NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1

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Phosphorylation regulates the function of ligand-gated ion channels such as the *N*-methyl D-aspartate (NMDA) receptor. Here we report a mechanism for modulation of the phosphorylation state and function of the NMDA receptor via an inhibitory scaffolding protein, RACK1. We found that RACK1 binds both the NR2B subunit of the NMDA receptor and the nonreceptor protein tyrosine kinase, Fyn. RACK1 inhibits Fyn phosphorylation of NR2B and decreases NMDA receptor-mediated currents in CA1 hippocampal slices. Peptides that disrupt the interactions between RACK1, NR2B, and Fyn induce phosphorylation and potentiate NMDA receptor-mediated currents. Therefore, RACK1 is a regulator of NMDA receptor function and may play a role in synaptic plasticity, addiction, learning, and memory.

The ionotropic glutamate receptor subtype, *N*-methyl D-aspartate (NMDA), plays an essential role in neuronal development, excitotoxicity, synaptic plasticity, and learning and memory (1, 2). The ligand-gated NMDA receptor channel is a heteromer comprised of NR1 and at least one of four NR2 subunits (A–D; ref. 2). A defining characteristic of the NR2 subunits is the long intracellular C-terminal tail required for channel function (3). Although NR2 subunits cannot independently form a functional NMDA receptor, they regulate the properties of the channel when coexpressed with NR1 subunits (4). Both NR2A and -B are differentially tyrosine phosphorylated (5), with NR2B being one of the most abundant tyrosine phosphorylated proteins in the postsynaptic density (PSD; ref. 6). Application of a tyrosine kinase inhibitor causes a progressive decrease in NMDA receptor-mediated currents, and inhibition of protein tyrosine phosphatases results in an increase in NMDA receptor-mediated currents (7). Subsequent studies have identified the Src family of protein tyrosine kinases (PTKs) as enzymes that phosphorylate the NR2 subunit regulating NMDA receptor activities (7, 8). Tyrosine phosphorylation of the NMDA receptor is increased in long-term potentiation (LTP; ref. 9), and both Src and Fyn are necessary for the induction of LTP (10–13). Fyn knock-out (K/O) mice have impaired synaptic plasticity and learning and memory (10). Introducing the Fyn gene onto the Fyn K/O background rescues the learning and memory phenotype (11). Therefore, the role of Fyn in the regulation of the NMDA receptor may be crucial to understanding such neuronal processes.

Compartmentalization of signaling proteins such as kinases is obtained by their association with scaffolding proteins (14). Recently, a number of signaling proteins were identified in the PSD (15–17). Many associate with the NMDA receptor complex via scaffolding proteins. However, the orchestration of these complex assemblies of proteins and the regulation of NMDA receptor channel function are only now beginning to be understood.

In this study, we identify a role for RACK1 as a scaffolding protein for NR2B and Fyn. RACK1 was originally identified as a protein kinase C-binding protein (18). In recent years, it has become apparent that RACK1 can interact with diverse signaling proteins such as kinases (18, 19), a phosphatase (20), a phosphodiesterase (21), and cell surface receptors (22–24). Here we present data that identify RACK1 as an inhibitory scaffolding protein for NMDA receptor phosphorylation and function.

Experimental Procedures

Reagents. Anti-Fyn, anti-NR2B, and anti-Lyn antibodies were from Santa Cruz Biotechnology. Anti-Src antibodies and Fyn kinase were from Upstate Biotechnology (Lake Placid, NY). Anti-RACK1, anti-NR2B, and anti-Yes antibodies were from Transduction Laboratories (Lexington, KY), picrotoxin and ifenprodil from Sigma, PP2 from Calbiochem, and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) and D-(−)-2-amino-5-phosphopentanoic acid (DAP5) from Tocris Neuramin (Bristol, U.K.).

Recombinant Proteins. MBP-RACK1 was expressed in *Escherichia coli* as described (18). Maltose-binding protein (MBP), MBP-ctNR2B [amino acids (a.a.) 1086–1482], MBP-ctNR2BΔN (a.a. 1172–1482), and MBP-ctNR2BΔC (a.a. 839–1170) and MBP-ctNR1 (a.a. 834–938) were expressed and purified as described above for MBP-RACK1. RACK1, and RACK1ΔN (a.a. 137–301) were subcloned into pTAT-HA and expressed in and purified from *E. coli*.

Peptides. R7 (RRFVGHTKDV, RACK1, a.a. 99–109); N1 (LYGKFSFKSDRYS, NR2B, a.a. 1038–1050); N2 (IYKERSDDFKRDSVS, NR2B, a.a. 1154–1168), F1 (LYGRFTIKSDVWS, Fyn, a.a. 436–448); scrambled F1 (WGTSSVDKIYFRL), and R14 (SRDKTIIMWKLTRD, RACK1, a.a. 35–48) were synthesized at Synpep (Dublin, CA).

***In Vitro* Binding Assay.** The assay was performed as described in ref. 25. Peptides were preincubated with RACK1, Fyn, or MBP-ctNR2B (a.a. 1086–1482) in overlay buffer for 30 min at room temperature. Membranes were probed with antibodies specific for overlay proteins. Purity of the recombinant proteins was verified by Coomassie staining.

