

Use of a modified bacteriophage to probe the interactions between peptides and ion channel receptors in mammalian cells

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Besides natural peptide ligands, screening of random peptide libraries has yielded novel bioactive peptides for cell surface receptors. A method is described that uses a modified bacteriophage as a detection reagent to monitor the expression of receptor channels in mammalian cells and to probe the molecular interaction between phage-tethered peptides (Φ T-peptides) and specific receptor targets. By taking advantage of a specific multivalent interaction between Φ T-peptides and the receptor target, assays have been developed that use Φ T-peptides specific for the N-methyl-D-aspartate glutamate receptor, an important ligand-gated ion channel in the nervous system, to monitor the receptor expression in cultured mammalian cells. Combining these Φ T-peptide binding assays with fluorescence-activated cell sorting, 10⁴ random glutamate receptor mutants were screened and candidate interaction residues were identified. This dual heterologous expression system offers a powerful approach to the molecular studies of protein-protein interactions.

Keywords: NMDA receptors, random peptide display, receptor detection

Molecular studies of the extracellular surface of membrane receptors and ion channels rely mainly on monoclonal antibodies, chemical modifications, and pharmacology. Selection of random peptides for various target proteins has yielded a variety of interacting peptides and provided potentially new probes for detailed structure and function studies of these proteins. The random peptide libraries have been generated either by chemical synthesis or by translation of random nucleic acid codons^{1,2}. The approach that displays random peptides as part of phage coat protein has been the most widely adapted. This method has provided interesting peptides for studying various soluble target proteins³⁻⁹. Bioactive peptides have been reported to interact with the hydrophilic portions of transmembrane proteins, including erythropoietin receptor (EPOR)¹⁰ and N-methyl-D-aspartate (NMDA) receptor channels¹¹.

By taking advantage of the specific multivalent interactions between the Φ T-peptides and NR1, an essential subunit of NMDA receptors, phage-mediated immunostaining and fluorescence-activated cell sorting (FACS) procedures have been developed to monitor the expression of NMDA receptor protein in mammalian cells. An extension of these assays allows the screening of random NR1 mutants to identify candidate interacting residues on the receptor. These procedures expand the usage of peptide-coated bacteriophages and facilitate molecular characterization of peptide-receptor interactions.

Results

In vitro selection of peptides that interact with the NMDA receptor.

Using the putative amino-terminal domain of NR1 (N-NR1, amino acids 1-561) as a target protein and several libraries consisting of more than 10¹¹ random peptides, we have isolated several classes of NR1-interacting peptides, including the Mag-1.1 (CDGLRHMWFC) (ref. 11) and Mag-4 peptides (Fig. 1A). These two classes differ in primary sequences. Similar to peptides in the Mag-1 family, Mag-4.1 phage binds selectively to N-NR1, but it fails to bind the N-terminal domains of homologous subunits including N-NR2A and N-GluR1

(Fig. 1B). The binding of Mag-4.1 phage was inhibited by synthetic Mag-4.1 peptide with IC₅₀ of approximately 100 μ M. By contrast, a mutated Mag-4.1 peptide showed no inhibition (Fig. 1C).

Monitoring the expression of NMDA receptor by the modified bacteriophage. Each phage particle contains about 400 copies of the phage-tethered (Φ T)-Mag-4 peptide. Specific multivalent interactions between N-NR1 and Mag-4.1 phage (Fig. 1) suggest that phage particles can be used for receptor detection just as primary antibodies are used in immunoassays.

To determine whether Mag-4.1 phage could interact with an intact receptor complex on a cell surface, we incubated purified Mag-4.1 phage with human embryonic kidney (HEK) cells that transiently express functional NMDA receptor through assembly of NR1 and NR2A subunits¹¹. After unbound phage was removed, cells were stained with anti-phage antibody (see Experimental protocol). Mag-4.1 phage bound to a subset of the cells (Fig. 2A and B). The binding is sequence specific, evidenced by the fact that a control phagemid lacking the Mag-4.1 peptide failed to bind (data not shown). In addition, the Mag-4.1 phage binding was completely inhibited in the presence of 500 μ M synthetic Mag-4.1 peptide (Fig. 2C). High expression of conducting NMDA receptor in tissue culture cells results in Ca²⁺-mediated cell death, which increases the background of fluorescent stain and reduces recovery of plasmid DNA (see below). To avoid the cell death that results from the expression of NMDA receptor, Chinese hamster ovary (CHO) cell lines expressing N-NR1 were used in the remaining experiments. The specificity of Mag-4.1 stain was further confirmed by double staining with a mouse antibody for N-NR1 (Fig. 2E) and a rabbit antibody for Mag-4.1 phage (Fig. 2F).

