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Targeting inhibition of GluR1 Ser⁸⁴⁵ phosphorylation with an RNA aptamer that blocks AMPA receptor trafficking

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Abstract

Phosphorylation of GluR1 Ser⁸⁴⁵ subunit has been widely accepted to be involved in AMPA receptor trafficking and behavioral actions of psychotimulants. However, it still remains to be directly established whether GluR1 phosphorylation plays a key role in AMPA receptor trafficking *in vivo* due to lack of effective methodologies for selectively targeting phosphorylation at single amino acid residue. In this study, we have successfully selected aptamers which effectively bind to phospho-Ser⁸⁴⁵ GluR1, but have no affinity for phospho-Ser⁸³¹ GluR1. Moreover, these aptamers inhibit protein kinase A-mediated phosphorylation at Ser⁸⁴⁵ via binding to the unphospho-GluR1. Importantly, representative aptamer A2 can effectively inhibit GluR1/GluR1-containing AMPA receptor trafficking to the cell surface and abrogate forskolin-stimulated phosphorylation at GluR1 Ser⁸⁴⁵ in both GFP-GluR1-transfected HEK293 cells and cultured rat cortical neurons. The strategy to use aptamer to modify single-residue phosphorylation is expected to facilitate evaluation of the potential role of AMPA receptors in various forms of synaptic plasticity including those underlying psychostimulant abuse.

Keywords

aptamer; GluR1; phosphorylation; trafficking; protein kinase A; protein kinase C

Introduction

AMPA receptors mediate a part of the excitatory synaptic transmission in the brain and their redistribution to synaptic membranes contributes to synaptic plasticity which underlies learning and memory, developmental maturation of neuronal circuits, and behavioral effects of drug addiction (Esteban et al., 2003; Boudreau and Wolf, 2005; Sun et al., 2005; Zhang et al., 2007). AMPA receptors are tetramers comprised of various combinations of four subunits, GluR1–GluR4 (Barry and Ziff, 2002). Among various mechanisms, phosphorylation of the GluR1 subunit at the Ser⁸³¹, Ser⁸⁴⁵, and Ser⁸¹⁸ residues in its

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intracellular carboxy-terminal domain plays a major role in regulating AMPA neurotransmission. Cell-based studies suggest that PKA-mediated phosphorylation of Ser⁸⁴⁵ can stabilize GluR1 in the plasma membrane, enhance the incorporation of GluR1-containing AMPA receptors into the synaptic membrane (Lee et al., 2000; Havekes et al., 2007) and decrease AMPA receptor internalization (Man et al., 2007), thus allowing for dynamic regulation of AMPA receptor trafficking. However, whether specific phosphorylation of the Ser⁸⁴⁵ residue serves the same function *in vivo* (intact animals) has not been directly addressed.

Recent studies have demonstrated that phosphorylation at Ser⁸⁴⁵ of GluR1 subunit, but not Ser⁸³¹, is significantly increased in the striatum and prefrontal cortex *in vivo* in response to the psychostimulant cocaine (Snyder et al., 2000; Edwards et al., 2007; Tropea et al., 2008). We have also demonstrated that: (1) long-term behavioral sensitization established by daily injections of high-dose cocaine and a chronic withdrawal increases phosphorylation of GluR1 Ser⁸⁴⁵ but not Ser⁸³¹ residue in the prefrontal cortex and nucleus accumbens; and (2) these increases are normalized when the established behavioral sensitization is reversed (Zhang et al., 2007). Furthermore, Boudreau and Wolf (2005) have shown that cocaine behavioral sensitization is associated with increased surface/intracellular ratios of GluR1 subunit in the nucleus accumbens (NAc). However, whether Ser⁸⁴⁵ phosphorylation of GluR1 subunit plays direct roles in cocaine sensitization remains to be elucidated. One of the main reasons for this shortcoming is that effective methodologies for selective suppression of *in vivo* phosphorylation at single amino acid residues have not been established. Thus, a protocol for specifically inhibiting single residue phosphorylation of GluR1 subunit or its functional consequences is expected to provide the first step toward elucidating roles of this and other protein phosphorylation events in physiology.

A technology has recently emerged that can evaluate the role of single residue phosphorylation events. Aptamers have been shown to possess the ability to bind targeted proteins with high affinity and specificity (Ellington and Szostak, 1990, 1992; Sullenger et al., 1990; Hermann and Patel, 2000), and, as nucleic acids, they exhibit increased stability, ease of generation, and simple modification which offers advantages over antibodies to artificially interfere with target gene function (Murphy et al., 2003; Hirao et al., 2004). More importantly, aptamers can be selected *in vitro* and the process can be tightly controlled (Griffin et al., 1993). In the present study we have developed and identified aptamers to selectively inhibit GluR1 phosphorylation at Ser⁸⁴⁵ and GluR1-containing AMPA receptor trafficking to the cell surface.

Materials and Methods

Production of a GST-fused C-terminal GluR1

The intracellular domain of the rat GluR1 subunit cDNA (deduced amino acids 808 to 889) was amplified by reverse transcriptase PCR from a rat RNA library with the forward primer: 5'-CGGGATCC *GGTGGCGGAGGGTCTGGAGAGTTCTGCTACAAATCCCG*-3' and reverse primer: 5'-GGAATTCCTTACAATCCTGTGGCTCCCAAG-3'. A 6-glycine hinge sequence (*italic*) and a *Bam*HI site (*underline*) were designed in the forward primer and an *Eco*RI site (*underline*) in the reverse primer. The amplified fragment was cloned into pGEX-2T vector at the *Bam*HI/*Eco*RI sites downstream of the GST, producing a fused GST/6 glycine/GluR1 protein (cGluR1). The plasmid was transformed into BL21 (DE3) *E. coli*. After the bacteria were grown in SOC medium to 1 unit optical density (OD) at 550 nm at 37°C, cGluR1 protein expression was induced with 0.4 mmol/L isopropyl thio-β-D-galactoside for 6 hrs at 22°C. Cells were sonicated in 10× volume of ice-cold PBS containing 1mmol/L phenylmethylsulfonyl fluoride (PMSF). The lysate was then centrifuged at 10,000 × g for 20 min and the supernatant was applied to a PBS pre-

equilibrated glutathione Sepharose column (GE Healthcare, San Diego, CA, USA). The bound protein was eluted with elution buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L reduced glutathione), and the concentration was determined by Bradford assay (Bradford, 1976).