***In Vitro* Kinase Assays.** Fyn (15 units, 0.32 pmol/min/unit) was incubated in the presence or absence of MBP-ctNR2B (a.a. 1086–1482, 750 ng) and increasing concentrations of RACK1 or MBP in kinase buffer [25 mM Tris·HCl, pH 7.2/31.5 mM $MgCl_2$/6.25 mM $MnCl_2$/0.5 mM EGTA/10 μCi $\gamma[^{32}P]$-ATP/125 μM cold ATP/protease and phosphatase inhibitors mix (Sigma)] at room temperature for 10 min. Samples were analyzed by autoradiography. Nonradiolabeled ATP (20 μM) was used for *in vitro* kinase assays carried out in the presence of the appropriate peptides. Samples were analyzed with antiphosphotyrosine antibodies.

Abbreviations: NMDA, *N*-methyl D-aspartate; PTK, protein tyrosine kinase; PSD, postsynaptic density; MBP, maltose-binding protein; EPSC, excitatory postsynaptic currents; a.a., amino acids.

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***In Vitro* Translation Assay.** [^{35}S]methionine-labeled proteins were generated in rabbit reticulocyte lysates (TNT Kit, Promega) programmed with RACK1, Fyn, and ctNR2B (a.a. 839–1482) cDNAs. The translation reactions were analyzed by SDS/PAGE and fluorography. Interaction of the proteins was determined by coimmunoprecipitation from the lysates with the antibodies indicated.

Two-Hybrid Interaction. Human Fyn and rat ctNR2B (a.a. 839–1482) cDNAs were cloned into pGADT7. RACK1ΔC (a.a. 1–60) was amplified and cloned into pGBKT7 (CLONTECH). Competent Y190 yeast was cotransformed with pGBK-RACK1ΔC and either pGAD-Fyn or pGAD-ctNR2B (a.a. 839–1482) and plated on synthetic dropout media lacking tryptophan and leucine. Negative controls were performed by replacing a binding partner with either pGBK alone or pGAD alone. After appropriate incubation, colonies were assayed for interaction by β-galactosidase freeze–fracture assay.

Preparation of Brain Homogenates. Three- to four-week-old male Sprague–Dawley rats were killed with Halothane, and whole brain or hippocampus was dissected and homogenized in buffer A (in mM: 320 sucrose/20 Tris·HCl, pH 7.5/2 EDTA/10 EGTA/12 β-mercaptoethanol and protease inhibitors). Homogenates were centrifuged at 4,500 $\times$ *g* for 30 min, and the pellet was resuspended in buffer B (1% deoxycholate/10 mM Tris·HCl, pH 7.4/10 mM EDTA/10 mM EGTA and protease inhibitors).

Preparation of Cell Homogenates. NG108–15 cells were cultured and processed as described (25).

Immunoprecipitation. Immunoprecipitation was performed with 5 μg of the appropriate antibodies as described in ref. 25.

Electrophysiology. Transverse hippocampal slices (400–450 μm) were prepared from 3- to 4-week-old male Sprague–Dawley rats. Slices were allowed to recover 1–2 h in an artificial cerebrospinal fluid saturated with 95% O_2/5% CO_2 containing (in mM): 126 NaCl, 1.2 KCl, 1.2 NaH_2PO, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose and maintained at room temperature, at pH 7.4, and at osmolarity of 300–320 mosmol. For all recordings, Picrotoxin (100 μM) was added to block $GABA_A$ receptor-mediated inhibitory postsynaptic currents. For NMDA receptor recordings, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) (10 μM) was added to block AMPA receptor-mediated excitatory postsynaptic currents (EPSCs). Similarly, DAP5 (50 μM) was added to block NMDA receptor-mediated EPSCs, while recording AMPA receptor currents. Somatic whole-cell voltage–clamp recordings were made from CA1 pyramidal cells by using 3–6 MΩ electrodes. The whole-cell solution contained (in mM): 117 cesium methansulfonic acid, 2.8 NaCl, 20 Hepes, 0.4 EGTA, 5 triethanolamine-Cl, 2.5 MgATP, and 0.25 MgGTP at pH 7.2–7.4 and osmolarity of 285–295 mosmol. The peptides were added to the whole-cell solution and introduced to the cells via the patching pipette by means of diffusional exchange. Cells were held at +40 mV (for NMDA) and −70 mV (for AMPA), and series and input resistances were monitored continuously. Schaffer collateral axons were stimulated every 10 sec by using steel bipolar microelectrodes placed at the stratum radiatum. Data were collected by using an Axopatch-1D amplifier, Axon Instruments (Foster City, CA), filtered at 2 kHz and digitized at 5–10 kHz. Peak amplitudes of EPSCs were measured, and the magnitude of the potentiation was calculated by comparing the average size of the EPSCs for each minute to the average size of the EPSCs of the first minute.

Results

RACK1 Directly Interacts with Fyn and NR2B. RACK1 is highly expressed in several brain regions such as the hippocampus (R.Y. and D.R., unpublished work). We therefore set out to identify neuronal binding partners for RACK1. We identified a 60-kDa tyrosine-phosphorylated protein that coimmunoprecipitated with RACK1 from the neuroblastoma-glioma cell line NG108–15 (data not shown). The protein was subsequently identified as the PTK, Fyn (Fig. 1*a*). To verify that RACK1 directly interacts with Fyn, we performed an *in vitro* binding assay. Immobilized RACK1 and a control protein (MBP) were overlaid with Fyn in a slot-blot assay. As shown in Fig. 1*b*, Fyn interacted directly with RACK1 but not with MBP. To determine whether the interaction between RACK1 and Fyn is shared with other members of the Src family of PTKs, RACK1 was immunoprecipitated from hippocampal slices and coimmunoprecipitation of Fyn, Src, Yes, and Lyn was assessed. RACK1 coimmunoprecipitated Fyn from hippocampal homogenates, but low or no detection of Src, Yes, and Lyn was observed (Fig. 1*c*). In summary, in NG108–15 cells, in hippocampal homogenates, and *in vitro*, RACK1 interacts specifically and directly with Fyn.

