Spatial and Temporal Regulation of RACK1 Function and N-methyl-D-aspartate Receptor Activity through WD40 Motif-mediated Dimerization*

Claire Thornton‡‡, Ka-Choi Tang‡, Khanhky Phamluong‡, Ken Luong‡, Alicia Vagts‡‡, Donna Nikanjam‡, Rami Yaka‡‡, and Dorit Ron‡‡**

From the ‡Department of Neurology, University of California San Francisco, San Francisco, California, 94143-0114 and the ‡‡Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel.

Efficient signaling requires accurate spatial and temporal compartmentalization of proteins. RACK1 is a scaffolding protein that fulfils this role through interactions of binding partners with one of its seven WD40 domains. We recently identified the kinase Fyn and the NR2B subunit of the N-methyl-D-aspartate receptor (NMDAR) as binding partners of RACK1. Scaffolding of Fyn near its substrate NR2B by RACK1 inhibits Fyn phosphorylation of NR2B and thereby negatively regulates channel function. We found that Fyn and NR2B share the same binding site on RACK1; however, their binding to RACK1 is not mutually exclusive (Yaka, R., Thornton, C., Vagts, A. J., Phamluong, K., Bonei, A., and Ron, D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5710–5715). We therefore tested the hypothesis that RACK1 forms a homodimer that allows the simultaneous binding of Fyn and NR2B. We found that RACK1 binds to itself both in vitro and in the brain. Deletion analyses identified a RACK1-RACK1 dimer-binding site within the 4th WD40 repeat, and application of the 4th WD40 repeat or a peptide derivative to hippocampal slices inhibited NMDAR activity. We further found that in hippocampal slices, both RACK1 and NR2B associated with another WD40 protein, the β-subunit of G protein (Gβ), previously shown to heterodimerize with RACK1 in vitro (Dell, E. J., Connor, J., Chen, S., Stebbins, E. G., Skiba, N. P., Mochly-Rosen, D., and Hamm, H. E. (2002) J. Biol. Chem. 277, 49888–49895). However, activation of the pituitary adenylate cyclase polypeptide 1–38 (–38) G protein-coupled receptor, previously found to induce the dissociation of RACK1 from the NMDAR complex (Yaka, R., He, D. Y., Phamluong, K., and Ron, D. (2003) J. Biol. Chem. 278, 9630–9638), attenuated the association of Gβ with RACK1 and NR2B. Based on these results, we propose that WD40-mediated homo- and heterodimerization of RACK1 mediate the formation of a transient signaling complex that includes the NMDAR, a G protein and Fyn.

RACK1 was originally identified as an anchoring protein for protein kinase C (4), but in the past decade, its role has been greatly expanded through structure prediction, localization, and identification of novel binding partners (reviewed in Ref. 5). RACK1 is a 36-kDa protein containing seven WD40 repeats that mediate its protein-protein interaction properties (6). Homology with the G protein subunit Gβ has led to the prediction of the structure of RACK1 as being a seven-bladed β-sheet propeller (7, 8), suggesting the availability of multiple protein interaction surfaces. RACK1 acts as a scaffolding/anchoring protein for a number of binding partners, ranging from signaling proteins such as kinases (1, 9, 10), a phosphodiesterase (11), and a phosphatase (12) to numerous intracellular tails of receptors (1, 10, 13, 14). In addition, recent data suggest that RACK1 binding may play a role in gene expression, translation, and ribosome assembly and activation (3, 15–17). Therefore, RACK1 is a multipurpose protein that regulates various biological functions.

We recently demonstrated that RACK1 acts as a novel inhibitory scaffolding protein associating in a tri-molecular complex with the tyrosine kinase Fyn and its substrate, the NR2B subunit of the glutamate-gated ion channel, NMDAR (1). We found that this association prevents the phosphorylation of NR2B by Fyn and inhibits NMDAR-mediated activity (1). However, removal of RACK1 from the NMDAR complex via the activation of the cAMP/PKA pathway by the adenylyl cyclase activator forskolin or PACAP(1–38), enables NR2B phosphorylation by Fyn and enhances NMDAR channel function (1, 3).

