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(54) Title: METHOD OF MEASURING PROTEIN–PROTEIN INTERACTIONS IN LIVING CELLS

(57) Abstract

A method of detecting protein–protein interactions in a living cell comprises (*a*) providing a cell that contains a first heterologous conjugate and a second heterologous conjugate, wherein the first heterologous conjugate comprises a first protein of interest conjugated to a detectable group, and wherein the second heterologous conjugate comprises a second protein of interest conjugated to a protein that specifically binds to an internal structure within the cell, and then (*b*) detecting the presence or absence of binding of the detectable group to the internal structure, the presence of the binding indicating that the first and second proteins of interest specifically bind to one another. Additional aspects of the invention include nucleic acids encoding fusion proteins as described above, cells containing and expressing such fusion proteins, kits useful for carrying out the methods described above, and nucleic acid libraries useful as screening tools for carrying out the methods described above. The invention is usefulfor screening compounds for the ability to disrupt or inhibit the binding of known binding pairs and thereby identifying competitive inhibitors thereof. The invention is useful for screening one known protein of interest against a library of other proteins of interest to identify compounds that bind to the known protein of interest.

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METHOD OF MEASURING PROTEIN-PROTEIN INTERACTIONS IN LIVING CELLS

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Field of the Invention

The present invention concerns methods of detecting or determining binding between two different proteins, or oligomer formation by the same protein, in living cells, along with cells and kits useful for carrying out such methods.

Background of the Invention

The binding of proteins to one another, or the disruption of binding of one protein to another by a competitive inhibitor, is typically measured *in vitro*. Such binding assays are typically used as a model for *in vivo*, including intracellular, binding events. While such techniques are well established, the *in vitro* binding conditions do not control for the vast number of variables introduced when a binding event occurs within a cell. Because of the importance of screening for new binding partners, or inhibitors of known binding partners, to the development of new therapeutic molecules, the development of techniques that measure binding within a cell is extremely important.

The "pull down" assay is known, in which the binding of a pair of proteins of interest is determined by forming a co-precipitate with an antibody *in vitro* and then centrifuging down, or "pulling down" the aggregate so formed. Disadvantages of this technique are that it is carried out *in vitro*, and that substantial nonspecific binding occurs.

The yeast "two hybrid" technique employs a pair of transcription factors that trigger the transcription of a selectable or detectable protein. The technique has been

adapted and extended to a number of situations, including examination of enzyme-substrate interactions. R. Sikorski and R. Peters, *Science* **281,** 1822-1823 (18 Sept. 1998). In general, a first hybrid is formed of a first protein of interest and one of the transcription factors; a second hybrid is formed of a second protein of interest and another of the transcription factors. If the two proteins of interest associate, then the two transcription factors associate and transcription of the detectable or selectable protein is initiated. Advantages of this technique are that binding occurs in a cell, and it can be readily adapted to the screening of combinatorial libraries by inserting various members of the library in different "two hybrid" cells and expressing the library transcription products therein. Disadvantages of this technique are that it is limited to the use of transcription factors, the binding reaction must be in a narrow range, is typically carried out in yeast, and the binding event must occur in the cell nucleus.

Accordingly, there is a need for new methods of screening for protein-protein interactions that can be carried out in living cells, that can be carried out in a variety of locations within a living cell, and that are readily adapted to the screening of combinatorial libraries.

Summary of the Invention

A first aspect of the present invention is a method of detecting a protein-protein interaction in a living cell. The method comprises *(a)* providing a cell that contains a first heterologous conjugate and a second heterologous conjugate, wherein the first heterologous conjugate comprises a first protein of interest conjugated to a detectable group, and wherein the second heterologous conjugate comprises a second protein of interest conjugated to a protein that specifically binds to an internal structure within the cell, and then *(b)* detecting the presence or absence of binding of the detectable group to the internal structure, the presence of the binding indicating that the first and second proteins of interest specifically bind to one another.

The proteins of interest may be the same or different; the proteins of interest may be members of a specific binding pair. Preferably, the detectable group is a protein, and the first protein of interest and the detectable group together comprise a fusion protein. Preferably, the second heterologous conjugate is also a fusion protein. If desired, the cell may contain and express a nucleic acid encoding either, or both,

fusion proteins, or the heterologous constructs may be administered exogenously to the cells. The cell is preferably a eukaryotic cell.

Additional aspects of the present invention include nucleic acids encoding fusion proteins as described above, cells containing and expressing such fusion proteins, kits useful for carrying out the methods described above, and nucleic acid libraries useful as screening tools for carrying out the methods described above.

The invention is useful for screening compounds for the ability to disrupt or inhibit the binding of known binding pairs and thereby identifying competitive inhibitors thereof. The invention is useful for screening one known protein of interest against a library of other proteins of interest to identify compounds that bind to the known protein of interest.

The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification below.

Brief Description of the Drawings

Figure 1. Cellular localization and mobility of GFP-tagged CaMKIIa and CaMKIIb

Figure 1A. Schematic representation of the domain organization of the GFP-tagged CaMKII isoforms. The catalytic-domain (C), regulatory-domain (R), variable-domain (V) and oligomerization-domain (A) are shown.

Figure 1B. Autophosphorylation of GFP-tagged CaMKII isoforms. Comparison of the baseline (left), calcium/CaM-dependent (middle) and burst (right) autophosphorylation activity of CaMKIIa, GFP-CaMKIIa and GFP-CaMKIIb. The kinase activity of the in vitro translated constructs are shown. Translated GFP alone was included as a control.

Figure 1C. Relative kinase activity corrected for the amount of expressed CaMKII or GFP-CaMKII protein (measured as the ratio of ^{32}P incorporation and ^{35}S-Met incorporation). The dark bars show CaMKII autophosphorylation after incubation with ^{32}P-ATP in high Ca^{2+}/CaM for 30 seconds. The light bars show the calcium independent "burst" autophosphorylation after 30 s in high calcium and 120 seconds in EGTA.

Figure 1D. Confocal image of GFP-tagged CaMKIIa (left) and CaMKIIb (right) expressed in living hippocampal CA1-CA3 neurons. Notice the homogenous distribution of GFP-CaMKIIa throughout the soma and major branches, while CaMKIIb is enriched in the dendritic branches.

Figure 1E. Confocal image of GFP-tagged CaMKIIa (left) and CaMKIIb (right) expressed in living RBL-cells. Notice the cortical staining and non-uniform internal staining of GFP-CaMKIIb and the homogenous distribution of GFP-CaMKIIa.

Figure 1F. Comparison of the calculated diffusion coefficients for GFP-CaMKIIa and GFP-CaMKIIb.

Figure 2. Development of a "Pull-Out" binding assay to study protein-protein interactions in living cells.

Figure 2A. Principle of the Pull-out binding assay. The binding interaction between a Protein X and Protein Y can be measured by tagging Protein X with an inducible plasma membrane binding domain (PM-domain) and Protein Y with GFP. If a significant fraction of the two proteins bind to each other, drug addition targets the GFP to the plasma membrane. In contrast, the cytosolic distribution remains unaltered if Protein X and Y do not bind to each other.

Figure 2B. Property of a minimal phorbol ester binding domain used as an inducible PM-domain in the Pull-Out binding assay. A fusion protein between GFP and the phorbol ester binding domain can be pulled from the cytosol to the plasma membrane by addition of phorbol ester. Left, distribution of the fusion protein before phorbol ester addition. Right, distribution of the fusion protein after phorbol ester addition. Bottom, line scans of the fluorescence intensity across the cell before and after phorbol ester addition.

Figure 2C. Demonstration that nearly all CaMKIIa molecules are part of oligomers. Phorbol ester addition to cells with co-expressed PM-CaMKIIa and GFP-CaMKIIa leads to the near complete plasma membrane translocation of GFP-CaMKIIa.

Figure 2D. Control measurements showing that expressed GFP itself is not affected by phorbol ester addition. Calibration bars are 10μm.

Figure 3. CaMKIIa forms larger oligomers than CaMKIIb.

Figure 3A. Schematic representation of the assay to measure the size of CaMKIIa and CaMKIIb oligomers in living cells.

Figure 3B. Quantitative comparison of the concentrations of expressed GFP-CaMKIIa and PM-CaMKIIa measured by in vitro translation. The relative concentration of the expressed proteins was compared by ^{35}S-Met incorporation into in vitro translated proteins. The same RNA was used for the in vitro translation and the RNA transfection of cells.

Figure 3C. Only for dilutions below 10% does PM-CaMKIIa begin to loose its potency to translocate GFP-CaMKIIa to the plasma membrane. Dilutions were achieved by mixing the RNA's for PM-CaMKIIa and GFP-CaMKIIa at decreasing ratios. Top and middle, plasma membrane translocation is still near maximal at dilutions of 1:1 and 1:5. Bottom, plasma membrane translocation is markedly reduced at a 1:40 dilution. Calibration bars are 10μm.

Figure 3D. Line scan profiles of three different dilution after phorbol ester addition.

Figure 3E. Schematic representations of the quantitative analysis used to measure the relative plasma membrane translocation. I_{pre} and DPM are measured before and after PMA addition, respectively.

Figure 3F. Plot of the relative plasma membrane translocation of CaMKIIa and CaMKIIb at decreasing ratios of expressed PM-CaMKII and GFP-CaMKII. Each point is an average of at least 10 experiments. The solid curves are best fits to the two set of data and the dashed lines show the confidence interval. Best fits were obtained assuming an average of 13.5 subunits for CaMKIIa and 4.2 subunits for CaMKIIb.

Figure 4. Requirement for more than one CaMKIIb subunits for targeting CaMKIIa/b hetero-oligomers to the actin cytoskeleton.

Figure 4A. Insertion of CaMKIIb into hetero-oligomers of mostly CaMKIIa is a stochastic process. Plot of the relative plasma membrane translocation of GFP-CaMKIIa at decreasing ratios of expressed PM-CaMKIIb. Each point is an average of at least 10 experiments. The dashed line shows the predicted curve for a stochastic insertion of PM-CaMKIIb into hetero-oligomers ($p = 1 - (R / (R+1))^N$ with R as the dilution ratio and N as the number of subunits; since the PM-domain induces a near

irreversible membrane binding interaction, it was assumed that one PM-domain per oligomer is sufficient for inducing translocation).

Figure 4B. The insertion of CaMKIIa into hetero-oligomers with mostly CaMKIIb is a stochastic process. Plot of the relative plasma membrane translocation of GFP-CaMKIIa at decreasing ratios of expressed PM-CaMKIIb. Each point is an average of at least 10 experiments. The predicted curve for a stochastic insertion of PM-CaMKIIa into CaMKIIb oligomers overlaps with the fit of the data.

