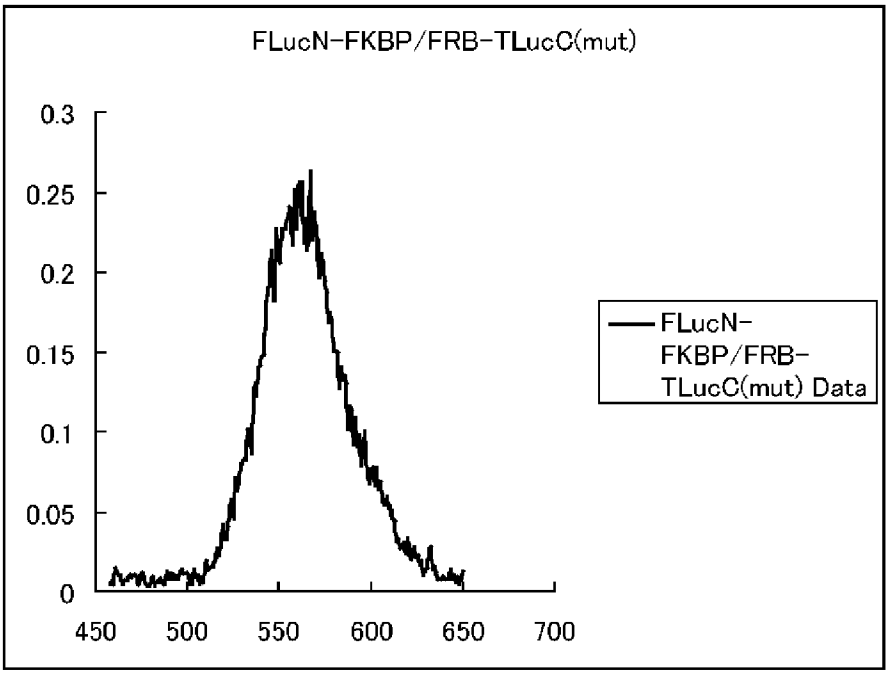

60
50
40
30
20
10
0
RLU (×10⁴)
Carrier
Forskolin
3-Isobutyl-1-methylxanthine
Diallyl trisulfide
Oliec acid

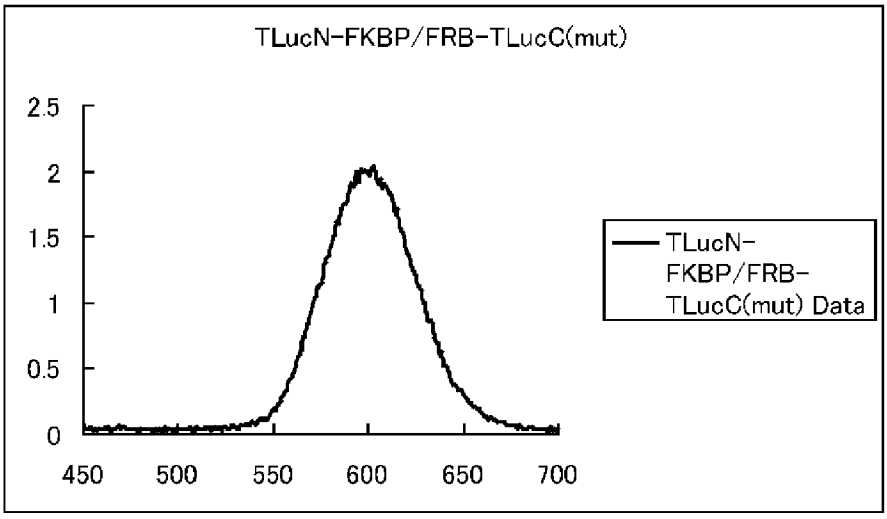
[Fig. 8]