It is interesting that Fyn and NR2B share a region of sequence homology that mediates their binding at a single RACK1 binding site located in the first WD40 repeat at amino acids 35–48 (1). Our previous in vitro data suggest that Fyn and NR2B interact with RACK1 simultaneously and that RACK1 serves as a physical bridge between the kinase and its

* This work was supported by funds provided by the State of California for medical research on alcohol and substance abuse through the University of California at San Francisco (to D. R.) and by the Department of the Army, Grant DAMD17-01-10740 (to D. R.) for which the United States Army Medical Research Acquisition Activity is the awarding and administering acquisition office. The content of the information represented does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Current address: MRC Clinical Sciences Centre, Imperial College of Science Technology and Medicine, London, UK.

‡ To whom correspondence should be addressed: 5858 Horton St., Suite 200, Emeryville, CA 94608. Tel.: 510-985-3150; Fax: 510-985-3101; E-mail: dorit@itsa.ucsf.edu.
substrate (1). As a possible explanation for the simultaneous interactions of Fyn and NR2B with RACK1 via a single binding site, we hypothesized that RACK1 can bind both Fyn and NR2B by existing as a homodimer.

Herein, we present data showing that RACK1 is indeed capable of forming both homo- and heterodimers with Gβ. We propose that the interaction of RACK1 with itself and with Gβ localizes RACK1 near the NMDAR and the appropriate GPCR, allowing the tight regulation of channel function via the GPCR-mediated Fyn phosphorylation of NR2B.

MATERIALS AND METHODS

Animals—All studies were conducted with approval from the Gallo Center Institutional Animal Care and Use Committee and were in accordance with PHS Policy on Humane Care and Use of Laboratory Animals, Office of Laboratory Animal Welfare, National Institutes of Health, revised 2002.

Reagents—The polyclonal anti-NR2B, anti-Gβ, and anti-glutathione S-transferase (GST) antibodies were purchased from Santa Cruz Biotechnologies. The monoclonal anti-maltose binding protein (MBP), anti-RACK1, and anti-NR2B antibodies were purchased from BD Transduction Laboratories. The polyclonal anti-GlutR1 antibodies were purchased from Chemicon International, Inc. The monoclonal anti-probe synaptosomal density (PSD) 95 antibodies were purchased from Upstate Biotech, Inc. Dithiobisuccinimidylpropionate (DSP) was purchased from Pierce. PACAP (1–38) was purchased from Calbiochem. Forskolin, sodium orthovanadate, and the phosphatase inhibitor mixture were purchased from Sigma. The monoclonal anti-HA antibodies, all secondary antibodies, and the protease inhibitor mixture were purchased from Roche. Restriction enzymes and TNT in vitro translation kit were purchased from Promega. [35S]methionine (15 μCi/ml; 3000 Bq) and Amersham Biosciences. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[fl]quinoline-7-sulfonamide and n(-)-2-amino-5-phosphonopentanoic acid were purchased from Tocris.

Recombinant Proteins—MBP, MBP-RACK1, GST, and GST-RACK1 were expressed in methods described previously (18). RACK1ΔC (N1) (aa 1–180) and RACK1ΔN (aa 138–317) (C2), and the 4D WD40 repeat of RACK1 (RPT4 (144–200)) and RACK1 (18). RACK1/H11011/H9004/H9252 were expressed in Escherichia coli using methods described previously (18). RACK1ΔC (N1) (aa 1–180) and RACK1ΔN (aa 138–317) (C2), and the 4D WD40 repeat of RACK1 (RPT4 (144–179)) were sub-cloned into pTat-HA and expressed and purified from E. coli as described previously (16) (see also Fig. 2a).

Peptides—RPT4-1 (QDESHSEWVSCVR, RACK1 aa 143–155), RPT-4-2 (CVRFSPNSSNPIIV, RACK1 aa 153–166), the RPT4-1 scrambled (16) (see also Fig. 2a).