Figure 4C. Relative cortical localization of GFP-CaMKIIa plotted as a function of increasing dilutions of CaMKIIb. Relative cortical localization is defined as D_{PM}/I_{av}, with D_{PM} as the intensity difference between the PM and the cytosol and I_{av} as the average fluorescence intensity of a particular cell. Each point is an average of at least 10 experiments.

Figure 4D. Measurement of the change in the diffusion coefficient as a function of an increasing dilution of CaMKIIb to GFP-CaMKIIa. The apparent diffusion coefficient of GFP-CaMKIIa increased from 0.2 to 1 mm^2/s as the ratio of CaMKIIb to CaMKIIa was lowered from 1:2 to 1:9. The outermost left and right data points show the diffusion coefficients of GFP-CaMKIIb and CaMKIIa, respectively.

Detailed Description of Preferred Embodiments

"Detectable groups" or "detectable proteins" used to carry out the present invention include fluorescent proteins, such as green fluorescent protein (GFP) and apoaequorin, including analogs and derivatives thereof. Green fluorescent protein is obtained from the jellyfish *Aequorea victoria* and has been expressed in a wide variety of microbial, plant, insect and mammalian cells. A. Crameri et al., *Nature Biotech.* **14,** 315-319 (1996). Any detectable group may be employed, and other suitable detectable groups include other fluorophores or fluorescent indicators, such as a fusion tag with any binding domain such as avidin, streptavidin and ligand binding domains of receptors. Coupling of biotin or other ligands to the fluorophore or indicator of interest may be achieved using a dextran matrix or other linker system. The detectable protein may be one which specifically binds a fluorophore, as in FLASH technology. Fluorescent detectable groups (including both fluorescent

proteins and proteins that bind a separate fluorophore molecule thereto) are currently preferred.

"Internal structure" as used herein refers to a separate, discreet, identifiable component contained within a cell. The term "structure" as applied to the constituent parts of a cell is known (*see, e.g.,* R. Dyson, Cell Biology: A Molecular Approach, pg, 10 (2d ed. 1978)), and the term "internal structure" is intended to exclude external structures such as flagella and pili. Such internal structures are, in general, anatomical structures of the cell in which they are contained. Examples of internal structures include both structure located in the cytosol or cytoplasm outside of the nucleus (also called "cytoplasmic structures"), and structures located within the nucleus (also called "nuclear structures"). The nucleus itself including the nuclear membrane are internal structures. Structures located within the cytoplasm outside of the nucleus are currently preferred. Thus the term "internal structure" is specifically intended to include any non-uniformly distributed cellular component, including proteins, lipids, carbohydrates, nucleic acids, etc., and derivatives thereof.

"Library" as used herein refers to a collection of different compounds, typically organic compounds, assembled or gathered together in a form that they can be used together, either simultaneously or serially. The compounds may be small organic compounds or biopolymers, including proteins and peptides. The compounds may be encoded and produced by nucleic acids as intermediates, with the collection of nucleic acids also being referred to as a library. Where a nucleic acid library is used, it may be a random or partially random library, commonly known as a "combinatorial library" or "combinatorial chemistry library", or it may be a library obtained from a particular cell or organism, such as a genomic library or a cDNA library. Small organic molecules can be produced by combinatorial chemistry techniques as well. Thus in general, such libraries comprise are organic compounds, including but not limited oligomers, non-oligomers, or combinations thereof. Non-oligomers include a wide variety of organic molecules, such as heterocyclics, aromatics, alicyclics, aliphatics and combinations thereof, comprising steroids, antibiotics, enzyme inhibitors, ligands, hormones, drugs, alkaloids, opioids, benzodiazepenes, terpenes, prophyrins, toxins, catalysts, as well as combinations thereof. Oligomers include peptides (that is, oligopeptides) and proteins, oligonucleotides (the term oligonucleotide also referred to simply as "nucleotide, herein) such as DNA and

RNA, oligosaccharides, polylipids, polyesters, polyamides, polyurethanes, polyureas, polyethers, poly (phosphorus derivatives) such as phosphates, phosphonates, phosphoramides, phosphonamides, phosphites, phosphinamides, etc., poly (sulfur derivatives) such as sulfones, sulfonates, sulfites, sulfonamides, sulfenamides, etc., where for the phosphorous and sulfur derivatives the indicated heteroatom for the most part will be bonded to C, H, N, O or S, and combinations thereof. *See, e.g.,* U.S. Patent No. 5,565,324 to Still et al., U.S. Patent No. 5,284,514 to Ellman et al., U.S. Patent No. 5,445,934 to Fodor et al. (the disclosures of all United States patents cited herein are to be incorporated herein by reference in their entirety).

"Nucleic acid" as used herein refers to both DNA and RNA.

"Protein" as used herein is intended to include protein fragments, or peptides. Thus the term "protein" is used synonymously with the phrase "protein or fragment thereof" (for the purpose of brevity), particularly with reference to proteins that are "proteins of interest" or members of a specific binding pair. Protein fragments may or may not assume a secondary or tertiary structure. Protein fragments may be of any length, from 2, 3, 5 or 10 peptides in length up to 50, 100, or 200 peptides in length or more, up to the full length of the corresponding protein.

"Specifically binds" and "specific binding" as used herein includes but is not limited to stereospecific binding, electrostatic binding, or hydrophlic binding interactions. Thus, specifically binds and specific binding are exhibited by at least a two or three fold (or two or three times), greater apparent binding affinity between the binding partners as compared to other proteins or binding partners within the cell in which binding is being detected.

"Specific binding pair" refers to a pair of molecules (e.g., a pair of proteins) that specifically bind to one another. A pair of molecules that specifically bind to one another, which may be the same or different, are referred to as members of a specific binding pair. A protein that is a member of a specific binding pair may be a protein that has been previously determined to be a member of a specific binding pair or a protein that is a putative member of a specific binding pair. Examples of the latter include members of a library, such as the products of a cDNA or combinatorial library, or a protein for which a binding partner has not yet been identified, where it is desired to identify a naturally occuring (*e.g.,* a product of a cDNA or genomic DNA library) or non-naturally occuring (*e.g.,* combinatorial) binding partner therefore.

"Translocation" as used herein refers to a change in distribution of a protein or conjugate (including a fusion protein) from one physical distribution within a cell to another, different, physical distribution within a cell. Preferably, translocation is from either a uniform or non-uniform distribution to a non-uniform distribution. Translocation could also be from a non-uniform to a uniform distribution.

As noted above, the present invention provides a method of detecting a protein-protein interaction. The method comprises first providing a cell that contains a first heterologous conjugate and a second heterologous conjugate. The first heterologous conjugate comprises a first protein of interest conjugated to a detectable group. The second heterologous conjugate comprises a second protein of interest (which may be the same as or different from the first protein of interest) conjugated to a protein that specifically binds to an internal structure within the cell. The binding of the protein that specifically binds to an internal structure may be immediate, may be induced (as discussed below), or may be a prior binding in the case of a protein that is previously localized to or permanently located at the internal structure of interest. The two conjugates are preferably each present in the cell at a total concentration between about 1 or 10 nM to about 1 or 10 mM.

The presence or absence of binding of the detectable group to the internal structure is then detected, the presence of the binding indicating that the first and second proteins of interest specifically bind to one another. Detection may be by any suitable means depending upon the detectable group employed, but preferably the detectable group is a fluorescent group and detection is carried out by optical or visual reading, which may be done manually, by an automated apparatus, or by combinations thereof.

If desired, the second heterologous conjugate can further comprise a detectable group, which detectable group is preferably different from the detectable group located on the first heterologous conjugate and fluoresces at a different wavelength therefrom. For example, both detectable groups could be a green fluorescent proteins, yet simply different mutants of green fluorescent protein that fluoresce at different wavelengths.

Either or both of the heterologous conjugates may be introduced directly in the cell by any suitable means, such as by electroporation or lipofection. In the alternative, when the heterologous conjugates are fusion proteins, a nucleic acid

(typically a DNA) may be stable introduced into the cell (for example, as a plasmid), which nucleic acid includes a suitable promoter segment that controls and causes the expression of a nucleic acid encoding the fusion protein. Again, either or both of the fusion proteins may be produced in the cell in this matter.

Binding events in the instant invention may be direct or indirect binding events. Indirect binding events are those mediated through an intermediate, or bridging, molecule or conjugate. Administration of such a bridge molecule can be a signal to induce translocation (discussed below). For example, the bridging molecule may be a covalent conjugate of FK506 and cyclosporin, to cause the indirect binding of FKBP12 and cyclophilin (both conventionally cytosolic proteins) to one another. Either of the FKBP12 or the cyclophilin can be modified so that it binds to the plasma membreane, such as by lipidating the protein or forming a fusion protein with the transmembrane domain of a transmembrane protein.

Cells used to carry out the present invention are typically eukaryotic cells, which may be yeast, plant, or animal cells. Yeast and animal cells, particularly mammalian cells, are currently preferred. Example plant cells include, but are not limited to, arabidopsis, tobacco, tomato and potato plant cells. Example animal cells include, but are not limited to, human, monkey, chimpanzee, rat, cat, dog, and mouse cells.

Any internal structure as defined above can be used to carry out the present invention, as long as the binding of the detectable group to the internal structure provides a different detectable signal from the cell than when the detectable group is not bound to the internal structure. In one preferred embodiment the internal structure is contained in the cell cytoplasm. Examples of internal structures include, but are not limited to, plasma membrane, cytoskeleton (including but not limited to actin cytoskeleton, tubulin cytoskeleton, intermediate filaments, focal adhesions, etc.), centromere, nucleus, mitochondria, endoplasmic reticulum, vacuoles, golgi apparatus, and chloroplasts. Preferably, the internal structure is either the plasma membrane or cortical cytoskeleton.

In a preferred embodiment of the invention, the protein that specifically binds to an internal structure is a translocatable protein. In this embodiment, the method further comprises the step of inducing translocation of the second heterologous conjugate prior to the detecting step. Induction of translocation may be carried out by

any suitable means, such as by administration of a physical or chemical signal (e.g., administration of a compound such as a phorbol ester). Such a protein may be selected from the group consisting of cytosolic protein kinases, protein phosphatases, adapter proteins, cytoskeletal proteins, cytoskeleton associated proteins, GTP-binding proteins, plasma transmembrane proteins, plasma membrane associated proteins, β-arrestin, and visual arrestin (including fragments thereof that specifically bind to an internal structure). Preferably, the protein is a protein kinase C isoform or a fragment thereof that specifically binds to an internal structure, such as a C1 domain fragment or a C2 domain fragment, where the induction signal is administration of a phorbol ester. In addition, induction of translocation may be induced by stimulation of a receptor, such as a glutamate receptor, beta-adrenergic receptor, or PAF receptor, with a receptor agonist to induce a signaling step which in turn induces translocation. Finally, numerous proteins may be modified to make them translocatable by employing bridging molecules, as discussed above.