To obtain a more quantitative assessment and detection sensitivity of phage staining conditions, FACS was used to monitor the phage binding. Wild-type CHO cells gave rise to background signal with the Mag-4.1 phage stain (Fig. 3A). This signal is similar to that obtained by staining the cells with secondary antibody alone

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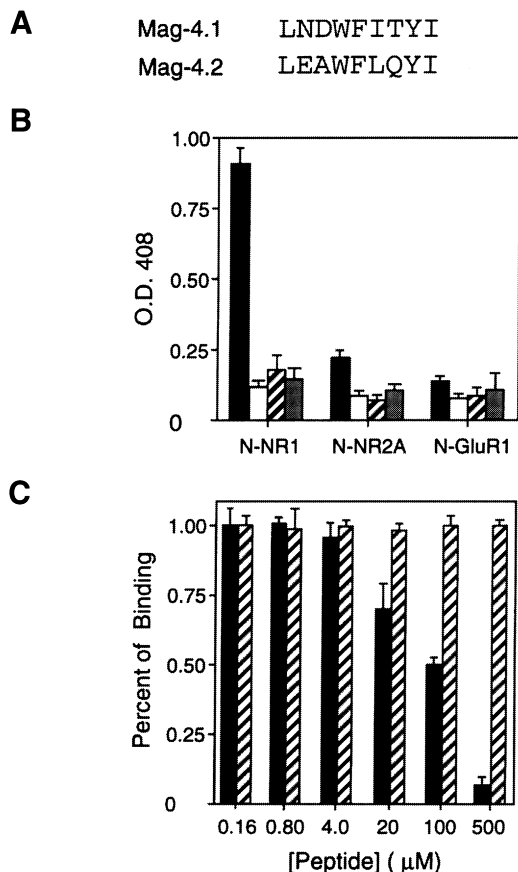


Figure 1. (A) Amino acid sequence alignment. Two phage clones encoding peptides Mag-4.1 and Mag-4.2, which specifically associate with N-NR1. (B) Binding specificity of Mag-4.1 peptides to N-NR1. Phage ELISA experiments¹¹ were carried out using N-NR1, N-NR2A, and N-GluR1 before (black bars) or after (gray bars) heat denaturation. The dashed bars indicate wells coated with bovine serum albumin (BSA) only; open bars, BSA and MabKT3. (C) Competition of Mag-4.1 phage binding by synthetic Mag-4.1 peptide (GGGLNDWFITYIGGG, black bars) or Mag-4.1 mutant peptide (GGGLNDGFITYIGGG, dashed bars). The vertical axis indicates the relative binding normalized against the signal obtained in the absence of Mag-4.1 peptide. The horizontal axis indicates peptide concentration. Error bars in panels B and C indicate standard errors of the mean ($n = 4$).

(data not shown). When CHO cells with a low density of N-NR1 protein (approximately 10^4 copies per cell) were incubated with Mag-4.1 phage, the fluorescence signal was elevated (Fig. 3B). Because the signal can be completely inhibited by the synthetic Mag-4.1 peptide (Fig. 3C), the staining was specific to N-NR1. Furthermore, when a 1:1 mixture of CHO cells expressing either 10^4 or 10^6 copies of N-NR1 (denoted as CHO-N-NR1L and CHO-N-NR1H) was subjected to the same staining procedure, the two populations of cells were clearly separated (Fig. 3D) and the staining of both populations of cells was similarly inhibited by synthetic Mag-4.1 peptide (Fig. 3E). Finally, the phage binding requires the Mag-4.1 peptide, since a phagemid lacking the recombinant peptide showed only background binding (Fig. 3F). Taken together, these results show that phage-mediated fluorescence stain can specifically detect expression of the corresponding receptors in cultured cells. The Φ T-Mag-4 peptide stain has a sensitivity of 10^4 copies of receptor per cell, which corresponds to an expression level that can be readily achieved by transient transfection.