In vitro overlay binding assay was performed as described in Ref. 18. In brief, proteins or peptides were blotted onto nitrocellulose membrane using a slot blot apparatus (Amersham Biosciences) and incubated in overlay buffer (0.1% bovine serum albumin, 50 mM Tris-HCl, pH 7.5, 0.1% polyethylene glycol (Mr 15,000), and 0.2 M NaCl). Bound proteins were eluted with 10 mM maltose and resolved by SDS-PAGE. Interactions were analyzed either by Western blot and immunoprecipitation was carried out using anti-RACK1 antibodies. RACK1 (lanes 1 and 2) or IgM immunoprecipitations (IP) (lane 4) were analyzed by Western blot using anti-RACK1 antibodies. Also shown is rat brain homogenate (lane 3) (n = 3).

FIG. 1. RACK1 forms a dimer in vitro and in brain. a, increasing concentrations of MBP-RACK1 and MBP were blotted onto nitrocellulose membrane and overlaid with GST-RACK1 (15 μg, top) or GST (15 μg, bottom). Binding was detected with anti-GST antibodies (n = 3). IB, immunoblot, b, MBP (1 mg) and MBP-RACK1 (1 mg) were immobilized on nitrocellulose membrane and overlaid with GST-RACK1 (15 μg). Bound proteins were eluted with 10 mM maltose and resolved by SDS-PAGE. Interactions were analyzed either by Western blot and immunoprecipitation (IP) or IgM immunoprecipitations (IP) (lane 4) were analyzed by Western blot using anti-RACK1 antibodies. Also shown is rat brain homogenate (lane 3) (n = 3).
RACK1 Dimerization Regulates NMDA Receptor Function

RACK1 dimerizes via the fourth WD40 domain.

FIG. 2. RACK1 dimerization regulates NMDA receptor function.

Electrophysiology—Transverse hippocampal slices (350–400 μm) were prepared from 3–4-week-old male Sprague-Dawley rats. The slices were allowed to recover for 2 h in a artificial cerebrospinal fluid perfusion medium that contained: 126 mM NaCl, 1.2 mM KCl, 1.2 mM NaH2PO4, 0.11 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, and 11 mM glucose, saturated with 95%O2/5%CO2 at 25 °C. After recovery, slices were submerged and continuously superfused with artificial cerebrospinal fluid at 25 °C.

Field excitatory postsynaptic potentials (fEPSPs) were recorded from stratum-radiatum of CA1 region with glass microelectrodes filled with 2 M NaCl. To obtain NMDA-mediated fEPSPs, picrotoxin (100 μM) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (10 μM) were added to the bath solution to block GABA_A receptor- and AMPA-mediated inhibitory postsynaptic potentials and fEPSPs, respectively. To evoke fEPSPs, Schaffer collateral/commissural afferents were stimulated with 0.1-Hz pulses using steel bipolar microelectrodes at intensities adjusted to produce an evoked response that was 50% of the maximum recorded fEPSP for each recording. The maximal rate of change in fEPSP within a time window selected around the rising phase was calculated.

Whole-cell, voltage-clamp recordings were made from CA1 pyramidal cells using a glass microelectrode (3–5 MΩ) filled with internal solution containing 117 mM cesium methanesulfonic acid, 2.8 mM NaCl, 20 mM HEPES, 0.4 mM EGTA, 5 mM tetraethylammonium chloride, 2.5 mM MgATP, and 0.25 mM MgGTP. The pH and osmolarity of the internal solution were 7.2–7.4 and 290–300 mOsm, respectively. Cells were held at +40 mV and −70 mV for NMDAR- and AMPA-mediated excitatory postsynaptic currents (EPSCs), respectively. Series and input resistances were continuously monitored with a 4-mV depolarizing step given with every afferent stimulus. The amplitudes of EPSCs were measured using a window at the peak of the event, relative to the baseline taken immediately before the stimulus artifact. Data were collected using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA), filtered at 2 kHz, and digitized at 5–10 kHz. Results are presented as mean ± S.E. Student’s t tests and one-way analysis of variance followed by Newman-Keuls multiple comparison test were used in data analysis.