As noted above, in one embodiment of the invention the first and second proteins of interest may together comprise members of a specific binding pair. In this embodiment, the invention may further include the step of administering a test compound to the cell prior to the detecting step, wherein the absence of binding of the detectable group to the internal structure indicates that the test compound inhibits the binding of the members of the specific binding pair. Any test compound can be used, including peptides, oligonucleotides, expressed proteins, small organic molecules, known drugs and derivatives thereof, natural or non-natural compounds, etc. Administration of the test compound may be by any suitable means, including direct administration such as by electroporation or lipofection if the compound is not otherwise membrane permeable, or (where the test compound is a protein), by introducing a heterologous nucleic acid that encodes and expresses the test compound into the cell. Such methods are useful for screening libraries of compounds for new compounds which disrupt the binding of a known binding pair.

The method may be used to quantitatively determine binding affinity by varying the concentration of either construct to measure the binding affinity of the constructs at different concentrations, or (where the members of the specific binding pair are the same) to establish the size of the oligomers formed.

As also noted above, in another embodiment of the invention, the method further comprises the step of: *(c)* repeating steps (a) and (b) a plurality of times with a library of proteins of interest, wherein one of the first and second proteins of interest is maintained the same and the other of the first and second proteins of interest (the variable protein or the protein being screened) is replaced with a different member of the library, so that the library is screened for proteins that specifically bind to one of the first or second proteins of interest. Repeating of the steps may be carried out serially, simultaneously, or both serially and simultaneously. Administration of the protein of interest that is varied may be by any suitable means, including direct administration such as by electroporation or lipofection if the compound is not otherwise membrane permeable, or (where the test compound is a protein), by introducing a heterologous nucleic acid that encodes and expresses the variable protein of interest into the cell. Such methods are useful for screening libraries of compounds for new candidates for binding to a known protein.

The invention provides fusion proteins comprising a protein that specifically binds to an internal structure within a cell and a protein of interest, such as a protein that is a member of a specific binding pair, along with nucleic acids encoding such fusion proteins and cells that contain and express such nucleic acids (the nucleic acid thus including regulatory sequences operative in the cell and operatively associated with the nucleic acid segment that encodes the fusion protein). Likewise the present invention provides fusion proteins comprising a protein that is a detectable group and a protein of interest, such as a member of a specific binding pair,

In one embodiment, a kit useful for detecting protein-protein interactions within a living cell, comprises *(a)* a cell as described above that contains and expresses a nucleic acid encoding a first fusion protein, the fusion protein comprising a protein that specifically binds to an internal structure within the cell and a first protein of interest; together with *(b)* a vector for the cell, the vector containing an expression cassette. The expression cassette comprises a promoter operable in the cell and operatively associated with a nucleic acid encoding a detectable protein, and has a splice site positioned adjacent the nucleic acid encoding a detectable protein so that a heterologous nucleic acid encoding a second protein of interest can be inserted therein to produce a nucleic acid segment encoding a second fusion protein. The second fusion protein comprising the detectable protein and the second protein of

interest. In an alternate embodiment, a kit useful for detecting protein-protein interactions within a living cell, comprises *(a)* a cell as described above that contains and expresses a nucleic acid encoding a first fusion protein, the fusion protein comprising a detectable protein and a first protein of interest; together with *(b)* a vector for the cell, the vector containing an expression cassette, the expression cassette comprising a promoter operable in the cell and operatively associated with a nucleic acid encoding a protein that specifically binds to an internal structure within the cell, as described above. The expression cassette likewise has a splice site positioned adjacent the nucleic acid encoding the protein that specifically binds to an internal structure within the cell (as described above; preferably a translocatable protein as described above) so that a heterologous nucleic acid encoding a second protein of interest can be inserted therein to produce a nucleic acid segment encoding a second fusion protein. The second fusion protein comprises the protein that specifically binds to an internal structure within the cell and the second protein of interest. Such kits can be provided in any suitable form, and are typically packed together with suitable instructions. Any vector may be employed, but the vector is typically a plasmid vector.

In using kits as described above, the vector is used in one embodiment to create, from a source nucleic acid library as described above, a product nucleic acid library comprising a plurality of separate nucleic acids, each of the separate nucleic acids encoding a fusion protein, the fusion protein comprising a protein of interest (encoded by the source library) and a detectable protein, wherein the protein of interest encoded by each of the separate nucleic acids is different from the protein of interest encoded by the other nucleic acids of the library. The product library, in the vector, can then be used to transform multiple cells so that the library constituents can be screened in the manner described above.

In the alternate embodiment described above, the vector is used to create, from a source nucleic acid library as described above, a product nucleic acid library comprising a plurality of separate nucleic acids, each of the separate nucleic acids encoding a fusion protein, the fusion protein comprising a protein of interest (from the source library) and a protein that specifically binds to an internal structure within a cell as described above (preferably a translocatable protein as described above), wherein the protein of interest encoded by each of the separate nucleic acids is

different from the protein of interest encoded by the other nucleic acids of the library. Again the product library, in the vector, can then be used to transform or transfect multiple cells so that the library constituents can be screened in the manner described above.

Screening of libraries, in either of the foregoing embodiments, may be carried out in accordance with conventional techniques. Typically, the screening will be carried out by transfecting pools (subsets of the members of the library) into different groups of cells to express the protein of interest. This allows one to identify pools that contain binding partners that interact with the protein of interest. Pools with binding partners can then again be divided into subpools until individual members (for example, individual cDNA sequences) of the library are identified that bind to the protein of interest.

The present invention is explained in greater detail in the following non-limiting examples.

Examples

Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) is a ubiquitous kinase which is expressed at high concentrations in neurons and at lower concentrations in most other cell types. Previous studies suggested that CaMKII is an essential mediator for long term potentiation and other forms of synaptic plasticity (reviewed in Braun and Schulman, 1995; Soderling, 1993) . Furthermore, CaMKII activity may have an important role in stabilizing the dendritic architecture (Wu and Cline, 1998). A critical neuronal function of the α-isoform of CaMKII (CaMKIIa) was directly demonstrated by studying mice which were either lacking CaMKIIa or which expressed mutated CaMKIIa. CaMKIIa deficient mice as well as transgenic mice expressing an autonomously active or an autophosphorylation deficient CaMKIIa showed impaired long term potentiation as well as defects in spatial learning and memory (Chapman et al., 1995; Glazewski et al., 1996; Gordon et al., 1996; Mayford et al., 1996; Mayford et al., 1995; Silva et al., 1992; Giese et al., 1998).

Since not only CaMKIIα but also CaMKIIβ is a prominent isoform in the central nervous system, the question arises whether CaMKIIα and CaMKIIβ have different roles in regulating neuronal functions. Such functional differences between

the two isoforms would have a direct impact on our understanding of cell type specific signaling processes since the relative expression of CaMKIIα and CaMKIIβ is markedly different in different brain regions and at different developmental stages. For example, the ratios of α and β subunits are about 3:1 and 1:4 in adult forebrain and cerebellum, respectively, while in 10-day postnatal mice, the forebrain a : b ratio is 1:1 (Miller and Kennedy, 1985). On a structural basis, recombinant CaMKIIα as well as purified brain CaMKII has been shown to form oligomers with approximately 8 to 12 subunits (Kanaseki et al., 1991, Bennett et al.,1983) . CaMKIIβ and CaMKIIα have a similar overall domain organization and corresponding autophosphorylation consensus sequences and even though the calmodulin binding affinity of CaMKIIβ is slightly higher than that of CaMKIIα, the regulation of different CaMKII isoforms by Ca^{2+}/CaM and autophosphorylation is overall similar (Miller and Kennedy, 1985; De Koninck and Schulman, 1998; GuptaRoy and Griffith, 1996). Despite these similarities, it has been controversial whether CaMKIIβ forms oligomers on its own (Yamauchi et al., 1989), whether CaMKIIα and CaMKIIβ form hetero-oligomers when expressed at the same time (Kanaseki et al., 1991) and whether the two isoforms are differentially localized within cells (Scholz et al., 1988, Nomura et al., 1997).

Here green fluorescent protein (GFP) - tagged CaMKIIα and CaMKIIβ isoforms are used to explore the subcellular localization and oligomerization of CaMKIIα and CaMKIIβ. It was found that dendritic spines and filopodia as well as the cortical cytoskeleton are the primary docking sites for expressed CaMKIIβ. In contrast, expressed CaMKIIα was uniformly distributed in the soma and processes and was largely absent from spines. However, when expressed in the same cell, CaMKIIβ targeted CaMKIIα to dendritic spines and the cell cortex. In vitro binding studies suggested that this targeting results from a direct binding interaction of CaMKIIβ with F-actin.

A GFP-based protein-protein interaction assay (Pull-Out binding assay) was then developed to explore the binding interactions between CaMKIIα and CaMKIIβ isoforms in living cells. When expressed alone, CaMKIIb was found to form homo-oligomers with an average size that is markedly smaller than the approximately thirteen subunits measured for CaMKIIa homo-oligomers. When expressed at the

same time, CaMKIIb isoforms incorporated equally well into either CaMKIIa or CaMKIIb oligomers (and vice versa). Half-maximal targeting of CaMKIIa oligomers to the cytoskeleton was achieved if at least 15% of CaMKIIb were present in the same cell, suggesting that a small number of CaMKIIb subunits are required to dock CaMKIIa/b hetero-oligomers with approximately thirteen subunits to F-actin. Our studies suggests that the synaptic localization of CaMKII activity is controlled by the relative expression of CaMKIIb F-actin docking modules.

1. PROCEDURES

a. Cloning of CaMKII fusion constructs.

The cDNA for rat CaMKIIα ,β and β' were generous gifts from Dr. Howard Schulman. The construction of the GFP-CaMKIIα vector was described previously (Shen and Meyer, 1998). To obtain the in vitro transcription vector for CaMKIIα without GFP, the CaMKIIα cDNA was amplified by PCR and cloned into the in vitro transcription vector dSHiro3. DNA sequencing were performed to exclude PCR errors. GFP-CaMKIIb and CaMKIIb were also cloned into the SHiro3 and dSHiro3 vectors using a similar PCR strategy. The construction of PM-GFP or Cys-GFP was described previously (Oancea et al., 1998). PM-CaMKIIα and β were made by replacing the GFP sequence with CaMKIIα and β coding sequence in the same SHiro3 vector.

b. In vitro translation.