Selection of interaction-defective NR1 mutants by phage-mediated FACS. The assays described above expand the use of phage clones obtained from random peptide screens, including

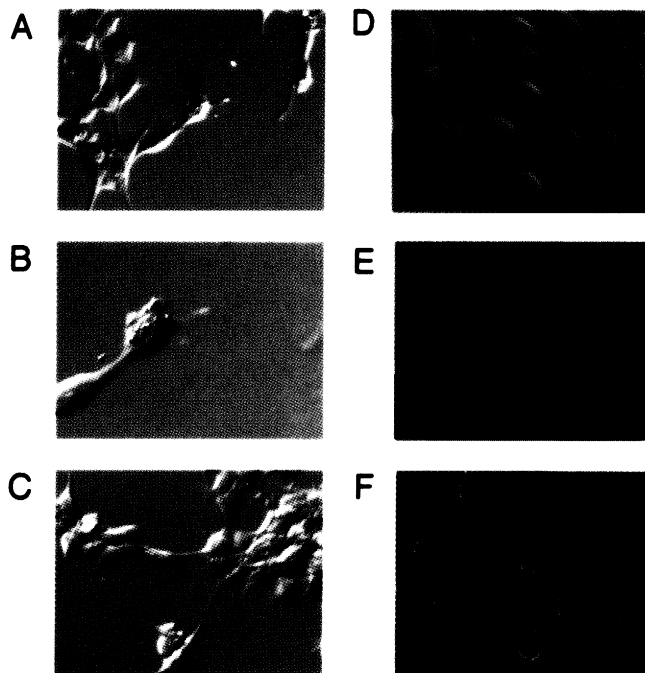


Figure 2. (A, B, C) Detection of NMDA receptor by Mag-4.1 phage. HEK 293 cells were cotransfected with plasmids expressing NR1 and NR2A cDNAs, which presumably formed functional NMDA receptor channels¹¹. At 48 h after transfection, the cells were incubated with Mag-4.1 phage. The phage binding was visualized by peroxidase-conjugated antibody. The same area of stained cells is shown at low (A) and high (B) magnification. (C) The phage stain in the presence of 500 μ M synthetic Mag-4.1 peptide. (D, E, F) Double stain of N-NR1 protein with MabKT3 and Mag-4.1 phage. N-NR1-positive Chinese hamster ovary (CHO) cell lines and wild-type CHO cells were mixed in a 1:1 ratio and allowed to grow on gelatin-coated cover glasses. The live cells were first incubated with Mag-4.1 phage. After fixation, they were incubated with MabKT3 (mouse) and anti-phage antibody (rabbit). The antibody binding was then detected with goat anti-mouse (using Texas red) and goat anti-rabbit (using fluorescein) antibodies. The same area of cells was visualized by (D) Nomaski, (E) MabKT3 binding, and (F) Mag-4.1 binding.

interacting peptides with relatively low affinity, such as Mag-4.1 (Fig. 1C). Because phage-mediated FACS is capable of identifying and separating a large pool of NR1 mutants that do or do not bind to Mag-4.1 phage, one may select NR1 mutants that no longer bind to Φ T-Mag-4 peptide. This would allow identification of candidate residues of the receptors that mediate the peptide-receptor interaction. We constructed a pool of N-NR1 mutants and cloned mutated coding fragments into an expression vector, which allows the N-NR1 to be expressed on the surface of CHO cells as fusion proteins anchored by glycosyl phosphatidylinositol (GPI)^{11,12}.

Because they carry the monoclonal epitope, the expressed mutants can be monitored by MabKT3 (see Experimental protocol). To identify N-NR1 mutants defective in binding to Mag-4.1, a pool of 10^5 independent clones (including an estimate of 10% mutants) was expressed transiently in CHO cells, which were subsequently stained with both MabKT3 (using phycoerythrin; PE) and Mag-4.1 (using fluorescein isothiocyanate; FITC). FACS purification of cells that were positive for MabKT3 but negative for Mag-4.1 allowed the isolation of Mag-4.1 binding-defective mutants that express full-length N-NR1 (Fig. 4A). The plasmid DNA was recovered from purified cells, amplified in *Escherichia coli*, and used for subsequent selection (see Experimental protocol). After three rounds of selection, individual plasmid was purified and tested by FACS for both the MabKT3 and Mag-4.1 binding.