RESULTS

RACK1 Forms a Dimer In Vitro and in Brain—We previously determined that Fyn and NR2B share the same binding site on RACK1. Our previous results also suggest that the binding of Fyn and NR2B to RACK1 is not mutually exclusive but simultaneous (1). We hypothesized, therefore, that RACK1 forms a homodimer that enables both Fyn and NR2B to bind. To test this hypothesis, RACK1 was expressed as MBP and GST fusion proteins, and binding of GST-RACK1 to MBP-RACK1 was detected using an overlay assay. As shown in Fig. 1a, GST-
RACK1 directly interacted with MBP-RACK1 (top) but not with the control protein MBP (top), and GST alone did not bind MBP-RACK1 (bottom), suggesting that RACK1 is capable of interacting with itself. Next, MBP-RACK1 or MBP was immunoblotted on a column, incubated with brain homogenate, and the association of endogenous RACK1 to recombinant MBP-RACK1 or MBP was determined by Western blot analysis using anti-RACK1 antibodies. As shown in Fig. 1b, a single immunoreactive band at the size of RACK1 was detected binding to MBP-RACK1 but not to MBP, suggesting that endogenous RACK1 can interact with itself. To test whether the RACK1 homodimer is present in brain in vivo, we treated rat brain homogenates with the membrane-permeable cross-linking reagent DSP, and carried out immunoprecipitations using anti-RACK1 antibodies. In DSP-treated homogenates, a RACK1-immunoreactive band was detected with a mass of 65 kDa (Fig. 1c, lane 2). The RACK1 monomer band immunoprecipitated from the untreated homogenate (Fig. 1c, lane 1) but was absent in the immunoprecipitation from the cross-linked homogenate (Fig. 1c, lane 2). These results imply that RACK1 can exist as a dimer in rat brain; however, we cannot exclude the possibility that the higher molecular weight complex also contains a different protein of similar size that interacts with RACK1. Nevertheless, we have found that RACK1 forms a homodimer in vivo and exists in a higher molecular mass complex in rat brain preparations.

Identifying the Binding Site for RACK1 Dimerization—Having determined that RACK1 is capable of forming a homodimer, we next set out to identify the dimer-binding site on RACK1. We expressed the N terminus fragment (aa 1–180, N1) and the C terminus fragment (aa 313–650, C1) of RACK1 (Fig. 2a, diagram) as HA-tagged fusion proteins and tested their ability to bind immobilized MBP-RACK1. It is interesting that significant RACK1 binding was detected with both deletion fragments (Fig. 2b, lanes 1 and 2), above the nonspecific binding to MBP alone (Fig. 2b, lanes 3 and 4). The RACK1 mutants N1 and C1 share a common sequence of 42 amino acids (aa 138–180), which encompasses the 4th RACK1 WD40 repeat (aa 144–179). Thus, sequences within this region could mediate at least part of the RACK1-RACK1 interaction. We therefore generated a series of RACK1 deletion mutants: (N2, C1, C2), and the 4th WD40 repeat (RPT4) bound to MBP-RACK1 (Fig. 2c, top, middle). No binding was observed for the N2 mutant (Fig. 2c, lane 1, top); however, the 4th WD40 repeat (RPT4) bound to MBP-RACK1 (Fig. 2c, lane 1, bottom), suggesting that the region between amino acids 144–179 contains a RACK1 dimerization site.