In vitro translation with SP6 RNA polymerase was performed according to the manufacturer's protocol using a commercial kit (TNT Coupled reticulocyte lysate system, Promega). In vitro transcription reactions were performed using mRNAs as templates. The relative molar concentration of the different translated proteins was calculated by calibration using ^{35}S-methionine incorporation and by counting the number of methionines in the respective protein. Non-radioactive methionine was used to obtain CaMKIIs and fusion constructs for the autophosphorylation assay.

c. Ca^{2+} dependent and independent autophosphorylation of CaMKII and GFP fusion proteins.

CaMKII autophosphorylation assay were performed as described previously (Hanson et al., 1994). Briefly, CaMKII isoforms and fusion constructs were autophosphorylated at 30°C in 25ml reactions containing 50 mM PIPES (pH 7.0), 10mM $MgCl_2$, 500 mM $CaCl_2$, 600 nM calmodulin, 50 mg/ml BSA, and 200 mM [g-^{32}P]ATP (6000cpm/pmol). The Ca^{2+} dependent autophosphorylation reaction was started by adding in vitro translation product into the reaction mix and stopped by addition of EDTA (16.7 mM final concentration) 30 seconds later. To measure the extent of the Ca^{2+}-independent autophosphorylation, a 30 second reaction at high Ca^{2+} was followed by a 120 second secondary incubation in the presence of added EGTA (3.3 mM final concentration). In control experiments, 3.3 mM EGTA were included in the initial reaction mix. The reaction mix was then resolved on a SDS-PAGE and subjected to Phosphorimager analysis. The densitometry of bands were measured and corrected by the amount of kinase which was determined in a separate in vitro translation reaction with ^{35}S-methionine as described above.

d. In vitro transcription and RNA processing.

In vitro transcription and RNA processing were performed as described before (Yokoe and Meyer, 1996; Shen and Meyer, 1998). Briefly, *in vitro* transcription with SP6 RNA polymerase was performed according to the manufacturer's protocol using a commercial kit (mMESSAGE mMACHINE™, Ambion). 10 mM EDTA was used to terminate the reaction. RNA was purified by column chromatography (RNeasy column, Qiagen) followed by the addition of a polyA tail. Poly adenylation was carried out for 30 minutes at 37°C in a 50 µl reaction mixture containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 0.25 mg / µl RNA, 250 mM ATP, 5 units poly(A) polymerase (Life Technologies). The reaction was terminated by addition of 20 mM EDTA. Unincorporated ATP and salts were removed by applying the mRNA to a RNeasy column. The eluent was dried and mRNA was dissolved at 1 µg / µl in the electroporation buffer (5 mM KCl, 125 mM NaCl, 20 mM HEPES pH 7.4 and 10 mM glucose).

e. Cell culturing and electroporation

RBL 2H3 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 20% fetal bovine serum (Life Technologies, Gaithersburg, MD) at 37°C with 5% CO_2. The cells were plated at 5 X 10^4 cells / cm^2 on glass cover slips and were allowed to attach to the coverslip for a minimum of 3 hours. Hippocampal neurons obtained from 2 to 4 days postnatal rats were cultured as described in Ryan and Smith (1995) and used 10 days to three weeks after plating. A self-built small volume electroporation device for adherent cells was used for electroporation (Teruel and Meyer, 1997). For the transfection of neurons, modified versions of the device and buffer conditions were used. After transfection, the electroporation buffers were replaced with the same culture medium.

f. Functional labeling of presynaptic terminals with FM 4-64

Functional presynaptic terminals were visualized with FM 4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium,dibromide; Molecular Probes). FM 4-64 (Henkel and Betz, 1995) is similar in structure and properties to FM 1-43 but its longer wavelength emission spectra make it more suitable for dual-channel fluorescence microscopy in conjunction with green fluorescent protein (Ziv and Smith, 1996). Cells were loaded with FM 4-64 by replacing the saline in imaging chamber with high potassium solution (100mM KCL, 20 mM HEPES, 1.5mM $CaCl_2$, 30mM NaCL, 1.5mMMgCL_2, pH 7.4 and 6 μM of FM 4-64) for 20 seconds and switch back to a saline solution for 5-10 min. After collecting a digital image of the labeled field, the cells were stimulated again by switching to the same high potassium solution. The spatial distribution of the active presynaptic terminals could then be determined from a difference image.

g. Diffusion analysis

The diffusion coefficients were determined using an analysis by which a photobleached area is produced by a focused laser pulse and the fluorescence recovery is fit to sequential 2-dimensional Gaussian distributions. Ratio images of the fluorescence distribution after the bleach pulse to the distribution before the pulse

were used for the analysis. This analysis follows at the same time the decrease in the bleach amplitude and the increase in the bleach radius. Assuming mass conservation, the resulting fluorescence distributions were fit by:

$F_n(x , y) = 1 - (Fo * a_o^2 / a_n^2) * \exp (- ((x - x_o)^2 + (y - y_o)^2) / a_n^2)$, with x and y as the pixel coordinates, a_n as the radius of the bleach diameter in the n-th image, and $F_n(x, y)$ as the local relative fluorescence intensity. A least square fit routine was used to fit at the same time Gaussian profiles to all images in the time series. An approximate diffusion coefficient was then determined from a graph of the square of the radius, a_n^2, versus time. The diffusion coefficient can be directly obtained from the slope of this graph ($Dy / D x = 4 * D$; with D as the diffusion coefficient, (Shen and Meyer, 1998)).

h. Model calculations of a stochastic insertion of CaMKIIb subunits into hetero-oligomers

The probability of having one or more subunits randomly inserting into a hetero-oligomer is [1-(probability to have no subunit inserted)]. The probability of having none inserted is $(R/(R+1))^N$ with R as the ratio of GFP-CaMKIIa to CaMKIIb and N as the number of subunits.

i. Immunofluorescence

NIH-3T3, RBL cells, and hippocampal neurons were cultured on glass coverslips and transfected with mRNA encoding GFP-CaMKIIb fusion construct. Seven to eight hours after transfection, the cells were fixed for 10 minutes at 4 °C with 4% paraformaldehyde in PBS (1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 138 mM NaCl and 2.7 mM KCl [pH 7.4]). NIH-3T3 cells and RBL cells were permeabilized for 5 minutes at 4 °C with 0.1% Triton in PBS. Hippocampal neurons were permeabilized for 10 minutes at 4 °C with 0.1% Triton. For F-actin staining, rhodamine phalloidin (Molecular Probes) was incubated with the cells for 30 min at room temperature at a dilution of 1:300. in PBS. For the staining of post-synaptic densities, hippocampal neurons were incubated with an monolonal PSD-95 antibody (Cat.# 05-428, Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C at a

dilution of 1:200 and then in secondary Cy3 labeled anti-mouse antibody (Cat.# 115-165-062, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. The cells were washed three times with PBS and coverslips were mounted onto glass slides using buffered glycerol mounting medium.

j. Fibroblast transfection and Western blotting assay

NIH-3T3 cells were plated in 35 mm dishes at a density of 1.0 X 10^5 per dish and incubated overnight at 37 ^oC in a humid atmosphere containing 5% CO_2. Cells were transfected with 1.5 -2.2 μg of pSRa-CaMKIIb or pSRa-CaMKIIa or cotransfected with pSRa-CaMKIIb and pSRa-CaMKIIa using lipofectin plus (GIBCO/BRL) according to manufacturer's instructions. Cells were harvested 48 hrs after transfection and extracted with 10 mM Tris-HCl, 50 mM KCl, 0.1 mM EGTA, 0.1% Triton, 2 mM PMSF and 5 μg/ml aprotinin, leupeptin and pepstatin at room temperature for 10 min and centrifuged at 30,000 X g for 30 min at 4 ^oC. Various fractions of the cell extract were resolved by electrophoresis on SDS-polyacrylamide gels (12%), transferred to nitrocellulose and blotted with monoclonal anti-CaMKIIa or anti-CaMKIIb antibody (GIBCO/BRL). The membranes were blotted using a secondary antibody conjugated to horseradish peroxidase and visualized by ECL (Amersham).

k. "Pull Out" protein-protein interaction assay

Poly-adenylated mRNA was made as described above. In many experiments, mRNA species were mixed and used for electroporation at a final concentration of typically 1 μg/μl total. The relative translation efficiency was determined by a separate in vitro translation reaction using the same mRNA as a template. Images of transfected RBL or NIH 3T3 cells were taken on a Zeiss confocal microscope 8-12 hours after electroporation. 1μM of PMA was added and images of single cells were taken under the same configuration. Images were taken before and after PMA addition and were analyzed using NIH-image software. A plasma membrane translocation factor was defined as D_{PM} / I_{pre} (**Figure 2E**).

2. RESULTS

a. Expressed CaMKIIb but not CaMKIIa is enriched in dendritic branches and cell cortex

The cellular localization of CaMKIIa versus CaMKIIb isoforms was investigated by constructing CaMKII fusion proteins with GFP (**Figure 1A**). An earlier study has shown that a GFP-CaMKIIa construct can phosphorylate substrate peptides as well as autophosphorylate itself at threonine 286 (Shen and Meyer, 1998). An additional criterion for functionally intact GFP-CaMKII is the preservation of secondary calcium-independent aut(**Figures 1B and 1C**).

When GFP-tagged CaMKIIa or b isoforms were expressed in cultured hippocampal neurons, CaMKIIa was largely homogeneous in the soma and main processes (**Figure 1D**, left) but was only minimally present in the finer branch structures. In contrast, CaMKIIb showed a striking enrichment in dendritic branches as well as at the cell cortex (**Figure 1D**, right). When expressed in RBL-cells, CaMKIIa was nearly homogeneously distributed in the cytosol and CaMKIIb had a distinct cortical localization (**Fig, 1E**, right).

The differential distribution of the two isoforms suggests that CaMKIIb has specific binding interactions in cells that do not occur for CaMKIIa.