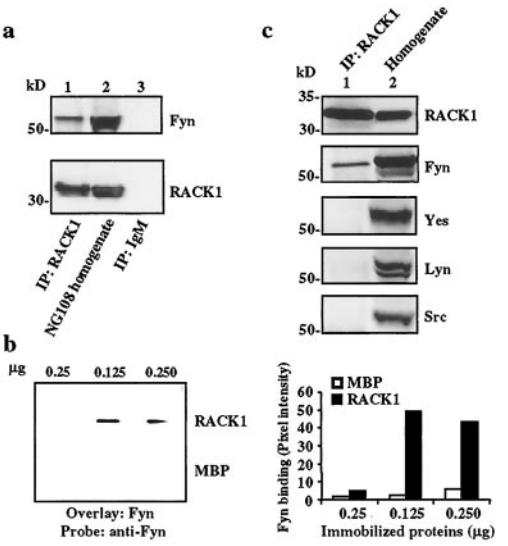

Fig. 1. RACK1 binds Fyn kinase. (*a*) Immunoprecipitation was performed with monoclonal IgM anti-RACK1 antibodies (lane 1) or with mouse IgM antibodies (lane 3). Membranes were probed with anti-RACK1 (*Lower*) or anti-Fyn antibodies (*Upper*). The presence of Fyn and RACK1 in NG108–15 homogenate was verified by Western blot analysis (50 μg, lane 2) (n = 3). (*b*) Increasing concentrations of RACK1 or MBP were blotted onto nitrocellulose membrane by using a slot-blot apparatus and overlaid with Fyn (75 units, 0.32 pmol/min/unit). Binding was detected with anti-Fyn antibodies (n = 3). Histogram (*Right*) shows densitometric analysis of binding. (*c*) Immunoprecipitations were carried out by using anti-RACK1 antibodies (lane 1). Membranes were probed with the antibodies indicated. The presence of the proteins in hippocampal homogenate was verified by Western blot analysis (50 μg, lane 2) (n = 3).

One of the identified substrates for Fyn in brain is the cytoplasmic tail of the NR2B (ctNR2B) subunit of the NMDA receptor (26–28). Interestingly, we identified sequences of homology between Fyn and its substrate ctNR2B. We found that ctNR2B contains two regions of similarity (a.a. 1038–1050 and 1154–1168) with the kinase domain of Fyn (a.a. 436–448; Fig. 2*a*).

Because ctNR2B contains sequences with homology to a sequence on Fyn, we speculated that these sequences might be RACK1-binding site(s) not only for Fyn but also for ctNR2B. If so, RACK1 should also directly interact with ctNR2B. To test this hypothesis, the binding of ctNR2B to RACK1 was determined in a slot-blot assay. As shown in Fig. 2*b*, recombinant RACK1 interacted directly with ctNR2B (a.a. 1086–1482). However, the cytoplasmic tail of NR1 (ctNR1 (a.a. 834–938), a

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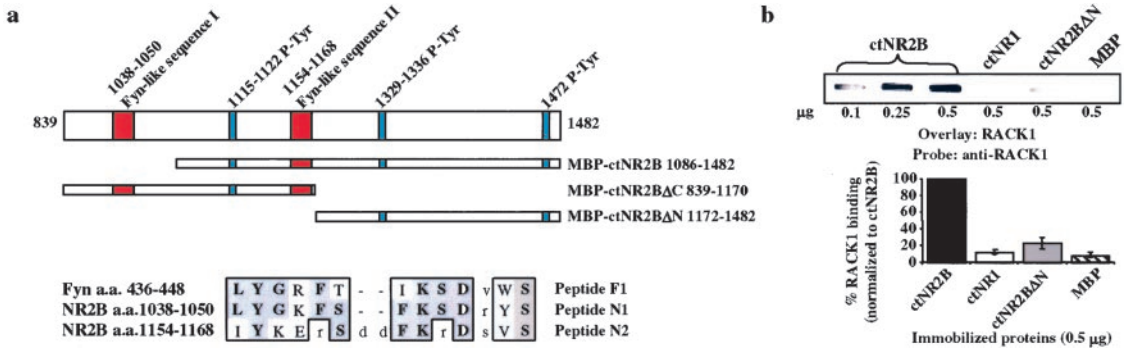

Fig. 2. ctNR2B shares sequences of homology with Fyn and interacts with RACK1. (*a*) Sequences of homology between NR2B and Fyn were identified with BLAST search (National Center for Biotechnology Information) and confirmed by the alignment of both sequences (NR2B: rat, accession no. Q00960; Fyn: rat, accession no. NP_036887) with MACVECTOR Ver. 6.5.3 (Oxford). Lower-case letters represent amino acids that are different (nonidentical and nonhomologous). (*b*) 0.5 μg of MBP-ctNR2B (a.a. 1086–1482), MBP-ctNR1, MBP-ctNR2BΔN (a.a. 1171–1482), and MBP were blotted onto a nitrocellulose membrane by using a slot-blot apparatus and overlaid with RACK1 (500 ng). RACK1 binding was detected with anti-RACK1 antibodies. Histogram (*Bottom*) shows densitometric analysis of binding normalized to binding of RACK1 to ctNR2B ± SD (n = 3).

deletion mutant of ctNR2B (ctNR2BΔN) and a control protein, MBP, did not bind RACK1 (Fig. 2*b*). Taken together, our results suggest that RACK1 associates not only with the kinase Fyn but also with its substrate NR2B.