After testing 10 randomly chosen clones, we found six clones

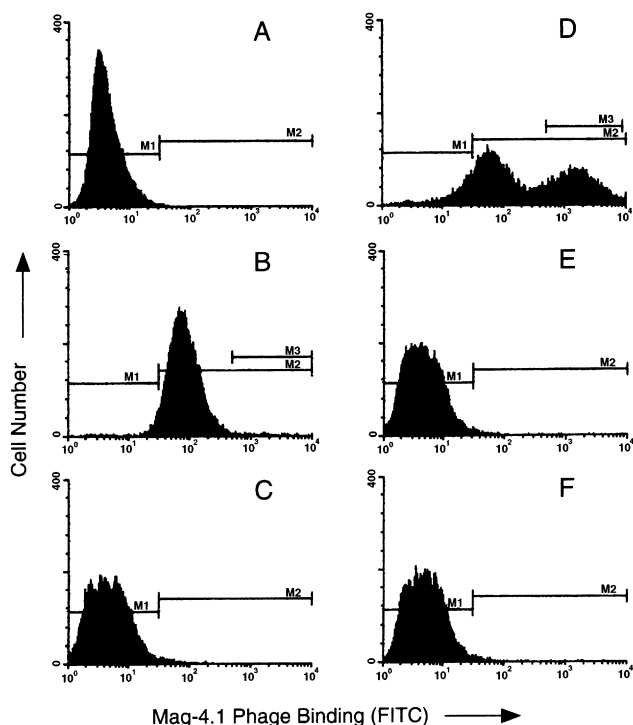


Figure 3. Detection of the N-NR1 protein in mammalian cells by FACS. Stable CHO cell lines expressing high (CHO-N-NR1H) and low (CHO-N-NR1L) levels of N-NR1 were used in phage binding, that was subsequently detected by anti-phage antibodies and FITC-conjugated anti-rabbit antibody. (A) wild-type CHO cells + Mag-4.1 phage; (B) CHO N-NR1L + Mag-4.1 phage; (C) CHO-N-NR1L + Mag-4.1 phage + 500 μ M Mag-4.1 peptide. (D to F) A 1:1 mixture of CHO-N-NR1H and CHO-N-NR1L was used. (D) cells were stained with Mag-4.1 phage alone; (E) cells were stained with Mag-4.1 phage in the presence of 500 μ M Mag-4.1 peptide; (F) cells were stained with wild-type phagemid.

that are positive for MabKT3 but negative for Mag-4.1 stain (Fig. 4B). Sequencing analysis revealed that five of the six clones have single base substitutions and the remaining one has two substitutions; all of these result in amino acid substitutions (Fig. 5A). A phage-mediated ELISA was used to test the mutated N-NR1 proteins individually for their ability to bind Φ T-Mag-4 peptide. Five of the six had significantly reduced binding, suggesting that these mutations perturb the peptide-receptor interaction (Fig. 5B). One mutant (A75P) showed binding similar to that of the wild-type. A difference in binding thresholds for ELISA and FACS might explain this discrepancy. The E188A and E192V substitutions are located near the insertion site of a splice variant of NR1, NR1.splice (Fig. 5A), suggesting that Mag-4.1 may distinguish between the two spliced forms of NR1 subunits. To examine this possibility, the amino-terminal domains of NR1 and NR1.splice (N-NR1 and N-NR1.splice) were expressed, and ELISA analysis was used to test them for binding to Mag-4.1 and Mag-1.5 (SDWCEGLQHMWFCSSL) (ref. 11) phages. Mag-1.5 binds to both the N-NR1 and N-NR1.splice¹¹ (Fig. 5B). In contrast, Mag-4.1 binds to N-NR1 but fails to interact with the N-NR1.splice. This result provides direct evidence that Mag-1.5 and Mag-4.1 recognize two distinct sites on NR1.

Mutations may cause the loss of Mag-4.1 binding by several mechanisms including major conformational changes independent of the Mag-4.1-receptor interaction. Such changes are likely to lead to nonfunctional receptors. To test whether the six mutants have resulted in large conformational changes, intact NR1 subunits carrying the corresponding mutations were coexpressed with NR2A. The channel opening was assayed by [³H]-MK-801 binding¹³. W56C,