In vitro phosphorylation of GluR1 protein

To produce phospho-Ser⁸⁴⁵ GluR1 protein, 1.6 mg of purified cGluR1 protein was incubated with reaction buffer 1 (40 mmol/L Tris-HCl, pH 7.4, 20 mmol/L MgAc₂), 200 μ mol/L ATP, and 268 units of PKA in 5 ml reaction volume for 2 hrs at 30°C. After reaction, the protein was isolated using the glutathione Sepharose column and phosphorylation of the protein was verified by Western blot using a site-specific anti-GluR1 Ser⁸⁴⁵ antibody (1:500 dilution, Upstate Inc., Lake Placid, NY, USA).

To produce phospho-Ser⁸³¹ GluR1 protein, 1 mg of cGluR1 protein was incubated with reaction buffer 2 (8 mmol/L MOPS, pH 7.0, 200 μ mol/L EDTA, pH 8.0, 15 mmol/L MgCl₂), lipid activator, 5 μ g active protein kinase C- θ (PKC- θ ; Millipore, Bedford, MA, USA), and 100 μ mol/L ATP in a 3 ml reaction volume at 30°C for 30 min. Following the reaction, the protein was isolated using the glutathione Sepharose column and phosphorylation was verified by Western blot using site-specific anti-GluR1 Ser⁸³¹ antibody (1:500, Upstate Inc., Lake Placid, NY, USA).

Selection procedure

In vitro selection was performed as previously described with modifications (Ishizaki et al., 1996; Rusconi et al., 2002). Library RNA (a random pool of RNA oligonucleotides) was generated by *in vitro* transcription with 2'-fluoro CTP and UTP (TriLink Biotech, San Diego, CA), 2'-hydroxy GTP and ATP (Roche, Florence, SC), and mutant T7 RNA polymerase (Sousa and Padilla, 1995) from synthesized DNA oligos (Oligos Etc. Inc., Wilsonville, OR, USA). The sequence of RNA library is as follows: 5'-GGGAGGACGAUGCGG(N40)CAGACGACUCGCUGAGGAUCCGAGA-3'. N40 represents 40 random nucleotides with equal molar A,G,C,U. Prior to each round selection, 1000-pmol RNA was pre-cleared with nitrocellular disc, then incubated with unphospho-cGluR1 protein for 15 min at 37°C. After passing the solution through a nitrocellulose membrane, the RNA bound to the unphospho-cGluR1 and disc was then removed. The flow-through was incubated with phospho-Ser⁸⁴⁵ cGluR1 at 37°C for 15 min and was then loaded onto a nitrocellular disc with vacuum. Free RNA was removed and phospho-protein bound RNA was bound on the disc. After washing with selection buffer F (20 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 2 mmol/L CaCl₂, 0.1g/L BSA), the nitrocellulose membrane was cut into small pieces for purification with phenol/chloroform extraction and reverse transcriptase PCR amplification. The PCR products were T7 transcribed and the majority of RNA were used for next round selection. One-hundred pmol RNA was retained for determination of the binding affinity. The fraction of bound RNA was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). For desired rounds, selected RNAs were reverse-transcribed, amplified, cloned, and sequenced as described previously (Ishizaki et al., 1996; Rusconi et al., 2002).

Binding assay

We determined RNA-protein equilibrium dissociation constants (i.e., K_d) by the double-filter binding method (Wong and Lohman, 1993). For all binding assays, RNA was dephosphorylated by incubating with bacterial alkaline phosphatase at 50°C for 1 hr. After phenol/chloroform extraction and ethanol precipitation, purified RNA was 5'-end labeled using T4 polynucleotide kinase and [γ -³²P] ATP. Binding assays were performed by incubating 10,000 counts per minute (CPM) ³²P-labeled RNA with unphospho-cGluR1 and

phospho-Ser⁸⁴⁵ cGluR1 protein (0.03 to 2 μ mol/L) in selection buffer F at 37°C for 15 min and then loaded onto the double-layer binding system where the upper nitrocellulose membrane traps the RNA-protein complex and the lower nylon membrane traps the free RNA. The K_d and maximal binding values (B_{max}) were calculated using Prism software (Irvine, CA, USA). Binding data were corrected for nonspecific background binding of radiolabeled RNA to the nitrocellulose filter to obtain adjusted fraction binding.

Co-immunoprecipitation

To determine whether the aptamers bind to the mammalian GluR1 subunit, 10 pmoles of aptamer or library RNA were ³²P-labeled and pre-incubated at 70°C for 5 min, then at 37°C for 15 min to assure proper folding. The pre-treated RNA was incubated with 100 μ g rat brain tissue homogenate and 30 μ g of tRNA at 37°C for 30 min, and then incubated with 2.5 μ g of anti-GluR1 antibody (Upstate Inc., Lake Placid, NY, USA) for 1 hr. This antibody recognizes the carboxy terminal region (amino acids 877-889: SHSSGMPLGATGL) of rat GluR1 subunit. The antibody/protein/aptamer complex was precipitated by incubating it with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 37°C for 1 hr. After washing the agarose 6 $\times$ with 1 ml washing buffer (10g/L SDS, 5 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, 10 μ g/ml aprotinin, 0.5 mmol/L PMSF, 50 mmol/L sodium fluoride, 50 mmol/L sodium pyrophosphate, 20 mmol/L 2-glycerol phosphate, 1 mmol/L p-nitrophenyl phosphate, 2 μ mol/L microcystin LR), samples were boiled in 2 $\times$ SDS sample buffer (160 ml/L glycerol, 20g/L SDS, 5.76 mmol/L β -mercaptoethanol, 4 mmol/L EDTA, 100 mmol/L Tris-HCl pH 6.8, 0.1g/L bromophenol blue) for 8 min and the supernatant (10 μ l) was separated on 12% SDS-PAGE gel for autoradiography.

The binding of aptamers to the rat GluR1 subunit was also examined with biotin-labeled oligos. Two hundred pmoles of aptamer or library RNA were annealed with 2.5 μ g of biotin-labeled oligo to form a biotin-oligo/aptamer complex. This complex was then incubated with 100 μ g rat brain tissue homogenate or lysate from HEK293 cells (transfected with a plasmid that expresses the full length rat GluR1 subunit) and 60 μ l Streptavidin Gel (Pierce, Rockford, IL, USA) at 37°C for 60 min. The gel was washed with washing buffer as described above. After boiling in 2 $\times$ SDS sample buffer for 8 min, the supernatant (10 μ l) was separated on 7% SDS-PAGE gel and subjected to Western blot using anti-GluR1 and site-specific anti-GluR1 Ser⁸⁴⁵ antibodies.