To address the possibility that Fyn and ctNR2B interact with RACK1 simultaneously, we performed triple *in vitro* translation assays with Fyn, ctNR2B (a.a. 839–1482) and RACK1. Rabbit reticulocyte lysates containing all three translated proteins were used in immunoprecipitation experiments with anti-Fyn, anti-NR2B, and anti-RACK1 antibodies. RACK1, Fyn, and ctNR2B (a.a. 839–1482) were coimmunoprecipitated regardless of which antibody was used for the immunoprecipitation (Fig. 3*a*). No interaction between ctNR2B (a.a. 839–1482) and Fyn was observed in the absence of RACK1 (Fig. 3*b*), and no competition between Fyn and ctNR2B (a.a. 839–1482) for RACK1 binding was observed (Fig. 3*c*). Taken together, the results suggest that *in vitro* RACK1 is required to bridge the interaction between Fyn and ctNR2B. Next, we determined whether the association between RACK1, Fyn, and NR2B occurred in brain. Anti-NR2B antibodies coimmunoprecipitated RACK1 and Fyn, and anti-RACK1 antibodies coimmunoprecipitated NR2B and Fyn from hippocampal homogenates (Fig. 3*d*). These results, together with cell fractionation experiments that identified Fyn, RACK1, and NR2B in the PSD fraction (data not shown), suggest that RACK1, Fyn, and NR2B associate *in vitro* and in brain.

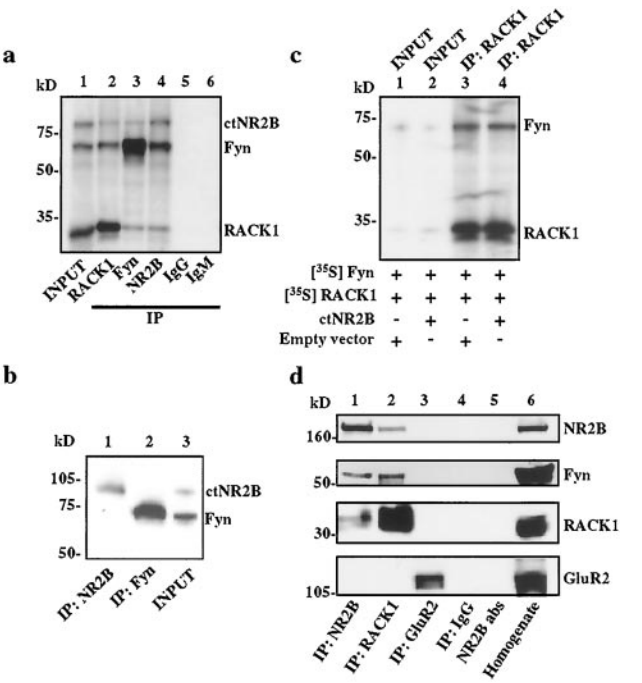

Fig. 3. RACK1 associates with ctNR2B and Fyn. (*a*) [^{35}S]methionine-labeled proteins were generated in rabbit reticulocyte lysates programmed with the appropriate cDNA [RACK1 (a.a. 1–317), Fyn (a.a. 1–537), ctNR2B (a.a. 839–1482)]. Interaction of the proteins was determined by coimmunoprecipitation from the lysates with anti-RACK1 (lane 2), anti-Fyn (lane 3), and anti-NR2B (lane 4) antibodies. An aliquot of the triple translation reaction is shown in lane 1. Control immunoprecipitations are shown in lanes 5 and 6 (n = 6). (*b*) [^{35}S]methionine-labeled proteins were generated as in *a*. Interactions were determined by coimmunoprecipitation from the lysates with anti-NR2B (lane 1) and anti-Fyn (lane 2) antibodies. An aliquot of the double translation reaction is shown in lane 3 (n = 2). (*c*) [^{35}S]methionine-labeled Fyn and RACK1 were generated as in *a*. Empty vector control (lanes 1 and 3) or unlabeled ctNR2B (a.a. 839-1482; lanes 2 and 4) were added to the reaction mixture. Complexes were immunoprecipitated with anti-RACK1 antibodies (lanes 3 and 4) (n = 2). (*d*) Immunoprecipitations were carried out by using mc anti-NR2B (lane 1), anti-RACK1 (lane 2), and anti-GluR2 (lane 3). Controls included monoclonal anti-NR2B antibodies alone (0.25 μg, lane 5) as well as immunoprecipitations using mouse IgG (lane 4) and mouse IgM antibodies (data not shown). Membranes were probed with the antibodies shown that included polyclonal anti-NR2B antibodies. The presence of RACK1, Fyn, NR2B, and GluR2 in hippocampal homogenate was verified by Western blot analysis (lane 6) (n = 6).