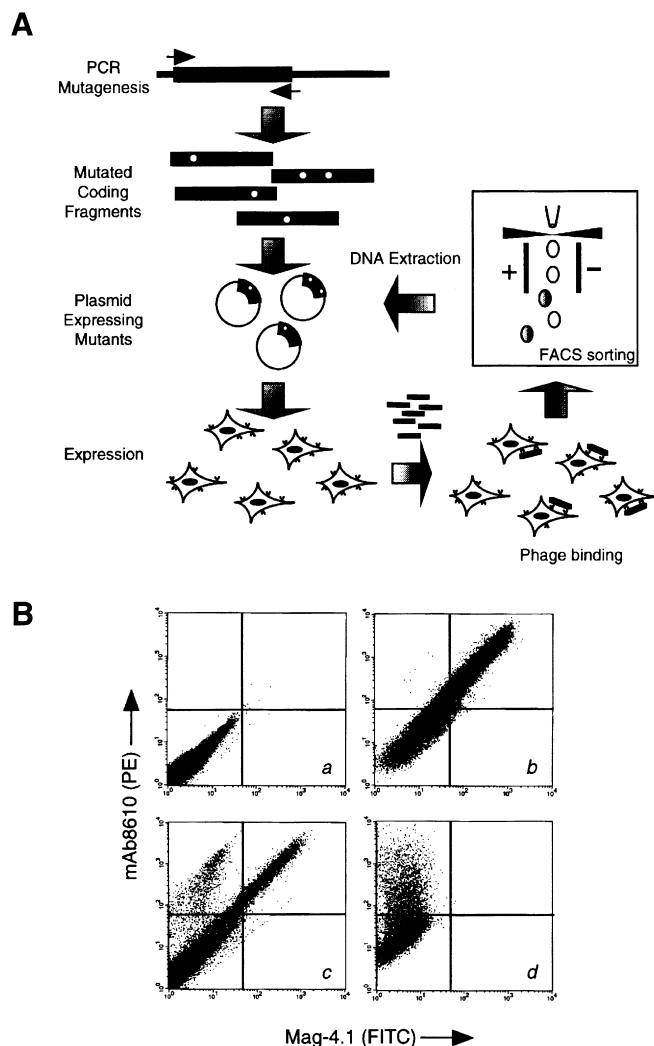


Figure 4. Selection of N-NR1 mutants from the randomly mutagenized library. (A) Scheme for the mutant selection. (B) Bivariate histograms for isolation of N-NR1 mutants defective for Mag-4.1 phage binding. CHO cells were transfected, and 48 h later, stained with both Mag-4.1 phage (using FITC) and MabKT3 (using PE): (a) mock transfected cells, (b) N-NR1 transfected cells, (c) mutagenized N-NR1 library (cells were sorted from those in the top left quadrant), (d) cells transfected with a plasmid encoding the N-NR1 (E188A) mutant.

A75P, E188A, and E192A had similar MK-801 binding to that of NR1 (Fig. 5C), consistent with the notion that these amino acid substitutions do not have detrimental effects on receptor conformation.

Discussion

Using a recombinant bacteriophage to monitor receptor expression is similar to using an antibody in an immunoassay¹⁴. Because the phage binding could be visualized by both enzyme-conjugated and fluorescent dye-conjugated secondary antibodies, the reported assays take advantage of existing signal amplification strategies. Thus, it is conceivable that Φ T-peptide binding may be extended to other antibody-mediated assays.

There is a key difference, however, between phage-mediated detection and the conventional primary antibody-mediated immunoassays. Generally, variable regions of immunoglobulin form a binding pocket that recognizes either the conformation or the primary sequence of a given epitope. Because the epitope can be a very small linear region, an immunoglobulin often will recognize a protein in both native and denatured conformations.