Western blotting

Samples were separated on 7% or 12% SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were rinsed with PBS and non-specific sites were blocked with PBST buffer (PBS - 0.05% Tween-20) containing 50g/L non-fat dry milk for 0.5-1 hr at 22°C. After incubation with primary antibody in the PBST buffer containing 50g/L milk for 2 hrs at 22°C, the membranes were sequentially washed with PBST for 4 $\times$ 15 min and re-blocked for 1 hr with PBST-milk, then incubated with peroxidase-labeled secondary antibody at a 1:5,000 dilution (Sigma, St. Louis, MO, USA) for 2 hrs at 22°C. After washing with PBST for 4 $\times$ 15 min, the blots were developed with Chemiluminescent substrate (Pierce Chemical Company, Rockford, IL, USA).

Inhibition of phosphorylation

To analyze whether the binding of selected aptamers to the cGluR1 protein could prevent PKA-mediated Ser⁸⁴⁵ phosphorylation, the aptamers were pre-heated at 70°C for 5 min, and then at 37°C for 15 min. Four hundred pmoles of pre-treated aptamer A1, A2, A3, or library, or different concentration of aptamer A2 (0, 50, 100, 200, 400 pmoles) was incubated with 100 pmols of unphospho-cGluR1 protein at 37°C for 15 min. The aptamer/cGluR1 mixture was subsequently incubated with reaction buffer 1, 200 μ mol/L ATP, and 0.5 units of PKA

in a 25 μ l reaction volume for 25 min at 30°C. The reaction was terminated by adding 5 μ l of 6 $\times$ SDS sample buffer and boiling for 8 min. The phosphorylation efficacy was determined by Western blot using anti-GluR1 and site-specific anti-GluR1 Ser⁸⁴⁵ antibodies.

Alternatively, the aptamer/cGluR1 mixture was incubated with reaction buffer 1, 200 μ mol/L ATP, 10 μ Ci of [γ -³²P]ATP, and 0.5 unit of PKA in a 25 μ l reaction volume for 25 min at 30°C. The reaction was transferred onto the center of a 2cm $\times$ 2cm P81 paper square (Millipore, Bedford, MA, USA), for a period of 30 seconds before transferring into a 50 ml tube. After washing the squares for 3 $\times$ 5 min with 40 ml of 0.75% (v/v) phosphoric acid, following by washing 5 min with 20 ml of acetone, the squares were then transferred to a scintillation vial containing 5 ml of scintillation cocktail. The CPM values were analyzed.

To analyze whether aptamers could prevent Ser⁸³¹ phosphorylation of the GluR1 subunit, 100 pmols of unphospho-cGluR1 protein were incubated with different concentrations of pre-treated aptamer A2 (0, 50, 100, 200, 400 pmols) at 37°C for 15 min. The aptamer/cGluR1 mixture was subsequently incubated with reaction buffer 2, 100 μ mol/L ATP, PKC lipid activator, and 25 ng active PKC- θ in a 25 μ l reaction volume for 10 min at 30°C. The reaction was stopped as described above and the phosphorylation efficacy was determined by Western blot using anti-GluR1 and site-specific anti-GluR1 Ser⁸³¹ antibodies.

Cell culture and transfection

HEK293 cells were grown in Dulbecco's Modified Eagle Media (Invitrogen Inc., Carlsbad, CA, USA) with 100ml/L fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂. Primary cortical neuron cultures were prepared as described (Sekine-Aizawa and Haganir, 2004). Briefly, prenatal (embryonic day 18) rat cortical neurons were plated into 12-well plates (2-3 $\times$ 10⁶ cells), pre-coated with poly-llysine, and maintained in medium that was free of 2-amino-5-phosphonovaleric acid for 2 weeks. Transfection was performed by using the lipofectamine 2000 according to the manufacture's manual (Invitrogen, Carlsbad, CA, USA).

Biotinylation assay of AMPA receptor-surface expression

The high-density cultured cortical neurons were transfected with 2'-fluoro-pyrimidine-modified library RNA or aptamer A2 for 36 hrs. The transfection efficacy was verified by Cy5-labeled aptamer A2 separately. The HEK293 cells were co-transfected with GFP-GluR1 expression plasmid (Shi et al., 1999) and library or aptamer A2 for 36 hrs. The transfection efficacy was verified by the expression of GFP. After treatments, cortical neurons or HEK293 cells were rinsed with CSF buffer (150 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L Hepes, 10 mmol/L glucose, pH 7.4) and treated with 20 μ mol/L forskolin plus 50 μ mol/L 3-isobutyl-1-methylxanthine in CSF buffer at 37°C for 15 min (Man et al., 2007). After washing with ice-cold CSF, cells were then incubated with 1 mmol/L sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) in CSF buffer at 10°C for 30 min and lysed with RIPA buffer (0.15 mmol/L NaCl, 0.05 mmol/L Tris-HCl, pH 7.4, 10 μ g/ml aprotinin, 0.5 mmol/L PMSF, 10g/L sodium deoxycholate, 10ml/L Triton X-100, 1g/L SDS). The sulfo-NHS-SS-biotin only binds to the proteins on cell surface. Biotinylated cell surface proteins were precipitated with immobilized Streptavidin beads, and the cell surface GluR1 expression was probed with anti-GluR1 antibody. The cell lysate was collected for phosphorylation assay with site-specific anti-GluR1 Ser⁸⁴⁵ or Ser⁸³¹ antibodies.

Results

Selection of aptamers to target the phospho-Ser⁸⁴⁵ GluR1 subunit

The GluR1 subunit of the AMPA receptor is phosphorylated by PKA at the Ser⁸⁴⁵ and by PKC or CamKII at the Ser⁸³¹ residue (Derkach et al., 1999; Lee et al., 2000). The intracellular domain of rat GluR1 was fused to GST by a polyglycine linker and the *E. coli* expressed protein was purified and phosphorylated by PKA or PKC. The efficacy of phosphorylation was evaluated by mixing different amounts (0, 20, 40, 60, 80, or 100%) of phospho-cGluR1 with unphospho-cGluR1 protein, following by Western blot analyses of the ratio of phospho-cGluR1 to total cGluR1 protein. The results showed that the Ser⁸⁴⁵ and Ser⁸³¹ residues of cGluR1 protein were effectively phosphorylated by PKA (Fig. 1A) and PKC (Fig. 1B), respectively. In vitro selection was performed with phospho-Ser⁸⁴⁵ cGluR1 and pre-cleared with unphospho-cGluR1 protein. After 12 rounds, the binding properties of the starting (library) and final pools were compared. The affinity of final pool for the target was significantly increased compared to the starting pool. Round 12 had a Bmax of 34% and Kd of 64 nmol/L for phospho-cGluR1. The starting library showed essentially no binding to the target (Fig. 1C). Although the selection was pre-cleared with unphospho-cGluR1, the round 12 pool still showed binding to the unphospho-cGluR1 protein with a relatively small Bmax (24%) and Kd (29 nmol/L) (Fig. 1D).