If RPT4 does indeed represent, at least in part, the dimerization site for RACK1, it should be possible to prevent RACK1 dimerization by preincubation of a RACK1 monomer with the RPT4 fragment. We therefore tested the ability of the radiolabeled C2 deletion mutant to bind with MBP-RACK1 in the presence and absence of the radiolabeled RPT4. RPT4 incubation reduced the binding of the C2 fragment to MBP-RACK1 (Fig. 2d) suggesting further that the 4th WD40 repeat is mediating, at least in part, the RACK1 homodimer formation. Based on these results, we generated two peptides spanning the 4th WD40 repeat, RPT4-1 (amino acids 143–155) and RPT4-2 (amino acids 153–166). To determine whether there was a preference in binding RACK1, we immobilized RPT4-1 and RPT4-2 onto nitrocellulose membrane and performed an overlay assay using MBP-RACK1 or MBP (Fig. 2e). Both peptides bound to MBP-RACK1 but not MBP; however, the binding between MBP-RACK1 and RPT4-1 (Fig. 2e) was much stronger than with RPT4-2 (Fig. 2e), suggesting that a dimer-binding site is contained within amino acids 143–155 of RACK1.

**Fig. 3.** The 4th WD40 repeat (RPT4) of RACK1 attenuated PACAP(1–38)-mediated enhancement of NMDAR function. a, rat hippocampal slices were incubated with artificial cerebrospinal fluid containing 1 μM HA-tagged Tat-HA-RPT4 for 2 h at room temperature. Cell homogenate (H) was fractionated to produce the Triton-soluble (TS) and Triton-insoluble (TIS) material. Samples (50 μg) were resolved by SDS-PAGE and analyzed by Western blot with anti-NR2B. Cell homogenate (H) was fractionated to produce the Triton-soluble (TS) and Triton-insoluble (TIS) material. Samples (50 μg) were resolved by SDS-PAGE and analyzed by Western blot with anti-NR2B.

Hatched bar indicates the period of PACAP(1–38) application. Data are presented as mean percentage of baseline ± S.E. Inset, traces of NMDAR-fEPSPs (average of 12 consecutive responses) obtained from 1) a slice recorded before (baseline, 8–10 min) and during PACAP(1–38) application (18–20 min); 2) pretreatment with Tat-HA-RPT4 followed by PACAP(1–38) application; or 3) pretreatment with Tat-peptide followed by PACAP application. c, histogram depicts the percentage change in fEPSP amplitude baseline compared with that 10–15 min after PACAP(1–38) application. One-way analysis of variance showed significant difference among different treatments (p < 0.0001). PACAP(1–38)-enhanced NMDAR-fEPSPs (black bar, 35.97% ± 5.18%) was attenuated in Tat-HA-RPT4 experiments (open bar, −3.98% ± 4.14%, **p < 0.001, Newman-Keuls multiple comparison test, n = 5), but not in the Tat-peptide experiments (hatched bar, 25.19% ± 2.22, p > 0.05, Newman-Keuls multiple comparison test).
RACK1 Homodimerization Is Important for NMDAR Function in the Hippocampus—In the hippocampus, RACK1 serves as an inhibitory scaffolding protein preventing the phosphorylation of the NMDAR by Fyn (1). Activation of the cAMP/PKA pathway by forskolin or PACAP(1–38) causes RACK1 to be removed from the NMDAR complex, which leads to an increase in Fyn phosphorylation of NR2B, and the enhancement of NMDAR-mediated activity (3) (Figs. 3b and 4c, open circle). We hypothesized that disruption of the RACK1 dimer by the 4th WD40 repeat fragment or a peptide derivative would interfere with RACK1 maintenance of the NR2B/RACK1/Fyn complex and consequently alter NMDAR-mediated function. To test the hypothesis, we first generated a Tat-HA-RPT4 fusion protein in which the Tat moiety allowed the transduction of the fragment into hippocampal slices (3). We measured the ability of PACAP(1–38) to enhance NMDAR activity in the presence and absence of Tat-HA-RPT4 and the Tat-peptide control. As shown in Fig. 3, Tat-HA-RPT4 was successfully transduced into hippocampal slices (Fig. 3a), and the transduction of 1 μM Tat-HA-RPT4 protein, but not the Tat-peptide alone, abolished PACAP(1–38)-mediated enhancement of NMDAR-mediated fEPSPs to baseline amplitude (Fig. 3, b and c). We also carried out whole-cell, patch-clamp experiments using 10 μM forskolin to enhance NMDAR activity in CA1 pyramidal cells, and introduced the RPT4-1 peptide through the recording electrode. We found that in the presence of 100 μM of RPT4-1, but not the scrambled peptide, forskolin-mediated increases in NMDAR-mediated EPSC amplitude were completely inhibited (Fig. 4, a and b). However, RPT4-1 did not alter AMPAR-mediated EPSCs (Fig. 4c). Because the RACK1/Fyn pathway does not alter AMPAR activity (1, 3), these results suggest that disruption of the RACK1 dimer affects the proper function of the NMDAR.