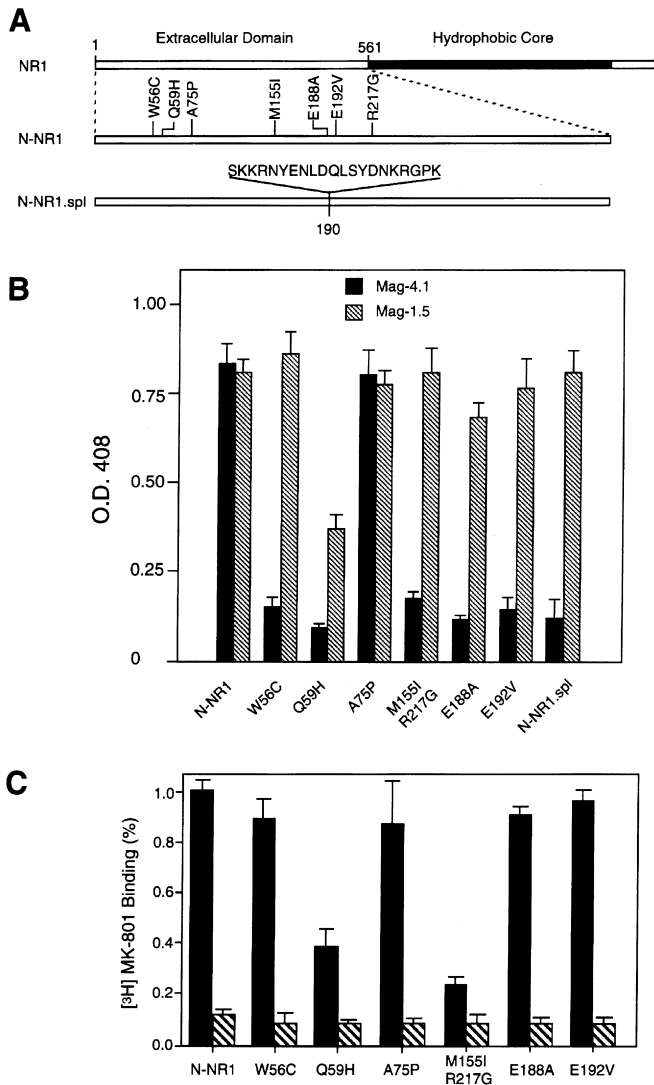


Figure 5. Selectivity of Mag-4.1 phage binding. (A) Schematic diagram of NR1 subunit. The black box indicates the hydrophobic core, which contains multiple transmembrane segments. Amino acids 1 to 561 represent the putative extracellular amino-terminal domain of NR1 (N-NR1). Amino acid substitutions in mutated N-NR1 are indicated. (B) ELISA test of N-NR1 mutants and N-NR1.splice for association with Mag-4.1 (black bars) and Mag-1.5 phage (dashed bars). (C) [³H]-MK-801 binding of transfected cells expressing NR1 mutants. The indicated NR1 mutants were coexpressed with NR2A subunit. The binding of [³H]-MK-801 was carried out in triplicate according to a published protocol^{11,19}. The signal (black bars) was normalized against NR1 wild-type. The dashed bars are signals obtained from cells that were transfected with the corresponding NR1 or NR1 mutants in the absence of NR2A. Error bars indicate standard errors of the mean (n = 3 in panel C, n = 4 in panel B).

By contrast, the phage binding described here requires that the corresponding receptor is in a certain conformation in order to bind peptides, although the conformation to which the peptides bind may not necessarily be native form. Because the conformation of the receptor binding pocket is critical for peptide binding, phage-mediated detection may be well suited for discriminating conformational differences in receptor variants. This notion is supported by the finding that Mag-4.1 peptide specifically interacts with NR1 but not its splice variant (Fig. 5).

As with antibody-antigen interaction, the specificity of phage staining may also depend on peptide sequence. For example, we have identified seven classes of N-NR1 binding peptides (Mag-1 to Mag-7) on the

basis of amino acid sequences. Except for Mag-1 peptides, which inhibit the channel activity, the biological activities of the other six classes have not been tested. Using the reported approach, we found that five out of the seven classes can also work in FACS staining, while the remaining two classes produced inconsistent staining results under the same conditions (data not shown). Furthermore, phage-mediated cell-binding assays can be negatively affected by other factors, such as nonspecific interactions between phage coat proteins and other cellular proteins. In contrast to antibody staining, recombinant phages are much less expensive to produce than antibody and do not require animals. In order to further reduce background binding, future experiments could focus on engineering phage variants with altered coat proteins. For example, one may consider mutating residues with positive charges into ones with neutral or negative charges in order to reduce potential nonspecific interaction of phage particles with carbohydrates on the cell surface.

The random peptide library has yielded novel peptides for important cellular receptors^{10,11,15,16}, and it promises more in the future. For recombinant or natural peptides with interesting biological or therapeutic activity, it is critical to understand their action at the molecular level. The method reported here is, in essence, to study ligand-receptor interaction by monitoring the association of cells with viral particles. Both receptor and ligand are heterologously expressed on the surface of either cells or viruses; this dual expression format offers many options including simultaneous genetic manipulations of both receptors and peptide ligands. In addition, natural peptide ligands, such as angiotensin II, may also be displayed on phage. Thus, this method should be applicable to other molecular studies of protein-protein interactions including ligand-receptor interactions and virus-host interactions.