Characterization of phospho-Ser⁸⁴⁵ GluR1 binding aptamers

We cloned and sequenced the round 12 pool and identified three predominant clones (A1, A2, A3). Their secondary structures were predicted by Mfold (<http://bioweb.pasteur.fr>) and are depicted in Fig 2. The A1, A2, and A3 clone exhibited a protein concentration-dependent increase in binding fraction to both the unphospho-cGluR1 (0 to 500 nmol/L) and phospho-Ser⁸⁴⁵ cGluR1 (0 to 1000 nmol/L) proteins. All aptamers exhibited a greater Bmax to the phospho-Ser⁸⁴⁵ cGluR1 than to the unphospho-cGluR1 (Fig. 3A & 3C). However, they showed larger Kds to phospho-Ser⁸⁴⁵ cGluR1 [120 nmol/L (A1), 121 nmol/L (A2), 119 nmol/L (A3)] compared to unphospho-GluR1 [46 nmol/L (A1), 57 nmol/L (A2), 28 nmol/L (A3)]. We further tested their binding to the PKC-phosphorylated cGluR1 subunit and an unrelated protein (IgG). Aptamer A1, A2, and A3 showed no binding to IgG protein (data not shown). Interestingly, these three aptamers possessed very low or no binding to the PKC-phosphorylated cGluR1 protein (no difference from the library) (Fig. 3B).

The binding of selected aptamers to mammalian GluR1

It is a common concern that aptamers selected with recombinant protein might not effectively bind to mammalian protein in its native conformation due to post-translation modifications (Durand and Seta, 2000). To confirm that the selected aptamers bind to native GluR1, the fused GFP-GluR1 protein was expressed in HEK293 cells. An equal amount of cell lysate was co-incubated with equal amount of biotin-labeled complementary 3' flank sequence and aptamer A1, A2 and A3. As expected, aptamer A1, A2, and A3 effectively precipitated the GFP-GluR1 protein (Fig. 4A). Given the fact that native AMPA receptors are tetramers composed of GluR1-4 subunits, the equal amount of aptamer A1, A2 and A3 and biotin-labeled compatible oligos were incubated with equal amount of rat brain tissue homogenate to answer whether the aptamers could bind to GluR1 in the native AMPA receptor. Aptamer A1, A2, and A3 effectively precipitated phospho-Ser⁸⁴⁵ and total GluR1 subunits (Fig. 4B). Among them, aptamer A2 showed most effective binding to both the total GluR1 and phospho-Ser⁸⁴⁵ GluR1. Incubation of rat brain tissue homogenate with anti-GluR1 antibody also co-precipitated ³²P-labeled aptamers detected by autoradiography (Fig. 4C). Collectively, these data demonstrate that aptamer A1, A2, and A3 exhibit specific affinities for the mammalian GluR1 protein in its natural context.

Preventing GluR1 Ser⁸⁴⁵ phosphorylation by selected aptamers

Although aptamers were selected with the phospho-Ser⁸⁴⁵ cGluR1 and pre-cleared with the unphospho-cGluR1 protein, they still showed affinity for the latter protein. We therefore hypothesized that pre-binding of the aptamers to unphospho-GluR1 protein may prevent the PKA-dependent Ser⁸⁴⁵ phosphorylation. Indeed, the aptamers decreased PKA-mediated cGluR1 phosphorylation (Fig. 5A). Among them, aptamer A2 appeared most efficient and was selected for further testing. Aptamer A2 inhibited PKA-mediated Ser⁸⁴⁵ phosphorylation in a concentration-dependent manner (Fig. 5B, C). In contrast, aptamer A2 did not block PKC-mediated Ser⁸³¹ phosphorylation (Fig. 5D). These results demonstrated that the binding of aptamer A2 to GluR1 can selectively block PKA-dependent phosphorylation.

Inhibiting GluR1 and GluR1-containing AMPA receptor expression on the cell surface by aptamer A2

Ser⁸⁴⁵ phosphorylation of the GluR1 subunit is thought to play a key role in GluR1-containing AMPA receptor trafficking (Gao et al., 2006). The selected aptamers can bind to the phospho-Ser⁸⁴⁵ GluR1 and inhibit PKA-dependent GluR1 phosphorylation. Thus, we hypothesize that these two events will affect GluR1 insertion into the cell membrane. Among these aptamers, A2 showed the biggest Bmax for binding to phospho-Ser⁸⁴⁵ GluR1, no affinity for PKC-phosphorylated cGluR1, and effective binding to mammalian GluR1. Thus, we chose aptamer A2 to evaluate its role in GluR1 trafficking. HEK293 cells were co-transfected with the 2'-fluoro-pyrimidine-modified library RNA or aptamer A2 and a plasmid which expresses the GFP-GluR1 fusion protein (Shi et al., 1999). Up to 85-90% transfection efficacy was observed by GFP expression (data not shown). Parenthetically, the GFP-GluR1 protein retains constitutive membrane surface insertion and internalization properties (Lee et al., 2000; Man et al., 2007). Following incubation with forskolin and 3-isobutyl-1-methyl-xanthine (forskolin treatment), appearance of the GluR1 on the cell surface was increased by 128% over that of the unstimulated control. Transfection of aptamer A2 abolished this increase (Fig. 6A, B). Conversely, forskolin treatment and aptamer A2 transfection exerted no effects on total GFP-GluR1 levels (Fig. 6A). The changes in cell surface GFP-GluR1 expression are closely related to the Ser⁸⁴⁵ phosphorylation on the GFP-GluR1 protein (Fig. 6A, C).