Identification of the Sites of Interaction Between RACK1, Fyn, and NR2B. If RACK1 association with Fyn and ctNR2B is important for the phosphorylation of the subunit by Fyn and/or for the function of the NMDA receptor, then interfering with the binding should alter channel phosphorylation and function. To assess these possibilities, we first identified the binding sites on the three proteins. Because Fyn and ctNR2B share sequences of similarity (Fig. 2*a*), we hypothesized that these sequences represent RACK1 interaction site(s) on the two proteins. Deletion mutants of ctNR2B with (ctNR2BΔC) and without (ctNR2BΔN) the putative binding sites for RACK1 were prepared and tested for RACK1 binding in a slot-blot overlay assay. As shown in Fig. 4*a*, ctNR2BΔC bound RACK1, whereas ctNR2BΔN did not. On the basis of these results, ctNR2B-derived peptides (N1 and N2) covering the regions of similarity (Fig. 2*a*) and the corresponding Fyn-derived peptide (F1) were synthesized and tested for their ability to affect the interactions between RACK1, ctNR2B, and Fyn. Peptides N1, N2, and F1 inhibited the interaction between ctNR2B (a.a. 1086–1482) and RACK1, and between Fyn and RACK1 in a dose-dependent manner (Fig. 4 *b* and *c*, and data

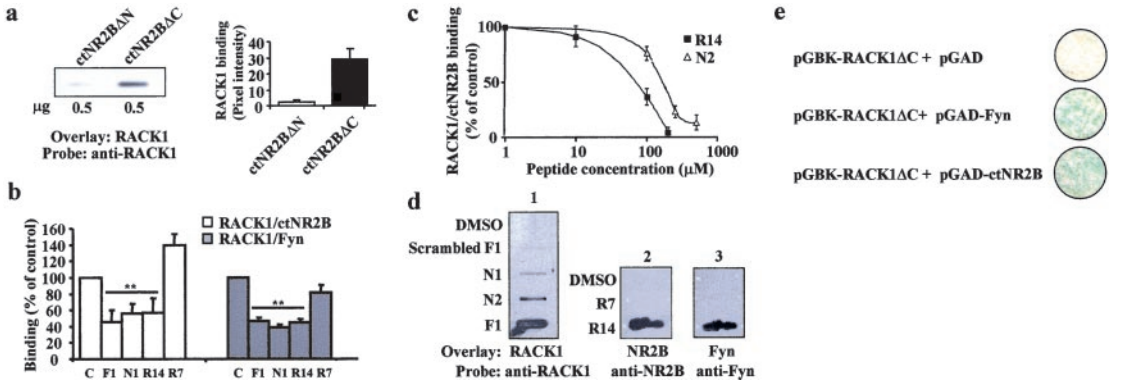

Fig. 4. Identification of the binding sites of RACK1, Fyn, and NR2B. (*a*) Five hundred nanograms of MBP-ctNR2BΔN (a.a. 1172–1482) and MBP-ctNR2BΔC (a.a. 839–1170) were blotted onto nitrocellulose membrane by using a slot-blot apparatus and overlaid with 500 ng of RACK1. Binding was detected with anti-RACK1 antibodies. Histogram (*Right*) shows densitometric analysis of binding ($n = 3$). (*b*) RACK1 (500 ng) was blotted onto nitrocellulose membrane and incubated with MBP-ctNR2B (a.a. 1086–1482; 500 ng) or Fyn (75 units, 0.32 pmol/min/unit) in the absence (*c*) or presence of peptides F1, N1, R14, and R7 (100 μM). Membranes were probed with anti-NR2B or anti-Fyn antibodies. Binding is presented as mean percent of control ± SD of three experiments. Results compared with R7 are statistically significant ($P < 0.01$). (*c*) MBP-ctNR2B (a.a. 1086–1482; 500 ng) was blotted as described in *a* and overlaid with RACK1 (500 ng) and increasing concentrations of peptides R14 (0–250 μM) and N2 (0–500 μM). Binding was detected with anti-RACK1 antibodies and presented as mean percent of control ± SD of three experiments. (*d*) R14, R7, F1, scrambled F1, N1, and N2 (10 μl of 10 mM) or vehicle (10 μl of 20% DMSO) were blotted onto nitrocellulose membrane. The membranes were incubated with RACK1 (3 μg, *1*), MBP-ctNR2B (a.a. 1086–1482; 3 μg, *2*), and Fyn (25 units, 0.32 pmol/min/unit, *3*). The membranes were probed with anti-RACK1 (*1*), anti-NR2B (*2*), and anti-Fyn antibodies (*3*) ($n = 3$, except for N2 peptide, $n = 2$). (*e*) Yeast coexpressing RACK1ΔC (1–60) and ctNR2B (a.a. 839–1482) or RACK1ΔC (a.a. 1–60), and Fyn gave a positive result compared with yeast containing RACK1ΔC (1–60) and pGAD alone ($n = 3$).