It was tested more directly whether CaMKIIb has more binding interactions than CaMKIIa by comparing the local fluorescence recovery after photobleaching of GFP-CaMKIIb to that of GFP-CaMKIIa. A 2 μm diameter laser photobleach spot was generated in the cell by a short laser pulse and the fluorescence recovery was monitored by rapid confocal imaging. Consistent with the hypothesis that CaMKIIb but not CaMKIIa undergoes binding interactions, the recovery after photobleaching was significantly more rapid for CaKIIa compared to that for CaMKIIb. This could be quantitatively shown by a calculated average diffusion coefficient of CaMKIIb that was 5 times lower than that of CaMKIIa (**Figure 1F**, see Procedures above for a description of the analysis). Nevertheless, the binding interactions of CaMKIIb were reversible, since most of the GFP-CaMKIIb fluorescence recovered on the time scale of 15 seconds after the laser bleach pulse. Together, these measurements suggest that CaMKIIa expressed alone is a highly mobile protein that has only limited cytosolic binding interactions, while CaMKIIb is bound in a reversible manner to dendritic and

cortical structures.

b. CaMKIIb is an F-actin docking module enriched in dendritic spines

What are the structures in the dendritic branches and cell cortex targeted by CaMKIIb? The markedly punctuate staining suggested that CaMKIIb is enriched in dendritic spines. Indeed, an antibody against the postsynaptic protein PSD-95 showed a clear co-localization between GFP-CaMKIIb and PSD-95 (data not shown). The arrows in the right panel point to presumed dendritic spines which were highly enriched in PSD-95 and GFP-CaMKIIb. Some of the neurons also showed an enriched staining of GFP-CaMKIIb in filopodia like branches (not shown) that were reminiscent of developing dendritic spines (Ziv and Smith, 1996). A magnified image of an arbor of such filopodias is shown in the right panel.

It was verified that the dendritic spines marked by anti-PSD-95 antibodies and GFP-CaMKIIb corresponded to mature postsynaptic terminals by comparing the distribution of GFP-CaMKIIb to that of functional pre-synaptic terminals. The terminals were marked with the fluorescent synaptic vesicle marker FM 4-64 using a double depolarization protocol (Ziv and Smith, 1996; see Methods). Since this type of co-localization study can be performed in livine of an arbor of such filopodian artifacts that may arise during the fixation of neurons can be excluded. When comparing the distribution of GFP-CaMKIIb to the location of active presynaptic terminals, the overlayed image showed a marked juxtaposed localization of GFP-CaMKIIb and loaded FM 4-64 (not shown). This suggests that CaMKIIb is indeed enriched in mature dendritic spines. In contrast, the uniformly distributed GFP-CaMKIIa was not enriched near active synapses (not shown).

Since actin is highly enriched in dendritic spines and cell cortex (Fisher et al., 1998; Landis and Reese, 1983, Caceres el al, 1983), it is conceivable that the localization of CaMKIIb to dendritic spines is mediated by a direct or indirect binding interaction of CaMKIIb with F-actin. FM 4-64 was tested. This suggests that CaMKIIb is indeed enriched in mature dendritic spines. In contrast, the uniformly distributed GFP-CaMKIIa was not enriched near active synapses (data not shown).

Since actin is highly enriched in dendritic spines and cell cortex (Fisher et al., 1998; Landis and Reese, 1983, Caceres el al, 1983), it is conceivable that the localization of CaMKIIb to dendritic spines is mediated by a direct or indirect binding

interaction of CaMKIIb with F-actin. The possible co-localization of CaMKIIb and F-actin in two model cell lines was tested by using rhodamine-phalloidin as a marker for polymerized actin (F-actin). In RBL-cells, which has predominant cortical F-actin structures, the cortical rhodamine-phalloidin closely co-localized with GFP-CaMKIIb. The same near complete overlap was also observed in NIH-3T3-cells which are rich in actin stress fibers (not shown). This suggests that the actin cytoskeleton co-localization of CaMKIIb is not cell type specific.

Further support for the co-localization of CaMKIIb with F-actin in living cells was obtained by comparing cells before and after treatment with the actin depolarizing drug latrunculin (Spector et al. 1989) (not shown). In RBL-cells (top) and fibroblasts (bottom), addition of latrunculin led to a near complete loss in the cortical as well as stress fiber staining of GFP-CaMKIIb.

We then determined biochemically whether expressed CaMKIIb can bind directly to purified F-actin. Indeed, Met-^{35}S-labeled CaMKIIb could be effectively sedimented by polymerized actin. In contrast, CaMKIIa is much less sedimentable by polymerized actin using the same assay. This suggests that the co-localization of CaMKIIb with the actin cytoskeleton observed in living cells is the result of a direct and reversible binding interaction between CaMKIIb and F-actin.

c. CaMKIIa is targeted to dendritic spines when co-expressed with CaMKIIb

It was then tested whether the co-expression of CaMKIIa and CaMKIIb in the same cell affects their respective localization. An effective co-expression of both isoforms was made possible by using an RNA transfection method. In this approach, a large number of translation competent RNA molecules are directly introduced into the cytosol of adherent cells by microporation (Teruel and Meyer, 1997; Yokoe and Meyer, 1996). Thus, RNA encoding different proteins can be mixed and expressed at a defined ratio within each transfected cell. Strikingly, when GFP-CaMKIIa was expressed together with CaMKIIb (without a GFP-tag) in hippocampal neurons, GFP-CaMKIIa became associated with the same dendritic spine and cortical structures (data not shown). A largely cortical localization of GFP-CaMKIIa was also observed in RBL-cells in the presence of CaMKIIb. In contrast, expression of CaMKIIa (without a GFP-tag) together with a similar amount of GFP-CaMKIIb did not affect the cortical localization of GFP-CaMKIIb (data not shown). Using the same co-

localization protocols as described above, we also found a marked co-localization between GFP-CaMKIIa, co-expressed with CaMKIIb, and anti PSD-95 antibodies (not shown). In living neurons, GFP-CaMKIIa, coexpressed with CaMKIIb, showed a marked localization juxtaposed to the presynaptic marker FM 4-64 (not shown).

Can this effect of CaMKIIb on CaMKIIa localization be confirmed biochemically? A detergent extraction procedure of NIH-3T3-cells was used to test whether CaMKIIb and/or CaMKIIa could be found in an actin enriched pellet fraction. This extraction protocol has been shown to significantly enrich for actin and actin binding proteins (Egelhoff et al., 1991). Expressed CaMKIIb and CaMKIIa isoforms without a GFP-tag were used for these measurements. In agreement with the in vivo data, CaMKIIa, when expressed alone, was largely absent from the actin pellet while CaMKIIb was highly enriched in the actin pellet (data not shown). When both isoforms were expressed together, a significant fraction of CaMKIIa was found in the actin cytoskeletal fraction. Taken together, these studies are consistent with a targeting mechanism by which CaMKIIa is localized to F-actin if expressed together with a sufficient amount of CaMKIIb.

How does CaMKIIb target CaMKIIa to the actin cytoskeleton? A likely hypothesis is that CaMKIIa does not undergo cytoskeletal binding interactions of its own but binds to CaMKIIb which then anchors the complex to F-actin. To understand this heterologous CaMKII targeting mechanism, several important questions about the binding interaction and oligomerization of CaMKIIa and CaMKIIb have to be answered: 1. Can CaMKIIb form oligomers on its own and if it does, how large are these oligomers compared to those formed by CaMKIIa?, 2. do co-expressed CaMKIIa and CaMKIIb isoforms assemble into hetero- or homo-oligomers in living cells?, 3. if they form hetero-oligomers, does the incorporation of CaMKIIb into CaMKIIa oligomers occur as a stochastic process?, and 4. what minimal ratio of CaMKIIb to CaMKIIa is required for targeting CaMKIIa to the actin cytoskeleton?

d. Oligomer formation can be explored in living cells using a GFP-based "Pull-Out" binding assay

To address the question of homo- versus hetero-oligomer formation, an assay was developed to quantitatively study protein-protein binding interactions in living

cells ("Pull-Out" binding assay, **Figure 2A**). The strategy was to use a protein-domain (PM-domain) that translocates to the plasma membrane in response to the addition of a drug. The potential binding interaction between the investigated Protein X and Protein Y could then be investigated by fusing this PM-domain to Protein X and a GFP-tag to Protein Y (or vice versa). Both fusion proteins could then be expressed in the same cell and their binding interaction investigated. 1. If the initially cytosolic GFP-Protein Y remains cytosolic after drug addition, no significant binding interaction occurs between Proteins X and Y. 2. If the drug addition leads to the GFP-Protein Y translocation to the plasma membrane (along with the non-fluorescent PM-Protein X), Proteins X and Y bind to each other.

We used the first phorbol-ester binding domain of protein kinase C as such a PM-domain. This small 6 kDa domain is an initially cytosolic protein that binds nearly irreversibly to the plasma membrane after phorbol ester addition (Oancea et al., 1998). The distinct property of this domain is shown in **Figure 2B**. Before phorbol ester addition, a fusion protein of the phorbol ester binding domain with GFP is a cytosolic protein (left panel) that is "pulled" from the cytosol to the plasma membrane after addition of phorbol ester (right panel). This translocation process occurs in less than a minute and is mediated by a diffusion-dependent high affinity binding interaction of the fusion protein with plasma membrane localized phorbol ester (Oancea et al., 1998). It should be noted that the also visible nuclear localized GFP fusion protein translocates to the plasma membrane much slower due to its slow diffusion through nuclear pores.

This PM-domain was first used to determine whether most of the expressed CaMKIIa isoforms is present in an oligomeric state. To test for oligomerization in vivo, PM-tagged CaMKIIa and GFP-CaMKIIa were expressed in the same cell (**Figure 2C**, left). As expected, addition of phorbol ester led to a marked translocation of the initially cytosolic GFP-CaMKIIa to the plasma membrane (**Figure 2C**, right). This change in the distribution before and after addition of phorbol ester can be more quantitatively measured in a line profile analysis comparing the fluorescence intensity across the cell before and after phorbol ester addition (Figure 5C, bottom). In control experiments, GFP was expressed alone and no plasma membrane translocation of GFP was observed after phorbol ester addition (**Figure 2 D**). The finding that most GFP-CaMKIIa is pulled to the plasma membrane by PM-

CaMKIIa strongly suggests that most of the expressed CaMKIIa is present in the cell in an oligomeric state.

e. CaMKIIb homo-oligomers are significantly smaller than CaMKIIa homo-oligomers

While CaMKIIa has been proposed to be an homo-oligomer with 8 to 12 subunits, it is controversial if CaMKIIb forms oligomers and how large these potential oligomers are (Yamauchi et al., 1989). To measure the apparent oligomer sizes of CaMKIIa and CaMKIIb in living cells, we expressed GFP-CaMKII together with a decreasing amount of PM-CaMKII and measured the phorbol ester induced translocation to the plasma membrane (a schematic view of the expected oligomer translocation process is shown in **Figure 3A**). Since the phorbol ester-induced plasma membrane affinity of the PM-domain is nearly irreversible, it is likely that a single subunit of PM-CaMKIIa is sufficient to induce the plasma membrane translocation of a CaMKIIa oligomer. As discussed above, the RNA transfection method allows one to quantitatively titrate the amount of the two CaMKII fusion proteins in the same cell. To determine the respective expression levels for the two microporated RNA species, the concentration of the translated proteins was measured in parallel by in vitro translation of the same RNAs in the presence of ^{35}S-Met (**Figure 3B**).