Although aptamer A2 can inhibit the surface expression of single GFP-GluR1 subunit, native AMPA receptors are tetramers composed of GluR1-4 (Barry and Ziff, 2002). To test whether aptamer A2 can bind to GluR1 in the AMPA receptors, we examined AMPA receptor trafficking in cultured cortical neurons detected by cell surface biotinylation techniques (Man et al., 2007). Forskolin treatment increased GluR1 plasma membrane expression by 91% compared with the control. Transfection of aptamer A2 suppressed the increase (Fig. 7A, B). About 80-90% transfection efficacy was observed by Cy5-labeled aptamer A2 (data not shown). Conversely, forskolin treatment plus aptamer A2 transfection exerted no effects on total GluR1 levels (Fig. 7A). These findings demonstrated that the changes in cell surface GluR1 expression are closely related to the GluR1 Ser⁸⁴⁵ phosphorylation (Fig. 7A, C) and are not associated with Ser⁸³¹ phosphorylation (Fig. 7A).

Discussion

Protein phosphorylation regulates most aspects of cell life, whereas abnormal phosphorylation is now recognized as causes or consequences of many human diseases (Cohen, 2002a). The relevance of phosphorylation to diseases has stimulated intensive efforts in the biomedical research community to develop inhibitors that regulate the activities of protein kinases and phosphatases (Cohen, 2002a; Cole et al., 2003; Shen et al.,

2005). However, it is indeed a difficult task to develop compounds that selectively inhibit mere one particular protein kinase, without affecting other related enzymes. The main obstacle comes from the structural and sequence similarity between the members of the protein kinase family (Bain et al., 2000; Hunter, 2000; Lochner and Moolman, 2006). The kinase- or phosphatase-based inhibitors might not provide us with accurate information about the functions of specific site phosphorylation *in vivo* because a kinase commonly has a variety of substrates, whereas a substrate, even a single residue could be phosphorylated by different kinases (Carvalho et al., 2000; Havekes et al., 2007). In the present study, we have successfully selected RNA aptamers against the phospho-Ser⁸⁴⁵ cGluR1 protein. The selected aptamers effectively bind to the phospho-Ser⁸⁴⁵ cGluR1, also the unphospho-cGluR1 protein. The latter binding selectively inhibited PKA-mediated GluR1 phosphorylation at Ser⁸⁴⁵, but not PKC-mediated phosphorylation at Ser⁸³¹. Importantly, the combined effects of aptamer A2 on these processes reduce GluR1/GluR1-containing AMPA receptor expression on the cell surface. Our study now provides a novel means to evaluate function of single residue phosphorylation, and furthermore, to interfere with site-specific phosphorylation of proteins *in vivo*.

AMPA receptor is one of the major receptors that mediate excitatory synaptic transmission in the mammalian brain and it undergoes constant trafficking between the plasma membrane and the cytosolic compartments (Ehlers, 2000; Man et al., 2007). Phosphorylation of the Ser⁸⁴⁵ plays a key role in AMPA receptor trafficking (Lee et al., 2000; Havekes et al., 2000; Man et al., 2007), in contrast, phosphorylation at the Ser⁸³¹ residue increases channel conductance (Derkach, et al., 1999; Suzuki et al., 2005). Phosphorylation at the same region of the c-terminus of GluR1 molecule (residues 831-845) controls distinct determinants of channel functions: receptor trafficking and channel conductance that may reflect differences in phosphorylation-induced conformation change in the receptor (Banke et al., 2000), substitute difference on agonist binding, or the binding to anchoring and trafficking proteins (Banke et al., 2000; Ehlers, 2000; Suzuki, et al., 2005). Our selected aptamers bind to the GluR1 subunit phosphorylated at the Ser⁸⁴⁵ residue but not at the Ser⁸³¹ residue (see below for discussion on the binding to the unphosphorylated form) (Fig. 3, 5). These two sites are so close as to that phosphorylation at these two residues should cause significant differences in GluR1 conformation; subsequently forming different binding epitope for our aptamers. The aptamers were selected with phospho-Ser⁸⁴⁵ GluR1 protein. It is therefore not surprising that the aptamers only bind to the phospho-Ser⁸⁴⁵ GluR1 protein.

Classically, aptamers can be selected to inhibit critical protein interactions by sterically blocking their active sites and interaction surfaces (Templin et al., 2002; Murphy et al., 2003). In the present study, pre-binding of the selected aptamers to unphospho-cGluR1 can inhibit PKA-mediated phosphorylation at Ser⁸⁴⁵, but not PKC-mediated phosphorylation at Ser⁸³¹ (Fig. 5). We therefore hypothesize that these aptamers cover a small surface of the GluR1 and only block the interaction between PKA and Ser⁸⁴⁵ residue sterically; subsequently inhibit Ser⁸⁴⁵ phosphorylation. Interestingly, the aptamers described here recognize the unphospho-cGluR1 with a small K_d value (Fig. 1, 3). This can be explained because phosphorylation increases negative charge in the epitope. Generally, tight binding aptamers preferentially evolve to recognize the epitopes that contain positively charged amino acid residues, presumably utilizing electrostatic interactions (Rentmeister et al., 2006). Notably, binding to the phospho-Ser⁸⁴⁵ cGluR1 showed a bigger B_{max} than to the unphospho-cGluR1. Although it is unclear whether phosphorylation at one site (Ser⁸⁴⁵) will affect other residue (Ser⁸³¹) exposure on the molecule surface and consequently result in its inability to be recognized by the kinase, the phosphorylation-caused conformation alteration can produce aptamers to differentiate two phospho-residues, but not the unphospho- and phospho-state at single residue. However, phosphorylation is certainly necessary for restricting aptamer-GluR1 interaction to the small epitope surrounding the phospho-residue.

Thus, no matter whether the selected aptamers could differentiate unphospho-GluR1 from phospho-Ser⁸⁴⁵ GluR1 due to sharing the small epitope, our selection strategy with single residue-phosphorylated protein can produce site-specific anti-phosphorylation aptamers. Additional experiments for identifying the epitope will provide direct evidence for how the aptamers worked.