not shown). A scrambled F1 peptide did not affect the binding of RACK1 to either protein (data not shown). Furthermore, peptides N1, N2, and F1, but not scrambled peptide F1, bound RACK1 in an overlay assay (Fig. 4*d*). It is therefore likely that these peptides represent at least part of the RACK1 interaction site on both Fyn and ctNR2B. Next, we identified the binding site(s) for Fyn and ctNR2B on RACK1. Five RACK1-derived peptides were tested for their ability to alter the interaction between ctNR2B (a.a. 1086–1482) or Fyn and RACK1. Peptide R14 (RACK1, a.a. 35–48) inhibited the interaction of RACK1 with either ctNR2B (a.a. 1086–1482) or Fyn in a dose-dependent manner (Fig. 4 *b* and *c*). Furthermore, another RACK1-derived peptide (R7, RACK1, a.a. 97–109) did not affect interactions between RACK1 and ctNR2B or Fyn (Fig. 4*b*). Moreover, peptides R14 but not R7 bound to Fyn or ctNR2B (a.a. 1086–1482) in an overlay assay (Fig. 4*d*). To further confirm the interactions, we conducted a series of yeast two-hybrid experiments. A GAL4 (binding domain)-RACK1ΔC fusion protein (pGBK-RACK1ΔC) containing the putative N-terminal binding site on RACK1 (a.a. 1–60) was expressed in yeast strain Y190. Fusion proteins of the activation domain of GAL4 and pGAD-Fyn, pGAD-ctNR2B (a.a. 839–1483) were coexpressed with RACK1ΔC in Y190. Yeast cotransformed with RACK1ΔC and Fyn or ctNR2B gave a positive result by β-galactosidase freeze fracture assay (Fig. 4*e*). No interaction was detected for cotransformations using the vector alone and RACK1ΔC, pGAD-Fyn, pGAD-ctNR2B (a.a. 839–1482). Therefore, the N-terminal region of RACK1 that contains the sequence of R14 is likely to be the binding site for both Fyn and ctNR2B.

RACK1 Inhibits Fyn-Mediated Phosphorylation of ctNR2B. Because Fyn phosphorylates ctNR2B, we assessed whether the interaction of RACK1 with Fyn and ctNR2B alters the ability of Fyn to phosphorylate ctNR2B. We performed an *in vitro* kinase assay in the absence or presence of RACK1. As has previously been reported (26, 28), Fyn phosphorylates ctNR2B (a.a. 1086–1482) (Fig. 5*a*, lane 1). Increasing concentrations of RACK1 added to the kinase reaction resulted in a dose-dependent decrease in the phosphorylation of ctNR2B (a.a. 1086–1482) (Fig. 5*a*, lanes 2–6). However, RACK1 did not inhibit Fyn phosphorylation of a peptide substrate and enolase (data not shown), suggesting that the binding of RACK1 to ctNR2B confers substrate specificity. The inhibition of Fyn phosphorylation of ctNR2B (a.a. 1086–1482) was also specific for RACK1, because the control protein, MBP, had no effect (Fig. 5*a*, lane 8). On the basis of these results, we hypothesized that RACK1 is an inhibitory scaffolding protein that interacts with Fyn and ctNR2B, preventing Fyn from phosphorylating the NMDA channel. If this is correct, then peptides that inhibit the interaction between RACK1 and ctNR2B or Fyn (Fig. 4 *b* and *c*) should restore the ability of Fyn to phosphorylate ctNR2B. Indeed, when the phosphorylation assay was performed in the presence of RACK1, together with the inhibitory peptides (N1, N2, F1, or R14), but not the inactive

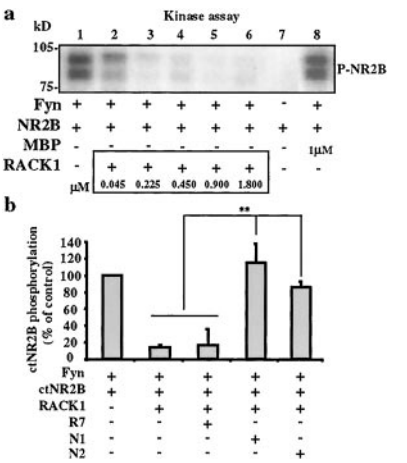

Fig. 5. RACK1 inhibits the phosphorylation of ctNR2B by Fyn, and peptides derived from RACK1, Fyn, or ctNR2B restore phosphorylation. (*a*) MBP-ctNR2B (a.a. 1086–1482; 750 ng) was incubated in the absence (lane 1) or the presence (lanes 2–6) of increasing concentrations of RACK1 or 1 μM MBP (lane 8) and 15 units of Fyn kinase (lanes 1–6 and 8). The presence and concentrations of MBP-ctNR2B (a.a. 1086–1482), Fyn, and RACK1 were verified by Western blot analysis (data not shown) ($n = 5$). (*b*) Kinase assays were performed in the presence of RACK1 (500 ng) and in the presence or absence of R7, N1 (100 μM), and N2 (500 μM). Data presented as mean percent of control ± SD ($n = 3$). Results compared with N1 or N2 are statistically significant ($P < 0.01$).

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peptide (R7), RACK1 was unable to inhibit the ctNR2B (a.a. 1086–1482) phosphorylation (Fig. 5*b* and data not shown). The peptides had no effect on ctNR2B (a.a. 1086–1482) phosphorylation when incubated in the absence of RACK1 (data not shown). Our results suggest that peptides that inhibit the binding between RACK1 and ctNR2B or Fyn release RACK1 from the complex and free Fyn to phosphorylate ctNR2B.

RACK1 Inhibits NMDA Receptor-Mediated EPSCs in Hippocampal Slices. Because tyrosine phosphorylation of NMDA subunits enhances the function of the channel (7, 8, 29), inhibition of phosphorylation by RACK1 should have functional consequences. Therefore, NMDA receptor-mediated EPSCs were measured in CA1 hippocampal neurons by using standard whole-cell patch–clamp configuration. We confirmed the presence of high levels of NR2B-containing NMDA receptors in the hippocampus by measuring EPSCs in the presence of ifenprodil, an NR2B-specific channel blocker. Ifenprodil (100 μM) inhibited NMDA receptor-mediated EPSCs by 85% ($n = 5$, data not shown). NMDA receptor-mediated EPSCs were measured in the presence of recombinant RACK1 that was perfused into cells through the recording pipette. Application of RACK1 resulted in a 50% decrease in NMDA receptor-mediated EPSCs (Fig. 6*a*), whereas a RACK1 fragment that does not contain the putative Fyn/NR2B-binding site (RACK1ΔN) had no effect on EPSCs (Fig. 6*a*). Furthermore, the inhibitory effect of RACK1 was specific to the NMDA receptors, because intracellular application of RACK1 did not alter AMPA receptor-mediated EPSCs (Fig. 6*e*).