Figure 3C shows the GFP-CaMKIIa plasma membrane translocation at increasing dilutions of co-expressed PM-CaMKIIa. The left images show the distribution before and the right images after phorbol ester addition. Interestingly, the phorbol ester induced targeting to the plasma membrane was still measurable when PM-CaMKIIa was diluted to less than 3% of GFP-CaMKIIa. The sequential reduction in plasma membrane translocation can be seen more clearly in fluorescence line intensity traces (**Figure 3D**). The loss in phorbol ester-mediated plasma membrane targeting at increasing PM-CaMKIIa dilutions was analyzed by dividing the relative plasma membrane fluorescence intensity of GFP-CaMKIIa (DPM) by the average cytosolic fluorescence intensity before phorbol ester addition (I_{pre}) (Figure 6E). A DPM / I_{pre} ratio of 0 indicates that no plasma membrane translocation

occurred and a DPM / I_{pre} ratio of ~ 3 corresponds to the translocation observed for the PM-GFP construct itself.

Based on this analysis, a titration curve can be obtained, showing the relative plasma membrane translocation at decreasing dilution ratios of PM-CaMKIIa to GFP-CaMKIIa (**Figure 3F**). A 50% reduction in plasma membrane targeting was observed at an approximate dilution ratio of one PM-CaMKIIa per fourteen GFP-CaMKIIa. A Poisson distribution model would predict that the probability (p) to introduce at least one subunit into an oligomer with N subunits is $p = 1 - (R/(R+1))^N$, with R as the dilution ratio. Using this model, a best fit to the data was obtained for N = 13.5, in close agreement with a previous estimate of 12 subunits for purified CaMKIIa (Kanaseki et al., 1991; Yamauchi et al., 1989).

We then used the same approach to determine if CaMKIIb forms oligomers. Although GFP-CaMKIIb has a partial cortical and internal F-actin localization, this binding interaction was reversible and a much more pronounced plasma membrane localization can be induced by addition of phorbol ester to a PM-tagged CaMKIIb. Using this phorbol ester triggered increase in plasma membrane translocation of GFP-CaMKIIb, a titration curve was obtained for the relative plasma membrane translocation at decreasing dilution ratios of PM-CaMKIIb to GFP-CaMKIIb. Interestingly, the apparent average size of CaMKIIb oligomers was 4.2, significantly smaller than that of the a-isoform (**Figure 3F**). Thus, these in vivo measurements clearly show that CaMKIIb can form oligomers, albeit with a significantly smaller apparent size than those formed by CaMKIIa. This apparent size of CaMKIIb oligomers was consistent with our finding that CaMKIIb purfied from Baculovirus transfected Sf9 cells had a size much smaller than that of CaMKIIa expressed by the same method. (unpublished results by Kang Shen).

f. CaMKIIa and b form stochastic hetero-oligomers

Since the relative expression of CaMKIIa to b is highly variable between different types of neurons, next determined was how efficient is hetero-oligomer formation compared to homo-oligomer formation. The same dilution approach as described in **Figure 3** was pursued but now for the hetero-oligomers. The calculated line plots in **Figure 4A** show the curves expected for the insertion of PM-CaMKIIb

into GFP-CaMKIIa oligomers for a stochastic insertion mechanism (dashed curve). The measured relative plasma membrane translocation for decreasing ratios of PM-CaMKIIb to GFP-CaMKIIa closely matched a predicted stochastic insertion mechanism. The same measurements were then made for PM-CaMKIIa insertion into GFP-CaMKIIb oligomers by dilution of the PM-CaMKIIa fusion protein at a constant concentration of CaMKIIb (**Figure 4B**). The calculated curve for a stochastic insertion mechanism is overlapping with the fitted one.

Together, this titration approach suggests that if CaMKIIa and CaMKIIb isoforms are expressed at the same time and place, they form mixed oligomers with a stochastic probability for the insertion of either one of the isoforms. This also suggests that most CaMKII oligomers in neurons contain a variable fraction of CaMKIIb that is defined by the relative expression level of locally translated CaMKIIb versus CaMKIIa. For the physiological situation, it is then important to know how many CaMKIIb isoforms have to be inserted into mostly CaMKIIa hetero-oligomers to still effectively target CaMKII to its cytoskeletal docking site.

g. A small number of CaMKIIb isoforms are sufficient to target CaMKII hetero-oligomers to the actin-cytoskeleton

While the previous studies were useful to dissect the oligomer formation of CaMKIIa and CaMKIIb, they did not resolve whether individual or multiple CaMKIIb subunits are required for the targeting of CaMKIIa/b hetero-oligomers to the actin cytoskeleton. The same RNA dilution strategy was used to determine at which ratio of GFP-CaMKIIa to CaMKIIb the cytoskeletal localization still occurs. The distinct cortical actin cytoskeleton localization of CaMKIIb in RBL-cells was used in this assay (**Figure 4C**). While CaMKIIb was less potent in targeting GFP-CaMKIIa to the plasma membrane than the PM-CaMKII constructs, 50% translocation to the cortical actin cytoskeleton required a ratio of approximately 6.5 : 1 of GFP-CaMKIIa to CaMKIIb. Since CaMKIIa isoforms contain approximately 13 subunits, this suggests that a small number of CaMKIIb subunits are sufficient to target CaMKIIa/b hetero-oligomers to the actin cytoskeleton.

In a second independent approach to understand the cytoskeletal targeting of CaMKIIa by CaMKIIb, we measured the binding interactions of the hetero-oligomers by measuring their diffusion coefficients. As shown in **Figure 1F**, GFP-CaMKIIa has

a 5-fold faster apparent diffusion than GFP-CaMKIIb. Diffusion coefficients were then measured at increasing dilutions of GFP-CaMKIIa to CaMKIIb. Similar to the results with the localization to the cortical cytoskeleton, the diffusion coefficient of CaMKIIa was reduced by 50% when the concentration of CaMKIIb exceeded 15% of that of GFP-CaMKIIa (**Figure 4D**). Together, these measurements show that CaMKIIb is a potent targeting domain that can localize a much larger number of CaMKIIa isoforms to the actin cytoskeleton.

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The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

What is Claimed is:

1. A method of detecting a protein-protein interaction, comprising:

(a) providing a cell that contains a first heterologous conjugate and a second heterologous conjugate,

wherein said first heterologous conjugate comprises a first protein of interest conjugated to a detectable group,

and wherein said second heterologous conjugate comprises a second protein of interest conjugated to a protein that specifically binds to an internal structure within said cell; and then

(b) detecting the presence or absence of binding of said detectable group to said internal structure, the presence of said binding indicating that said first and second proteins of interest specifically bind to one another.

2. A method according to claim 1, wherein said detectable group is a protein, and said first protein and said detectable group together comprise a fusion protein.

3. A method according to claim 1, wherein said cell contains and expresses a nucleic acid encoding said fusion protein.

4. A method according to claim 1, wherein said second heterologous conjugate is a fusion protein.

5. A method according to claim 5, wherein said cell contains and expresses a nucleic acid encoding said fusion protein.

6. A method according to claim 1, wherein said first and second proteins of interest together comprise members of a specific binding pair.

7. A method according to claim 6, further comprising the step of administering a test compound to said cell prior to said detecting step,

and wherein the absence of binding of said detectable group to said internal structure indicates that said test compound inhibits the binding of said members of said specific binding pair.

8. A method according to claim 1, further comprising the step of:

(c) repeating steps (a) and (b) a plurality of times with a library of proteins of interest, wherein one of said first and second proteins of interest is maintained the same and the other of said first and second proteins of interest is replaced with a different member of said library, so that said library is screened for proteins that specifically bind to one of said first or second proteins of interest.

9. A method according to claim 8, wherein said library is a combinatorial library.

10. A method according to claim 8, wherein said library comprises the expression product of a cDNA library.

11. A method according to claim 1, wherein said second heterologous conjugate further comprises a detectable group.

12. A method according to claim 1, wherein said cell is a eukaryotic cell.

13. A method according to claim 1, wherein said cell is a yeast, plant, or animal cell.

14. A method according to claim 1, wherein said cell is a mammalian cell.

15. A method according to claim 1, wherein said internal structure is the cell nucleus or a structure contained in the cell nucleus.

16. A method according to claim 1, wherein said internal structure is contained in the cell cytoplasm.

17. A method according to claim 1, wherein said internal structure is selected from the group consisting of plasma membrane, cytoskeleton, centromere, nucleus, mitochondria, endoplasmic reticulum, vacuoles, golgi apparatus, and chloroplasts,

18. A method according to claim 1, wherein said internal structure is selected from the group consisting of the plasma membrane and the cortical cytoskeleton.

19. A method according to claim 1, wherein said protein that specifically binds to an internal structure is a translocatable protein, and wherein said method further comprises the step of inducing translocation of said second heterologous conjugate prior to said detecting step.

20. A method according to claim 1, wherein said first protein is a translocatable protein, and wherein said method further comprises the step of inducing translocation of said first protein prior to said detecting step.

21. A method according to claim 1, wherein said protein that specifically binds to an internal structure is selected from the group consisting of cytosolic protein kinases, protein phosphatases, adapter proteins, cytoskeletal proteins, cytoskeleton associated proteins, GTP-binding proteins, plasma transmembrane proteins, plasma membrane associated proteins, β-arrestin, visual arrestin, and fragments thereof that specifically bind to an internal structure.

22. A method according to claim 21, wherein said protein that specifically binds to an internal structure is a protein kinase C isoform or a fragment thereof that specifically binds to an internal structure.

23. A method according to claim 22, wherein said protein that specifically binds to an internal structure is a protein kinase C fragment selected from the group consisting of C1 domains and C2 domains.

24. A method according to claim 1, wherein said first and second proteins of interest are the same.

25. A method according to claim 1, wherein said first and second proteins of interest are different.

26. A fusion protein comprising a protein that specifically binds to an internal structure within a cell and a protein that is a member of a specific binding pair.

27. A nucleic acid encoding a fusion protein according to claim 26.

28. A cell that contains and expresses a nucleic acid of claim 27.

29. A fusion protein comprising a protein that is a detectable group and a translocatable protein.

30. A nucleic acid encoding a fusion protein according to claim 29.

31. A cell that contains and expresses a nucleic acid according to claim 30.

32. A kit useful for detecting protein-protein interactions within a living cell, comprising:

(a) a cell that contains and expresses a nucleic acid encoding a first fusion protein, said fusion protein comprising a protein that specifically binds to an internal structure within said cell and a first protein of interest; together with

(b) a vector for said cell, said vector containing an expression cassette;

said expression cassette comprising a promoter operable in said cell and operatively associated with a nucleic acid encoding a detectable protein;

said expression cassette further comprising a splice site positioned adjacent said nucleic acid encoding a detectable protein so that a heterologous nucleic acid encoding a second protein of interest can be inserted therein to produce a nucleic acid segment encoding a second fusion protein;

said second fusion protein comprising said detectable protein and said second protein of interest.