Phosphorylation can modify the function of a protein in almost every conceivable way for example: by increasing or decreasing its biological activity, by facilitating or inhibiting its movement between subcellular compartments, and by initiating or disrupting protein-protein interactions (Hunter, 1994; Cohen, 2002b). In the past several years, single residue phosphorylation leading to AMPA receptor relocation was extensively studied, but the topic remains controversial. Our study demonstrated that the combination of cAMP-elevating agent (forskolin) and phosphodiesterase inhibitor (3-isobutyl-1-methyl-xanthine) elevated phospho-Ser⁸⁴⁵ GluR1, but not Ser⁸³¹ GluR1 levels in GluR1-plasmid-transfected HEK293 cells (Fig. 6) and cultured cortical neurons (Fig. 7). The increased GluR1 Ser⁸⁴⁵ phosphorylation is accompanied by GluR1 and GluR1-containing AMPA receptor trafficking to the cell surface. This suggests that the PKA-mediated phosphorylation on GluR1 appears crucial in AMPA receptor trafficking. We further demonstrated that aptamer A2 inhibited forskolin-mediated increase in GluR1 Ser⁸⁴⁵ and GluR1/GluR1-containing AMPA receptor trafficking to the cell surface, and the latter is more effective. We therefore hypothesize that the decreased phosphorylation is a direct consequence of the binding of aptamer A2 to unphospho-GluR1, which sterically prevents PKA to recognize it. Conversely, the decreased surface expression of AMPA receptors might be a combined effect of the decreased GluR1 Ser⁸⁴⁵ phosphorylation and the effect of aptamer A2 in preventing the interaction between phospho-Ser⁸⁴⁵ GluR1 and its interacting proteins. Although we provided no direct evidence regarding how the aptamer affected AMPA receptor movement, the combined effects of aptamer A2 strongly inhibited AMPA receptor trafficking. Additional experiments to determine how the aptamers affect GluR1-containing AMPA receptor trafficking will help us to understand the regulation of AMPA receptor underlying synaptic plasticity.

In this study, one of our goals is to develop aptamers against phospho-Ser⁸⁴⁵ GluR1 for *in vivo* application. The aptamers described here inhibited Ser⁸⁴⁵ phosphorylation as well as GluR1/GluR1-containing AMPA receptor insertion into the cell surface. This provides the feasibility to use these aptamers for cocaine abstinence. Although aptamers have been successfully selected against a wealth of targets, such as small molecules, proteins, nucleic acids and even intact viruses and live cells (Burgstaller et al., 2002; Crawford et al., 2003; Toulme et al., 2003), they have only recently entered into clinical use (Perkins and Missailidis, 2007). A main technical hurdle to develop aptamers targeting intracellular protein for the clinical application is the delivery of aptamer across the plasma membrane of cells *in vivo* and the blood-brain barrier when applying to brain diseases. Actually, a number of approaches for delivering small nucleotides, including cationic lipids (Yano et al., 2004) and hydrodynamic injection (Lewis and Wolff, 2007), could be adopted as a nonspecific delivery of aptamers. We have recently developed a virus-based delivery of aptamer into a xenograft tumor in mice (Mi et al., 2006, 2008). Notably, a cell type-specific delivery of aptamer-siRNA chimeras has been developed (McNamara et al., 2006) which may open a new view for the aptamer *in vivo* delivery. Especially, the progress in nanoparticle-based delivery approaches (Guo, 2005; Farokhzad et al., 2006; Shiba, 2006) provided a new window. However, it is a long way before these delivery approaches enter into clinical use.

In conclusion, the selected aptamers can modify protein phosphorylation state at single residue in mammalian cells, which provides a novel tool for studying the biochemical and physiological role of single residue phosphorylation in isolated cell systems. Accompanying

with the successes in delivering aptamers in animal models, aptamers will become feasibility for targeting treatment of phosphorylation-related diseases.

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References

- Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. *Biochem J.* 2003; 371:199–204. [PubMed: 12534346]
- Banke TG, Bowie D, Lee H, Haganir RL, Schousboe A, Traynelis SF. Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *J Neurosci.* 2000; 20:89–102. [PubMed: 10627585]
- Barry MF, Ziff EB. Receptor trafficking and the plasticity of excitatory synapses. *Curr Opin Neurobiol.* 2002; 12:279–286. [PubMed: 12049934]
- Boudreau AC, Wolf ME. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J Neurosci.* 2005; 25:9144–9151. [PubMed: 16207873]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
- Burgstaller P, Jenne A, Blind M. Aptamers and aptazymes: accelerating small molecule drug discovery. *Curr Opin Drug Discov Devel.* 2002; 5:690–700.
- Carvalho AL, Duarte CB, Carvalho AP. Regulation of AMPA receptors by phosphorylation. *Neurochem Res.* 2000; 25:1245–1255. [PubMed: 11059799]
- Chen Q, Xiong X, Lee TH, Liu Y, Wetsel WC, Zhang X. Neural plasticity and addiction: Integrin-linked kinase and cocaine behavioral sensitization. *J Neurochem.* 2008 Aug 12. Epub ahead of print.
- Cohen P. The origins of protein phosphorylation. *Nat Cell Biol.* 2002a; 4:127–130.
- Cohen P. Protein kinases--the major drug targets of the twenty-first century? *Nat Rev Drug Discov.* 2002b; 1:309–315. [PubMed: 12120282]
- Cole AL, Subbanagounder G, Mukhopadhyay S, Berliner JA, Vora DK. Oxidized phospholipid-induced endothelial cell/monocyte interaction is mediated by a cAMP-dependent R-Ras/PI3-kinase pathway. *Arterioscler Thromb Vasc Biol.* 2003; 23:1384–1390. [PubMed: 12805072]
- Crawford M, Woodman R, Ferrigno PK. Peptide aptamers: tolls for biology and drug discovery. *Brief Funct Genomic Proteomic.* 2003; 2:72–79. [PubMed: 15243998]
- Derkach V, Barria A, Soderling TR. Ca²⁺/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc Natl Acad Sci U S A.* 1999; 96:3269–3274. [PubMed: 10077673]
- Durand G, Seta N. Protein glycosylation and diseases: blood and urinary oligosaccharides as markers for diagnosis and therapeutic monitoring. *Clin Chem.* 2000; 46:795–805. [PubMed: 10839767]
- Edwards S, Graham DL, Bachtell RK, Self DW. Region-specific tolerance to cocaine-regulated cAMP-dependent protein phosphorylation following chronic self-administration. *Eur J Neurosci.* 2007; 25:2201–2213. [PubMed: 17439498]
- Ehlers MD. Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron.* 2000; 28:511–525. [PubMed: 11144360]
- Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature.* 1990; 346:818–822. [PubMed: 1697402]
- Ellington AD, Szostak JW. Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures. *Nature.* 1992; 355:850–852. [PubMed: 1538766]