We hypothesized that the same peptides that inhibited the interaction of Fyn and NR2B with RACK1 and restored phosphorylation *in vitro*, would also increase NMDA channel function in a hippocampal slice preparation. To test this hypothesis, we measured NMDA receptor-mediated EPSCs in the CA1 region in the presence and absence of peptides R14, R7, N1, F1, and the scrambled F1 peptide. Peptides were applied directly into the cells by means of diffusional exchange during standard whole-cell patch–clamp recordings. Application of 500 μM of R14 and F1 (Fig. 6*b*) and N1 (data not shown) potentiated NMDA receptor-mediated EPSCs and thus acted as peptide agonists. A concentration of 100 μM of R14 was still sufficient to induce a 50% increase in NMDA receptor-mediated EPSCs (Fig. 6*c*). In contrast, R7, the RACK1-derived peptide that had little effect on binding and phosphorylation (Figs. 4 *b* and *c* and 5*b*), and the scrambled peptide F1, had no effect on the amplitude of the EPSCs (Fig. 6*b*). In addition, no effect on AMPA receptor-mediated EPSCs was observed in the presence of the RACK1-derived peptide R14 (Fig. 6*e*). In summary, Fyn, NR2B, and RACK1-derived peptides inhibited binding, restored phosphorylation and potentiated NMDA receptor-mediated EPSCs.

If the decrease in the binding of RACK1 to Fyn and NR2B allows Fyn to phosphorylate NR2B, which in turn results in potentiation of the NMDA receptor channel, then a kinase inhibitor of Fyn should decrease the activity of the peptide agonists. We therefore tested the ability of peptide R14 (100 μM) to increase NMDA receptor-mediated currents in the presence and absence of the PTK inhibitor, PP2 (50 nM). Bath application of PP2 abolished the increase in NMDA receptor-mediated EPSCs obtained with R14 (Fig. 6*c*). In addition, we found that bath application of PP2 precludes the ability of RACK1 to inhibit or R14 to increase NMDA receptor activity (Fig. 6*d*). These results suggest that the peptide R14 is enhancing NMDA receptor function via a PTK, presumably Fyn and the release of RACK1.