Gβ Interacts with RACK1 and NR2B in Brain—Recently, Gβ is structurally related to RACK1, and β-subunit of G protein, Gβ, was identified as a RACK1 binding partner in vitro (2). Gβ is structurally related to RACK1, and both proteins contain seven WD-40 repeat-containing proteins. In addition, a recent study by the same group suggests that in COS-7 cells, Gβ localizes RACK1 to the membrane (22). Therefore, we tested the hypothesis that in brain, the NR2B/Fyn/RACK1 trimer-molecular complex might be stabilized at the membrane by the interaction of RACK1 with Gβ. Gβ, together with Gα, associates with GPCRs and is primarily localized to the membrane, as is NR2B (Fig. 5a, top and bottom). Furthermore, a portion of Gβ is immunoreactive in the synaptosomal membranal fraction (LP1) where the majority of NR2B is localized (Fig. 5b).

Although RACK1 is mainly cytosolic, a portion of it is found in the membranal fraction (Fig. 5a, middle), and specifically in the synaptosomal membranal fraction (Fig. 5b). To determine whether RACK1 interacts with Gβ in brain, we immunoprecipitated RACK1 from whole-brain homogenates and tested for the presence of Gβ in the complex. We observed a significant amount of Gβ co-immunoprecipitating with RACK1 (Fig. 5c, lane 1). To determine whether the interaction of RACK1 with Gβ allows the

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**Fig. 4.** RPT4-1 peptide abolishes forskolin-enhanced NMDAR-EPSCs. a, NMDAR-mediated EPSCs plotted for recordings made from hippocampal CA1 slices in the presence of 10 μM forskolin (Fsk) alone (C, n = 6), forskolin + 100 μM RPT4-1 peptide ( ), n = 6), or forskolin + 100 μM scrambled RPT4-1 peptide ( , n = 6). The horizontal bar indicates the period of forskolin application. Peptides were applied via the patch electrode by means of diffusional exchange during whole-cell recordings as described previously (1). Data are presented as mean percentage of baseline ± S.E. Inset, traces of NMDAR-EPSCs (average of 12 consecutive responses) obtained from the following: 1) a neuron recorded before (baseline, 3–5 min) and during forskolin application (13–15 min); 2) infusion of RPT4-1 peptide followed by forskolin application; 3) infusion of scrambled RPT4-1 peptide followed by forskolin application. b, histogram depicts the percentage change in EPSC amplitude baseline compared with 10–15 min after forskolin application. One-way analysis of variance showed significant difference among different treatments (p < 0.0001). Forskolin-enhanced NMDAR-EPSCs (black bar, 35.12% ± 6.52, n = 6) was significantly abolished in RPT4-1 dialysis experiments compared with forskolin alone (open bar, 6.48% ± 3.83, **, p < 0.001, Newman-Keuls multiple comparison test, n = 6), but not in scrambled RPT4-1 peptide dialysis experiments compared with forskolin alone (hatched bar, 35.12% ± 6.52, p > 0.05, Newman-Keuls multiple comparison test, n = 6). c, AMPAR-mediated EPSCs plotted for recordings made from hippocampal CA1 slices in cells dialyzed with 100 μM RPT4-1 peptide. I/L1 [the average size of the AMPAR-EPSCs for each minute (I)] divided by the average size of the AMPAR-EPSCs of the first minute (L1) was plotted for recordings made by using standard whole-cell voltage-clamp configuration from CA1 pyramidal cells. The measurement of AMPAR-EPSCs started 3 min after the breakthrough of the recording pipette (n = 4). Data are presented as mean percentage of baseline ± S.E.
formation of a larger complex containing the NMDAR, we immunoprecipitated NR2B and found Gβ to be co-immunoprecipitated as well (Fig. 5d, lane 1). To determine whether the association between NR2B and Gβ was specific, we immunoprecipitated the GluR1 subunit of AMPAR and determined whether Gβ was co-immunoprecipitated. As shown in Fig. 5f, lane 1, no association between GluR1 and Gβ was observed. These results suggest that RACK1 is linking Gβ, and therefore its GPCR, to the NMDAR complex at the synapse. At this point, however, we cannot exclude the possibility that NR2B and Gβ directly interact.