- Esteban JA, Shi SH, Wilson C, Nuriya M, Haganir RL, Malinow R. PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat Neurosci.* 2003; 6:136–143. [PubMed: 12536214]
- Farokhzad OC, Karp JM, Langer R. Nanoparticle-aptamer bioconjugates for cancer targeting. *Expert Opin Drug Deliv.* 2006; 3:311–324. [PubMed: 16640493]
- Gao C, Sun X, Wolf ME. Activation of D1 dopamine receptors increases surface expression of AMPA receptors and facilitates their synaptic incorporation in cultured hippocampal neurons. *J Neurochem.* 2006; 98:1664–1677. [PubMed: 16800848]
- Griffin LC, Toole JJ, Leung LL. The discovery and characterization of a novel nucleotide-based thrombin inhibitor. *Gene.* 1993; 137:25–31. [PubMed: 8282198]
- Guo P. RNA nanotechnology: engineering, assembly and applications in detection, gene delivery and therapy. *J Nanosci Nanotechnol.* 2005; 5:1964–1982. [PubMed: 16430131]
- Havekes R, Timmer M, Van der Zee EA. Regional differences in hippocampal PKA immunoreactivity after training and reversal training in a spatial Y-maze task. *Hippocampus.* 2007; 17:338–348. [PubMed: 17315197]
- Hermann T, Patel DJ. Adaptive recognition by nucleic acid aptamers. *Science.* 2000; 287:820–825. [PubMed: 10657289]
- Hirao I, Harada Y, Nojima T, Osawa Y, Masaki H, Yokoyama S. In vitro selection of RNA aptamers that bind to colicin E3 and structurally resemble the decoding site of 16S ribosomal RNA. *Biochemistry.* 2004; 43:3214–3221. [PubMed: 15023071]
- Hunter T. 1001 protein kinases redux--towards 2000. *Semin Cell Biol.* 1994; 5:367–376. [PubMed: 7711285]
- Hunter T. Signaling--2000 and beyond. *Cell.* 2000; 100:113–127. [PubMed: 10647936]
- Ishizaki J, Nevins JR, Sullenger BA. Inhibition of cell proliferation by an RNA ligand that selectively blocks E2F function. *Nat Med.* 1996; 2:1386–1389. [PubMed: 8946842]
- Lee HK, Barbarosie M, Kameyama K, Bear MF, Haganir RL. Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature.* 2000; 405:955–959. [PubMed: 10879537]
- Lewis DL, Wolff JA. Systemic siRNA delivery via hydrodynamic intravascular injection. *Adv Drug Deliv Rev.* 2007; 59:115–123. [PubMed: 17442446]
- Lochner A, Moolman JA. The many faces of H89: a review. *Cardiovasc Drug Rev.* 2006; 24:261–274. [PubMed: 17214602]
- Man HY, Sekine-Aizawa Y, Haganir RL. Regulation of {alpha}-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking through PKA phosphorylation of the Glu receptor 1 subunit. *Proc Natl Acad Sci U S A.* 2007; 104:3579–3584. [PubMed: 17360685]
- McNamara JO 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotechnol.* 2006; 24:1005–1015. [PubMed: 16823371]
- Mi J, Zhang X, Rabbani ZN, Liu Y, Su Z, Vujaskovic Z, Kontos CD, Sullenger BA, Clary BM. H1 RNA polymerase III promoter-driven expression of an RNA aptamer leads to high-level inhibition of intracellular protein activity. *Nucleic Acids Res.* 2006; 34:3577–3584. [PubMed: 16855294]
- Mi J, Zhang X, Rabbani ZN, Liu Y, Reddy SK, Su Z, Salahuddin FK, Viles K, Giangrande PH, Dewhirst MW, Sullenger BA, Kontos CD, Clary BM. RNA aptamer-targeted inhibition of NF-kappa B suppresses non-small cell lung cancer resistance to doxorubicin. *Mol Ther.* 2008; 16:66–73. [PubMed: 17912235]
- Murphy MB, Fuller ST, Richardson PM, Doyle SA. An improved method for the in vitro evolution of aptamers and applications in protein detection and purification. *Nucleic Acids Res.* 2003; 31:110.
- Perkins AC, Missailidis S. Radiolabelled aptamers for tumour imaging and therapy. *Q J Nucl Med Mol Imaging.* 2007; 51:292–296. [PubMed: 17464274]
- Rentmeister A, Bill A, Wahle T, Walter J, Famulok M. RNA aptamers selectively modulate protein recruitment to the cytoplasmic domain of beta-secretase BACE1 in vitro. *RNA.* 2006; 12:1650–1660. [PubMed: 16888322]
- Rusconi CP, Scardino E, Layzer J, Pitoc GA, Ortel TL, Monroe D, Sullenger BA. RNA aptamers as reversible antagonists of coagulation factor IXa. *Nature.* 2002; 419:90–94. [PubMed: 12214238]

- Sekine-Aizawa Y, Haganir RL. Imaging of receptor trafficking by using alpha-bungarotoxin-binding-site-tagged receptors. *Proc Natl Acad Sci U S A*. 2004; 101:17114–17119. [PubMed: 15563595]
- Shen K, Hines AC, Schwarzer D, Pickin KA, Cole PA. Protein kinase structure and function analysis with chemical tools. *Biochim Biophys Acta*. 2005; 1754:65–78. [PubMed: 16213197]
- Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R. Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science*. 1999; 284:1811–1816. [PubMed: 10364548]
- Shiba K. Functionalization of carbon nanomaterials by evolutionary molecular engineering: potential application in drug delivery systems. *J Drug Target*. 2006; 14:512–518. [PubMed: 17046797]
- Snyder GL, Allen PB, Fienberg AA, Valle CG, Haganir RL, Nairn AC, Greengard P. Regulation of phosphorylation of the GluR1 AMPA receptor in the neostriatum by dopamine and psychostimulants in vivo. *J Neurosci*. 2000; 20:4480–4488. [PubMed: 10844017]
- Sousa R, Padilla R. A mutant T7 RNA polymerase as a DNA polymerase. *EMBO J*. 1995; 14:4609–4621. [PubMed: 7556104]
- Sun X, Zhao Y, Wolf ME. Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. *J Neurosci*. 2005; 25:7342–7351. [PubMed: 16093384]
- Suzuki T, Li W, Zhang JP, Tian QB, Sakagami H, Usuda N, Kondo H, Fujii T, Endo S. A novel scaffold protein, TANC, possibly a rat homolog of *Drosophila* rolling pebbles (rols), forms a multiprotein complex with various postsynaptic density proteins. *Eur J Neurosci*. 2005; 21:339–350. [PubMed: 15673434]
- Templin MF, Stoll D, Schrenk M, Traub PC, Vohringer CF, Joos TO. Protein microarray technology. *Drug Discov Today*. 2002; 7:815–822. [PubMed: 12546969]
- Toulme JJ, Darfeuille F, Kolb G, Chabas S, Staedel C. Modulating viral gene expression by aptamers to RNA structures. *Biol Cell*. 2003; 95:229–238. [PubMed: 12867086]
- Tropea TF, Kosofsky BE, Rajadhyaksha AM. Enhanced CREB and DARPP-32 phosphorylation in the nucleus accumbens and CREB, ERK and GluR1 phosphorylation in the dorsal hippocampus is associated with cocaine conditioned place preference behavior. *J Neurochem*. 2008; 106:1780–1790. [PubMed: 18554320]
- Wong I, Lohman TM. A double-filter method for nitrocellulose-filter binding: application to protein-nucleic acid interactions. *Proc Natl Acad Sci U S A*. 1993; 90:5428–5432. [PubMed: 8516284]
- Yano J, Hirabayashi K, Nakagawa S, Yamaguchi T, Nogawa M, Kashimori I, Naito H, Kitagawa H, Ishiyama K, Ohgi T, Irimura T. Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. *Clin Cancer Res*. 2004; 10:7721–7726. [PubMed: 15570006]
- Zhang X, Lee TH, Davidson C, Lazarus C, Wetsel WC, Ellinwood EH. Reversal of cocaine-induced behavioral sensitization and associated phosphorylation of NR2B and GluR1 subunits of NMDA and AMPA receptors. *Neuropsychopharmacology*. 2007; 32:377–387. [PubMed: 16794574]