Experimental protocol

Expression and mutagenesis of the amino-terminal domains of glutamate receptors. Procedures for cloning and expression of glutamate receptors were as described¹¹, except that the following amino acid sequence was fused to the carboxyl terminus of expressed coding regions: AAADYKSTTHEE-AAASYPYDVPDYASLGGPPPAGTTDAHPGRSVVPLLPLLAGTLLLETATAP. This fragment contains two epitope tags—DYKSTTHEE (KT3) and SYPYDVPDYASLGG (12CA5)—and a GPI anchor recognition sequence²². In order to generate mutations in the coding region between amino acid 1 and amino acid 306 of NR1, two primers (AGCTTGATATCGAATTCC and GTGTTGCCACGCAACCC) were used to amplify the DNA region under conditions of reduced *Taq* polymerase fidelity¹⁷. The resulting mutagenized PCR fragments span the EcoRV (5' in the vector) and SacII sites in the coding region. The mutant N-NR1 library was constructed by inserting the PCR-amplified products after digestion with EcoRV and SacII.

Phage selection and purification. The published protocol¹¹ was used for phage selection, except that the N-NR1 protein was first treated with endo-F and endo-H (1 unit per 10 μg protein) in the presence of 0.1% sodium dodecyl sulfate overnight at 37°C. The phage binding was carried out by incubating with a total of 10¹² transducing units (tu) of a random nonamer library with a complexity of 1.2 × 10¹⁰. The infected bacteria were cultured overnight, and the resultant culture medium containing phage was spun at 5000 × g for 30 min. To precipitate the phage in the supernatant, 0.2 volumes of 20% polyethylene glycol (average molecular weight 8000) with 2.5 M NaCl were added. After incubation on ice for 1 h, the precipitated phage was collected by centrifugation at 12,000 × g for 30 min. The pellet was resuspended in 1:10 of the starting culture volume and dialyzed for 48 h against phosphate-buffered saline (PBS) using dialysis membrane with a 12-kDa to 14-kDa cutoff (Spectrum Medical Industries, Los Angeles, CA). The resultant phage preparation was titered and stored in PBS supplemented with 0.1% bovine serum albumin (BSA).

Immunostaining and FACS. The cells were grown on cover glasses coated with 1% gelatin. Before immunoglobulin was added, the cells were first washed three times with cold PBS and fixed overnight with 2% paraformaldehyde (PFA). After being washed again with cold PBS, the fixed cells were incubated with MabKT3 (1 mg/ml) at room temperature for 1 h. The binding of MabKT3 was detected by incubating with anti-mouse antibody conjugated with Texas red (15 μg/ml, Vector Laboratories, Burlingame, CA) for 1 h at 4°C. The

immunostain was visualized with a Nikon Optiphot-2 fluorescent microscope, and images were collected with a 20× differential interference contrast lens.

Phage-mediated immunostaining, FACS detection, and plasmid recovery. The purified phage preparation was used to stain live cells. Cells were first rinsed three times with cold minimal essential medium. Phage binding was initiated by incubating the cells with purified phage (10^{10} tu/ml) on ice for 1 h. After incubation, the unbound phage was removed by three washes with cold PBS supplemented with 1% BSA. The fixation was carried out using 2% PFA overnight at 4°C. The phage binding was detected by first adding rabbit anti-phage antibody (1:5000 dilution; Pharmacia, Uppsala, Sweden) and then fluorescein-labeled antirabbit antibody (10 mg/ml, Vector Laboratories). The labeled cells were visualized under a fluorescence microscope.

For phage-mediated FACS, the transfected cells were first dislodged from their dishes at 48 h using PBS supplemented with 5 mM EDTA. The transfected cells were washed twice with cold PBS and stained with Mag-4.1 phage (10^{10} tu/ml) and MabKT3. The remaining steps were identical to the procedures described above except that PE-conjugated anti-mouse antibody was used to detect the MabKT3 binding. The cytometry of stained cells was analyzed on a FACScan or FACStar Plus Sorter (Becton Dickinson, San Jose, CA). For FACS, samples were analyzed by 488 nm argon excitation, with emission detected with a 525±30 nm bandpass filter for FITC and a 575±30 nm bandpass filter for PE after propidium-stained cells were electronically removed. Recovery of the plasmid DNA in transfected cells after sorting was carried out essentially according to a protocol by Hirt¹⁸.

Acknowledgments

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