33. A kit according to claim 32, wherein said vector is a plasmid.

34. A kit useful for detecting protein-protein interactions within a living cell, comprising:

(a) a cell that contains and expresses a nucleic acid encoding a first fusion protein, said fusion protein comprising a detectable protein and a first protein of interest; together with

(b) a vector for said cell, said vector containing an expression cassette,

said expression cassette comprising a promoter operable in said cell and operatively associated with a nucleic acid encoding a protein that specifically binds to an internal structure within said cell;

said expression cassette further comprising a splice site positioned adjacent said nucleic acid encoding a protein that specifically binds to an internal structure within said cell so that a heterologous nucleic acid encoding a second protein of interest can be inserted therein to produce a nucleic acid segment encoding a second fusion protein,

said second fusion protein comprising said protein that specifically binds to an internal structure within said cell and said second protein of interest.

35. A kit according to claim 34, wherein said vector is a plasmid.

36. A nucleic acid library comprising a plurality of separate nucleic acids, each of said separate nucleic acids encoding a fusion protein, said fusion protein comprising a protein of interest and a detectable protein, wherein said protein of interest encoded by each of said separate nucleic acids is different from the protein of interest encoded by the other nucleic acids of said library.

37. A nucleic acid library according to claim 36, wherein said detectable protein comprises a green fluorescent protein or a protein that specifically binds a fluorescent group.

38. A nucleic acid library according to claim 36, wherein said library is a cDNA library.

39. A nucleic acid library according to claim 36, wherein said library is a combinatorial library.

40. A nucleic acid library comprising a plurality of separate nucleic acids, each of said separate nucleic acids encoding a fusion protein, said fusion protein comprising a protein of interest and a protein that specifically binds to an internal structure within a cell, wherein said protein of interest encoded by each of said separate nucleic acids is different from the protein of interest encoded by the other nucleic acids of said library.

41. A nucleic acid library according to claim 40, wherein said protein that specifically binds to an internal structure within a cell is a translocatable protein.

42. A nucleic acid library according to claim 40, wherein said protein that specifically binds to an internal structure is a protein kinase C isoform or a fragment thereof that specifically binds to an internal structure.

43. A nucleic acid library according to claim 40, wherein said protein that specifically binds to an internal structure is a protein kinase C fragment selected from the group consisting of C1 domains and C2 domains.

44. A nucleic acid library according to claim 40, wherein said library is a cDNA library.

45. A nucleic acid library according to claim 40, wherein said library is a combinatorial library.

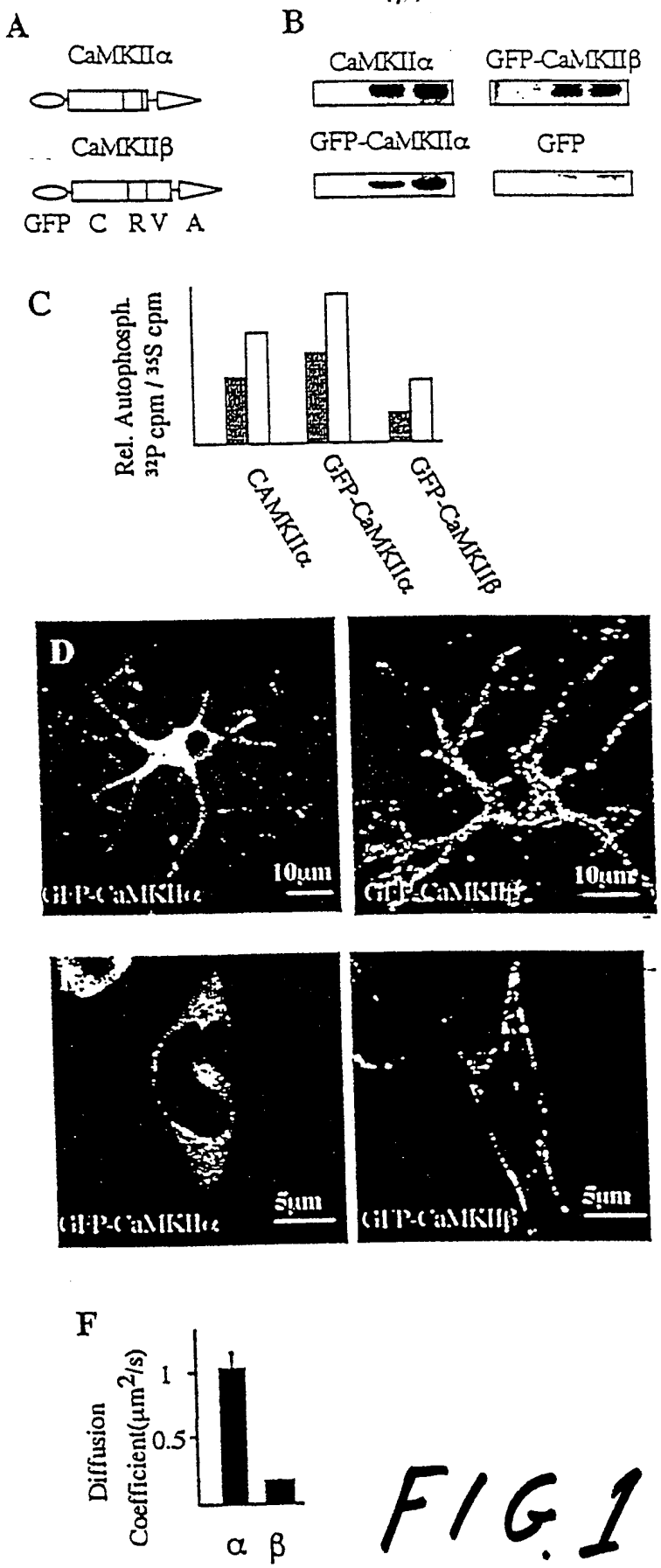
A
CaMKIIα
CaMKIIβ
GFP C RV A
B
CaMKIIα
GFP-CaMKIIβ
GFP-CaMKIIα
GFP
C
Rel. Autophosph.
32P cpm / 35S cpm
CAMKIIα
GFP-CaMKIIα
GFP-CaMKIIβ
D
10μm
10μm
5μm
5μm
F
Diffusion
Coefficient(μm2/s)
1
0.5
α β

FIG. 1

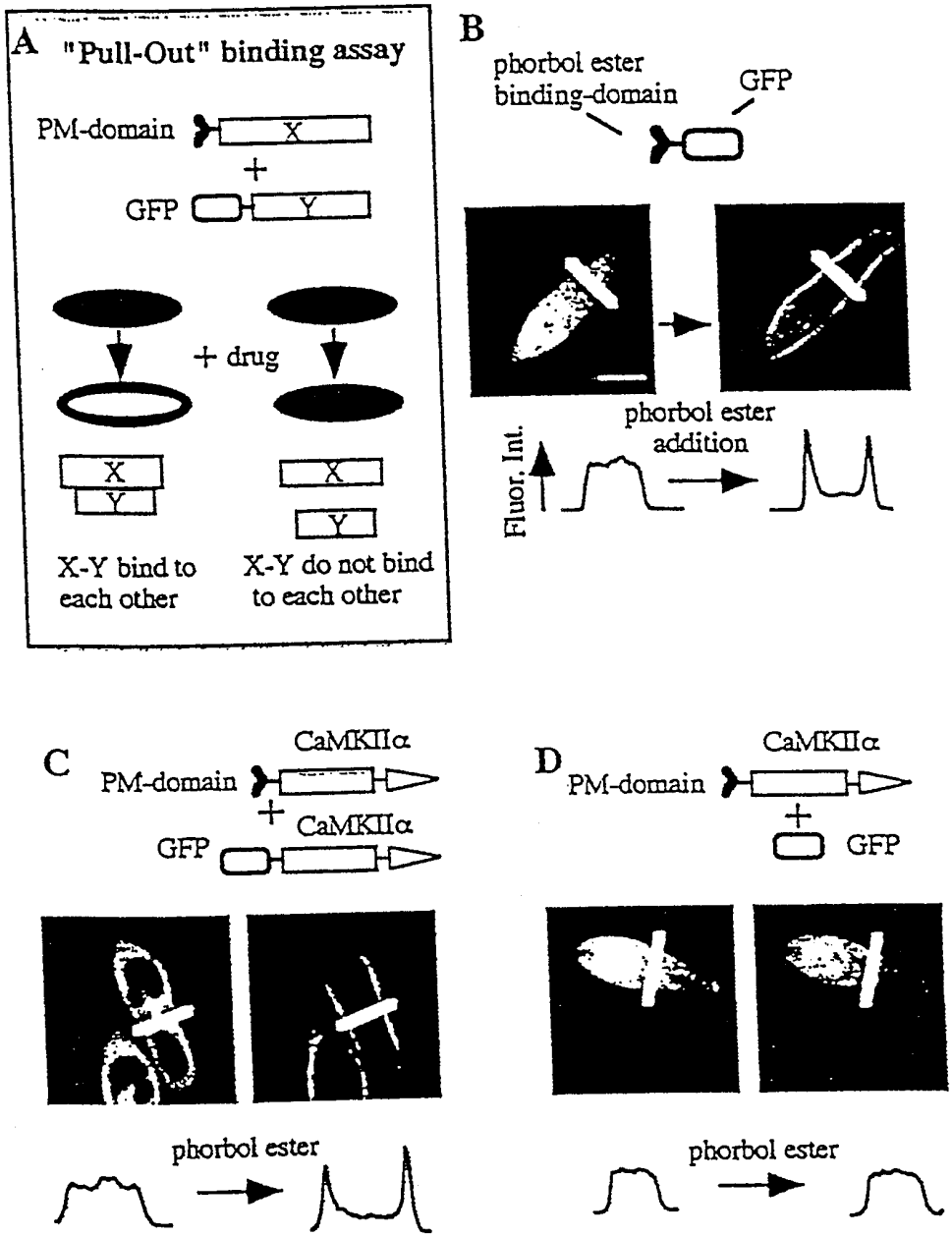
A "Pull-Out" binding assay
PM-domain
X
+
GFP
Y
+ drug
X
Y
X
Y
X-Y bind to each other
X-Y do not bind to each other
B
phorbol ester binding-domain
GFP
phorbol ester addition
Fluor. Int.
C
CaMKIIα
PM-domain
+
CaMKIIα
GFP
phorbol ester
D
CaMKIIα
PM-domain
+
GFP
phorbol ester