Abbreviations used

PKA	protein kinase A
PKC	protein kinase C
GluR1	glutamate receptor subunit 1
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
HEK293	Human Embryonic Kidney cells
GFP	green fluorescent protein
GST	glutathione-S-transferase
PCR	polymerase chain reaction
RNA	ribonucleic acid
PBS	phosphate buffered saline

Figure 1.

The phosphorylation efficacy and binding of RNA pools. A, The efficacy of PKA on Ser⁸⁴⁵ phosphorylation of the GluR1 subunit was detected by site-specific anti-GluR1 Ser⁸⁴⁵ antibody. PKA effectively phosphorylated cGluR1. B, The efficacy of PKC on Ser⁸³¹ phosphorylation was detected by site-specific anti-GluR1 Ser⁸³¹ antibody. C, The binding of RNA pools to phospho-Ser⁸⁴⁵. D, The binding of RNA pools to unphospho-GluR1 protein. The RNA pool 12 showed a preferential binding to the phospho-cGluR1 Ser⁸⁴⁵ protein.

Figure 2.
Secondary structure of selected aptamers. A1: Aptamer A1; A2: Aptamer A2; A3: Aptamer A3.

Figure 3.

The binding of selected aptamers. Aptamers showed high binding fraction for the phospho-Ser⁸⁴⁵ cGluR1 protein, lower binding fraction for unphospho-cGluR1 protein, and very low or no binding to the PKC-phosphorylated cGluR1 protein. A, The binding of aptamers to phospho-Ser⁸⁴⁵ cGluR1. B, The binding of aptamers to phospho-Ser⁸³¹ cGluR1. C, The binding of aptamers to unphospho-cGluR1. Lib: library; A1: aptamer A1; A2: aptamer A2; A3: aptamer A3.

Figure 4.

The binding of aptamers to the mammalian GluR1 subunit. A, GFP-GluR1 was co-precipitated with aptamer A1, A2, or A3 from GFP-GluR1 plasmid-transfected HEK293 cell lysate. B, The GluR1 and phospho-Ser⁸⁴⁵ GluR1 subunits were co-precipitated with the aptamers from rat brain tissue homogenate. C, The aptamers were co-precipitated with the anti-GluR1 antibody in the rat brain tissue homogenate. Aptamer A2 showed most effective in binding mammalian GluR1. Lib: library.

Figure 5.

Inhibition of Ser⁸⁴⁵ phosphorylation. A, Aptamers inhibited cGluR1 phosphorylation at the Ser⁸⁴⁵ residue. The RNA library (Lib), aptamer A1, A2 or A3 were pre-bound with unphospho-cGluR1 and then incubated with PKA and ATP. NC: neither RNA nor PKA applied. A2 showed a most effective inhibition in PKA-mediated phosphorylation at GluR1 Ser⁸⁴⁵. B, Aptamer A2 inhibited Ser⁸⁴⁵ phosphorylation in a concentration-dependent manner detected by site-specific antibody. C, Aptamer A2 inhibited Ser⁸⁴⁵ phosphorylation in a concentration-dependent manner detected by radiation assay. D, Aptamer A2 has no effect on Ser⁸³¹ phosphorylation detected by site-specific antibody.

Figure 6.

Inhibition of GluR1 trafficking in GluR1-transfected HEK293 cells. Cells were co-transfected with library RNA (Lib) or aptamer A2 RNA (A2) and GFP-GluR1 expression plasmid for 36 hrs, then treated with 20 μ mol/L forskolin plus 50 μ mol/L 3-isobutyl-1-methylxanthine (F) to increase GluR1 surface expression. A, Western blot of surface GluR1 expression (S-GluR1), GluR1 Ser⁸⁴⁵ phosphorylation (GluR1 Ser⁸⁴⁵), total GluR1 levels (T-GluR1), and α -tubulin. B, Densitometric analyses of cell surface GluR1. C, Densitometric analyses of phospho-Ser⁸⁴⁵ GluR1 levels. * $p < 0.05$ vs. other groups, $n = 4$.

Figure 7.

Inhibition of GluR1 trafficking and Ser⁸⁴⁵ phosphorylation in cultured cortical neurons. High-density cultured cortical neurons were transfected with library RNA (Lib) or aptamer A2 RNA (A2) for 36 hrs, and then treated with 20 μ mol/L forskolin plus 50 μ mol/L 3-isobutyl-1-methylxanthine (F). A, Western blot of surface GluR1 expression (S-GluR1), GluR1 Ser⁸⁴⁵ phosphorylation (GluR1 Ser⁸⁴⁵), GluR1 Ser⁸³¹ phosphorylation (GluR1 Ser⁸³¹), total GluR1 (T-GluR1), and α -tubulin levels. B, Densitometric analyses of cell surface GluR1. C, Densitometric analyses of phospho-Ser⁸⁴⁵ GluR1 levels. * $p < 0.05$ vs. other groups, $n = 4$.