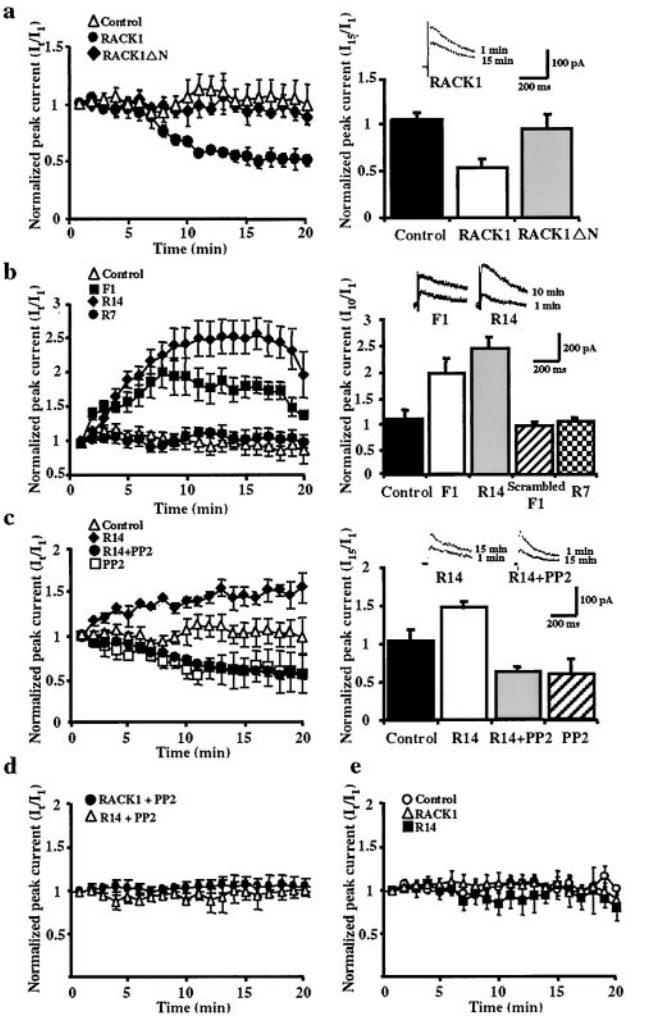

Fig. 6. RACK1 inhibits NMDA receptor-mediated EPSCs, whereas peptides enhance NMDA receptor-mediated currents. (*a*) I_t/I_1 [the average size of the EPSCs for each minute (I_t) divided by the average size of the EPSCs of the first minute (I_1)] was plotted for recordings made by using standard whole-cell voltage–clamp configuration from CA1 pyramidal neurons. EPSCs were measured in the absence (Δ; $n = 5$) or presence of recombinant Tat-RACK1 (●; 15 μg/ml, $n = 4$) or RACK1 fragment that does not contain the putative Fyn/NR2B-binding site Tat-RACK1ΔN (◆; 15 μg/ml, $n = 4$) in the intracellular solution. The bar histogram on the right is the mean ratio ± SD of amplitude of the current recorded at 15 min after the start of the recording (I_{15}) divided by that of the initial current (I_1). *Top Right* shows representative current tracers taken at the time indicated using one neuron perfused with Tat-RACK1. (*b*) EPSCs were recorded in the absence (Δ; $n = 5$), or presence of the Fyn-derived peptide, F1 (■; 500 μM; $n = 5$), the scrambled F1 peptide (500 μM; $n = 5$, shown only in the bar histogram) and the RACK1-derived peptides, R14 (◆; 500 μM; $n = 5$) and R7 (●; 500 μM; $n = 5$). The bar histogram on the right is the mean ratio ± SD of I_{10}/I_1. *Top Right* shows representative current tracers taken at the time indicated using one neuron perfused with F1 and another perfused with R14. (*c*) EPSCs were recorded in the absence (Δ, $n = 5$) or presence (◆, $n = 5$) of RACK1-derived peptide R14 (100 μM), and in the presence of R14 (100 μM) and PP2 (50 nM) (●, $n = 5$) or in presence of only PP2 (□, $n = 5$) (50 nM) in the bath solution. The bar histogram on the right is the mean ratio ± SD of I_{15}/I_1. *Top Right* shows representative current tracers taken at the time indicated using one neuron perfused with R14 and another perfused with R14 and PP2. (*d*) Before the recordings, PP2 (50 nM) was bath applied for 20 min. EPSCs were recorded in the presence of Tat-RACK1 (●; 15 μg/ml, $n = 5$) or RACK1-derived peptide R14 (500 μM; Δ; $n = 4$). (*e*) AMPA receptor-mediated EPSCs were measured in the absence (○; $n = 4$), or presence of recombinant Tat-RACK1 (Δ; 15 μg/ml, $n = 4$), or RACK1-derived peptide, R14 (■; 500 μM; $n = 5$) in the intracellular solution.

Discussion

We have identified RACK1 as a scaffolding protein for Fyn kinase and the NR2B subunit of the NMDA receptor and have revealed a molecular mechanism by which RACK1 affects the function of Fyn and the NMDA receptor. RACK1 directly bound to Fyn and ctNR2B. RACK1 inhibited the phosphorylation of ctNR2B by Fyn and decreased NMDA receptor-mediated EPSCs in the CA1 region of the hippocampus. We identified the binding sites on RACK1, Fyn, and ctNR2B. Peptides derived from these binding sites inhibited the interaction between RACK1 and Fyn, or RACK1 and ctNR2B, and restored the phosphorylation of the NMDA receptor subunit by Fyn. The same peptides increased NMDA receptor-mediated currents. On the basis of our results, we propose the following model: RACK1 binds ctNR2B and Fyn, allowing Fyn to be localized in close proximity to its substrate. However, if RACK1 is present, Fyn cannot phosphorylate ctNR2B. Once RACK1 is released from the complex, Fyn is free to phosphorylate ctNR2B, which in turn results in an increase in the function of the NMDA receptor channel.

Because Fyn and ctNR2B share sequences of homology and interact with RACK1 simultaneously, our results suggest that Fyn and ctNR2B bind RACK1 via the same binding site. One possibility suggests that RACK1 forms separate heteromers with Fyn, or with NR2B, and these heteromers are in equilibrium. Another possibility is that RACK1 forms a homodimer that allows both Fyn and NR2B to interact simultaneously.

Interestingly, RACK1 inhibition of Fyn phosphorylation was specific for NR2B. It is possible that RACK1 masks the phosphorylation sites on NR2B, or that the interaction between RACK1 and NR2B does not allow interaction with Fyn's active sites.

The sequences of homology shared between Fyn and ctNR2B were not found in ctNR1, ctNR2C, and ctNR2D receptor subunits, but the ctNR2A contains a smaller region of 9 a.a. (a.a. 1186–1194) that contain three homologous and three identical residues that shares similarity with Fyn. However, we were not able to detect the presence of NR2A in a trimolecular complex with Fyn and RACK1 (data not shown), suggesting that the interaction is specific for NR2B.

Other members of the Src family of tyrosine kinases, Src and Yes, also share the identified sequence of homology between Fyn and ctNR2B. Src has also been shown to phosphorylate the NMDA receptor (8, 29) and to be involved in processes such as long-term potentiation, learning and memory (12, 13). RACK1 was previously found to interact with Src in NIH 3T3 cells, causing an inhibition of its kinase activity (19). However, in brain, we did not detect interaction of RACK1 with other members of the Src PTKs (Src, Yes, Lyn). Because the RACK1-binding site identified in Fyn is identical with Src, other mechanisms and/or interaction sites are required to promote Fyn binding or prevent Src binding to RACK1.

Interestingly, interaction of Fyn with PSD95 was found to induce tyrosine phosphorylation of NR2A (30). Taken together, it is possible that, whereas PSD95 serves as an activator scaffold protein, RACK1 serves as an inhibitory scaffold protein for NMDA receptor channel function.

In summary, we present data to suggest that RACK1 is an inhibitory scaffolding protein that plays an important role in the regulation of function of the tyrosine kinase Fyn and the NMDA receptor channel. Our finding, therefore, may have implications for essential NMDA receptor-mediated processes such as development, synaptic plasticity, and learning and memory and in disease states such as excitotoxicity and addiction.

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