FIG. 2

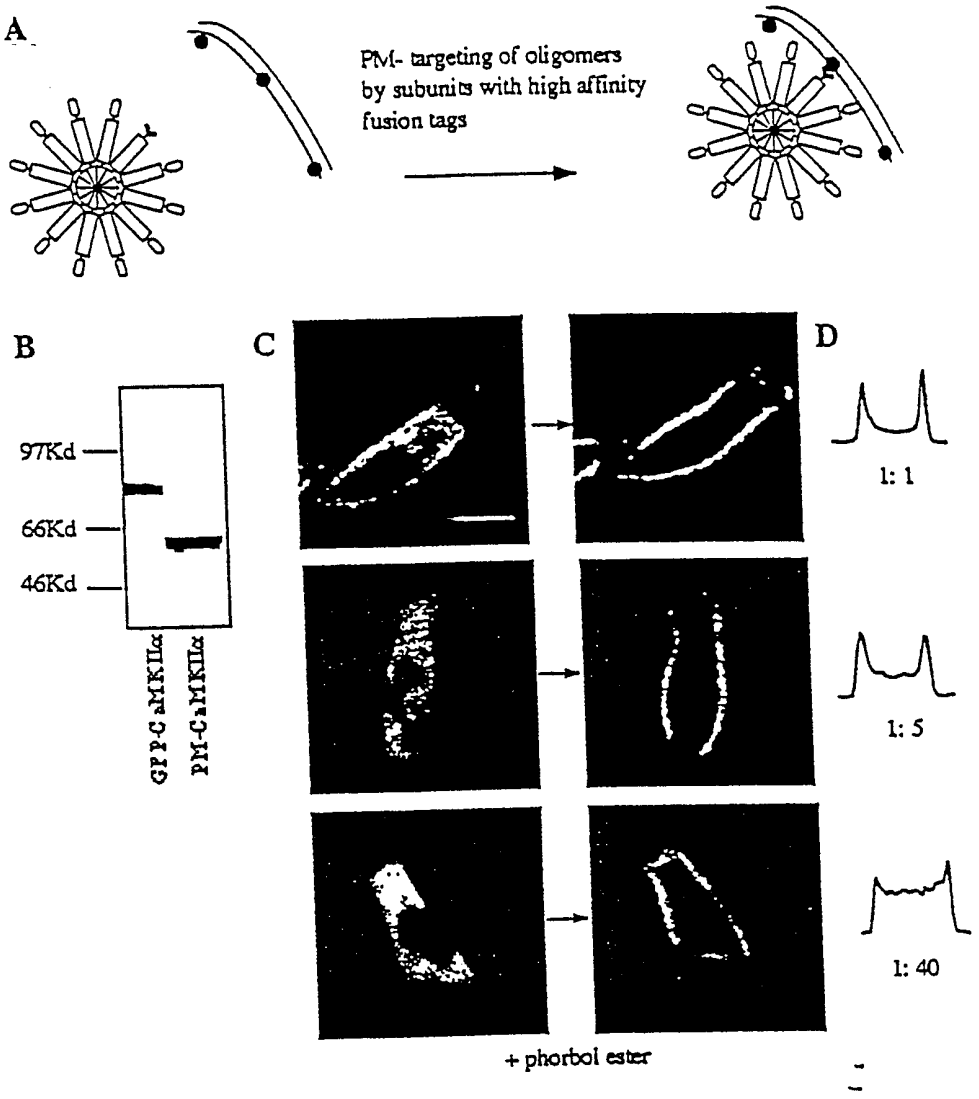
A
PM- targeting of oligomers
by subunits with high affinity
fusion tags
B
97Kd
66Kd
46Kd
GFP-CaMKIIα
PM-CaMKIIα
C
D
1: 1
1: 5
1: 40
+ phorbol ester
E
Ipre
+ phorbol
ester
ΔPM
F
Rel PM-translocation (ΔPM / Ipre)
X=CaMKIIα
X=CaMKIIβ
1:1
1:10
1:20
1:30
1:40
1:50
Protein dilution ratio (PM-X: GFP-X)

FIG. 3

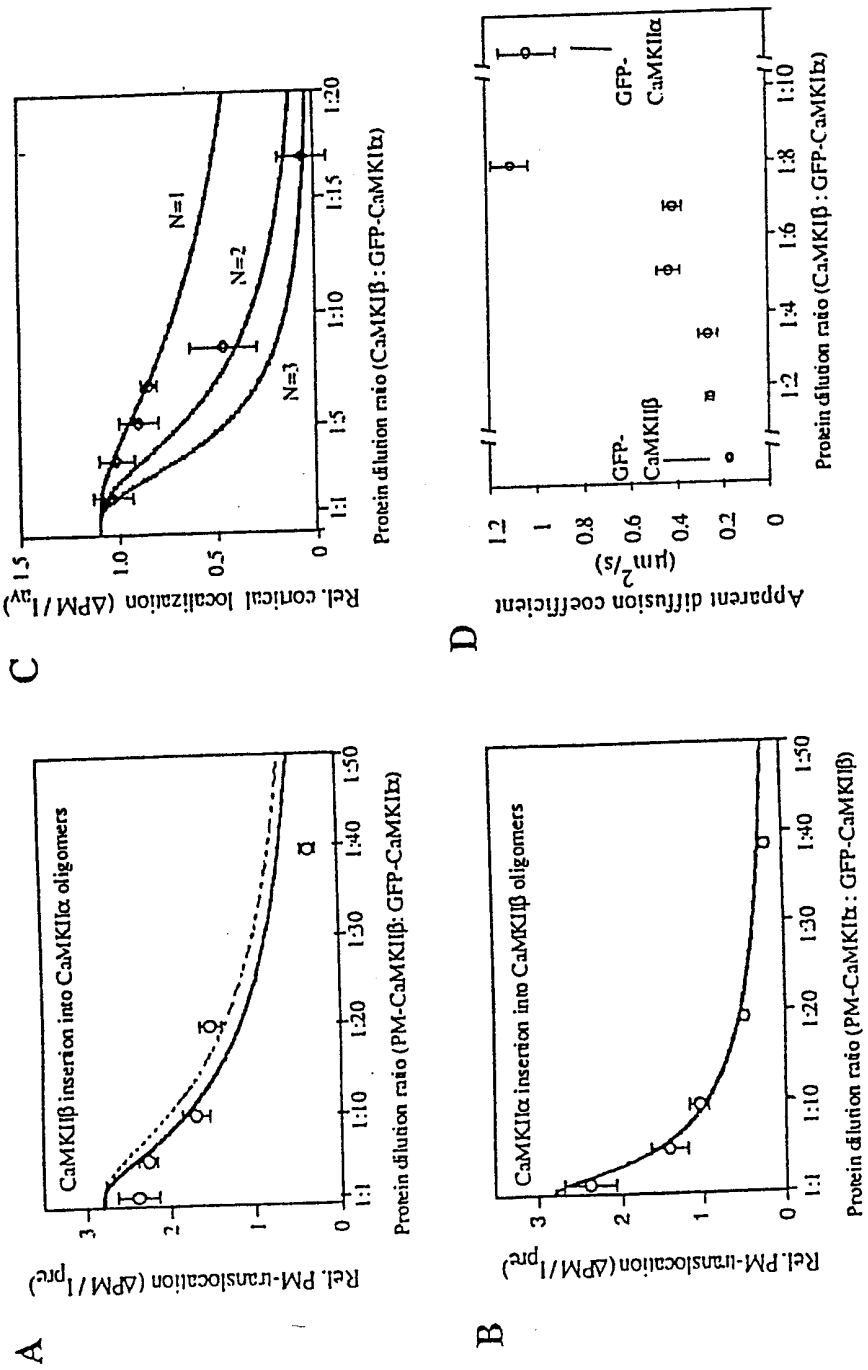
A
Rel. PM-translocation ($\Delta PM / I_{pre}$)
CaMKIIβ insertion into CaMKIIα oligomers
Protein dilution ratio (PM-CaMKIIβ: GFP-CaMKIIα)
B
Rel. PM-translocation ($\Delta PM / I_{pre}$)
CaMKIIα insertion into CaMKIIβ oligomers
Protein dilution ratio (PM-CaMKIIα : GFP-CaMKIIβ)
C
Rel. conical localization ($\Delta PM / I_{av}$)
N=1
N=2
N=3
Protein dilution ratio (CaMKIIβ : GFP-CaMKIIα)
D
Apparent diffusion coefficient ($\mu m^2/s$)
GFP-CaMKIIβ
GFP-CaMKIIα
Protein dilution ratio (CaMKIIβ : GFP-CaMKIIα)

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19118

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/04; C12Q 1/68
US CL :536/23.4; 435/6

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)

U.S. : 536/23.4; 435/6

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

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

EAST, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,871,907 A (WINTER et al) 16 February 1999, col. 13, lines 28-47; col. 16, line 47 - col. 17, line 52; col. 26, line 50.	1-6, 8-11, 21-22, 24-25, 26-41, 44-45
Y	US 5,580,717 A (DOWER et al) 03 December 1996, col. 2, lines 15-41; col. 3, line 55 up to col. 6, line 34.	1-6, 8-11, 15-18, 21, 24-41, 44-45
Y	BASS et al. Hormone Phage: an enrichment method for variant proteins with altered binding properties. Proteins: Structure, Function and Genetics. 1990, Vol. 8, pages 309-314, entire document.	1-11, 15-21, 24-41, 44-45.
Y	US 5,723,287 A (RUSSELL et al) 03 March 1998, entire document.	1-11, 15-21, 24-41, 44-45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"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
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 DECEMBER 1999	Date of mailing of the international search report 29 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer T. WESSENDORF Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19118

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BLOND-ELGUINDI et al. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell. 15 November 1993, Vol. 75, pages 717-728, entire document.	1-45
Y	US 5,804,604 A (FRANKEL et al) 08 September 1998, entire document.	1-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19118

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19118

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.
Group I, claims 1-25, drawn to a method of detecting protein-protein interaction.
Group II, claim 26, drawn to fusion protein comprising a protein that binds to an internal structure in a cell and protein of a member of a specific binding pair.
Group III, claims 27-28 and 32-33, drawn to nucleic acid, cell and kit.
Group IV, claim 29, drawn to a fusion protein comprising a protein that is a detectable group and a translocatable protein.
Group V, claims 30-31 and 34-35, drawn to nucleic acid, cell and kit.
Group VI, claims 36-39, drawn to nucleic acid library.
Group VII, claims 40-45, drawn to nucleic acid library.

The inventions listed as Groups I and II-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: as the different compounds of Groups II-VII can be used for other purposes such the nucleic acid as a probe or to produce a library of different probes.

The inventions listed as Groups II-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: as each of these compounds have different structures that can be produced by different methods.