100
80
60
40
20
0
RLU (×10⁴)
BAD/BCL-XL
Antimycin
HA 14-1

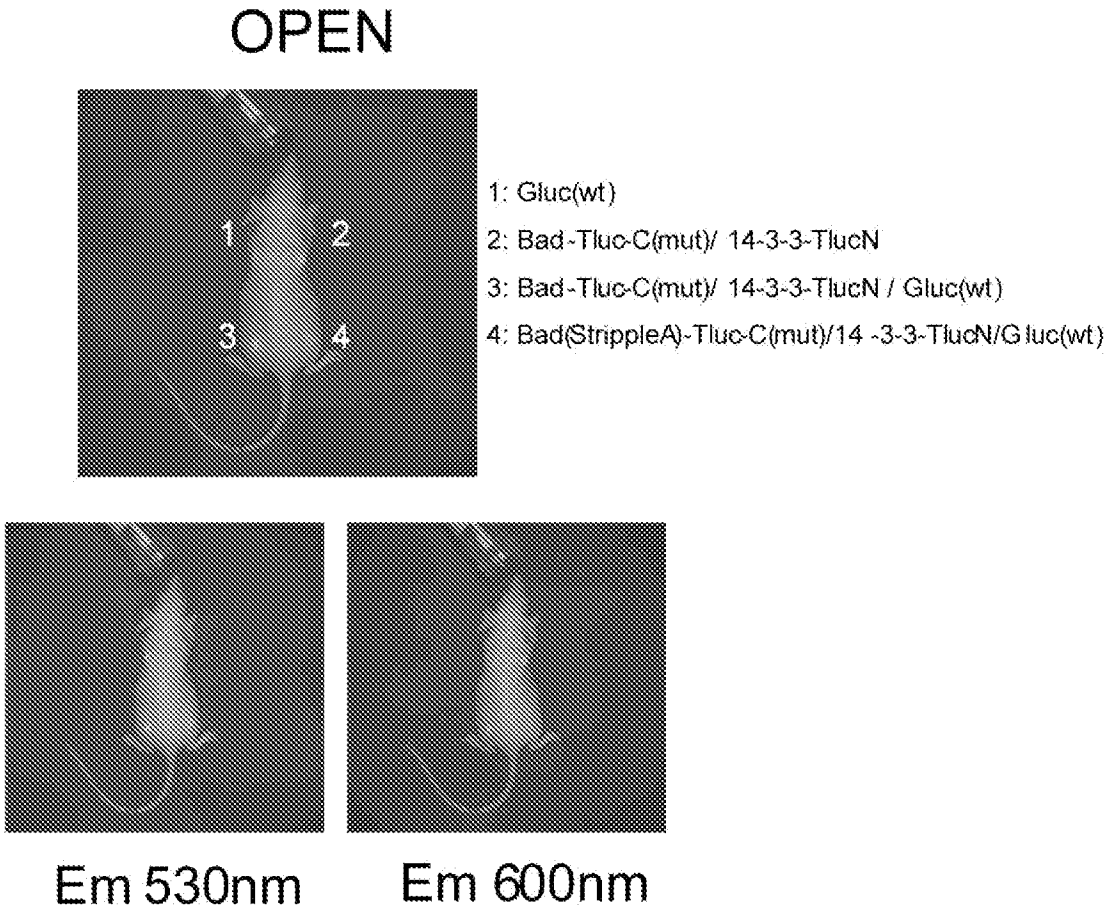
[Fig. 9]


GLucN4-FKBP/FRB-TLucC(mut)
0.12
0.1
0.08
0.06
0.04
0.02
0
400
450
500
550
600
650
GLucN4-FKBP/FRB-TLucC(mut) Data


FLucN-FKBP/FRB-TLucC(mut)
0.3
0.25
0.2
0.15
0.1
0.05
0
450
500
550
600
650
700
FLucN-FKBP/FRB-TLucC(mut) Data


TLucN-FKBP/FRB-TLucC(mut)
2.5
2
1.5
1
0.5
0
450
500
550
600
650
700
TLucN-FKBP/FRB-TLucC(mut) Data

[Fig. 10]


OPEN
1
2
3
4
1: Gluc(wt)
2: Bad-Tluc-C(mut)/ 14-3-3-TlucN
3: Bad-Tluc-C(mut)/ 14-3-3-TlucN / Gluc(wt)
4: Bad(StrippleA)-Tluc-C(mut)/14 -3-3-TlucN/Gluc(wt)
Em 530nm
Em 600nm

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/000033

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N15/09(2006.01)i, C12N9/02(2006.01)i, C12Q1/66(2006.01)i, G01N21/76(2006.01)i, G01N33/53(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N15/00-15/90, C12N9/02, C12Q1/66

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2009
Registered utility model specifications of Japan 1996-2009
Published registered utility model applications of Japan 1994-2009

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS/MEDLINE/CAplus/EMBASE(STN), UniProt/GeneSeq, JSTPlus/JMEDPlus/JST7580(JDreamII), G-search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WO 2007/027919 A2 (WASHINGTON UNIVERSITY IN ST.LOUIS) 2007.03.08 & US 2008/0233589 A1	2-7 1,8-11
Y A	WO 2002/016944 A2 (PROMEGA CORPORATION) 2002.02.28 & AU 200185278 A & EP 1341808 A2 & JP 2004-520807 A & US 2006/0127988 A1 & JP 2007-006910 A & US 2008/0090291 A1	2-7 1,8-11
A	Ozawa,T., Hikari probe no atarashii design to seitaikinou no kashika. Kagaku Kogyo(Nov.2007)Vol.58,No.11,p.860-864	1-11

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02.03.2009	10.03.2009

Name and mailing address of the ISA/JP	Authorized officer
Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	Kenji MIHARA 4N 2937 Telephone No. +81-3-3581-1101 Ext. 3488