As mentioned previously, exposure of hippocampal slices to PACAP(1–38) leads to NR2B phosphorylation by Fyn and enhancement of channel activity (3) (Fig. 3b, open circle). Activation of GPCRs such as the PACAP (1–38) receptor PAC1 results in the dissociation of Ga from Gβγ and the initiation of downstream signaling pathways. It is interesting that Dell and colleagues (2) found that the binding affinity of RACK1 for Gβγ is greater with the G protein holoenzyme Gaβγ than with Gβγ alone. Therefore, we hypothesized that exposure of hippocampal slices to PACAP(1–38) may release RACK1 not only from the NMDAR complex but also from Gβγ. To test this possibility, we treated hippocampal slices with PACAP(1–38) and examined the interaction between Gβ and RACK1 or NR2B. We observed a decrease in the co-immunoprecipitation of Gβ with RACK1 and NR2B after PACAP(1–38) treatment (Fig. 5, c, d, e, and f), suggesting that activation of the GPCR leads to the dissociation of the RACK1-Gβ heterodimer and the consequent loss of the association between a G protein and an ion channel.

DISCUSSION

Compartmentalization of proteins is recognized as a fundamental mechanism for introducing subtle control of signal transduction pathways (23). Thus, understanding the role of scaffolding proteins in signaling processes becomes essential. We found previously that RACK1 scaffolds the kinase Fyn to its substrate, the NR2B subunit of the NMDAR; surprisingly, however, the sites for these interactions on RACK1 overlap (1). We hypothesized that this signaling complex could be formed only if RACK1 exists as a dimer. Herein, we show that RACK1 is capable of forming homo- and heterodimers. We found that RACK1 binds to itself in vitro and in brain, and this interaction is mediated by the fourth of its seven WD40 repeats. It is noteworthy that our results further suggest that the RACK1 homodimer is important for the function of the NMDAR, because disruption of the RACK1 dimer, by application of the 4th WD40 repeat (RPT4) or a peptide derivative (RPT4-1), prevented PACAP(1–38)- and forskolin-induced increase in NMDAR-mediated channel activity. In addition, we found evidence to suggest that RACK1 forms a WD40 motif-
mediated heterodimer with Gβ in brain. The Gβ binding site on RACK1 was previously mapped to sequences within amino acids 1–217 (2). This region contains the 4th WD40 repeat; therefore, we cannot rule out the possibility that the 4th WD40 repeat also contributes to the RACK1-Gβ interaction. However, this is unlikely, because Dell et al. (2) reported that activated β1 protein kinase C competes with Gβ binding to RACK1, suggesting an overlap of binding sites between these two proteins; however, the β1 protein kinase C binding sites on RACK1 are not within the 4th WD40 repeat. Furthermore, the peptide RPT4-1 was found to inhibit the enhancement of NMDAR activity in the presence of forskolin. Because forskolin activates adenylate cyclase directly and bypasses the GPCR, it is highly likely that the 4th WD40 repeat-derived peptide specifically inhibits the RACK1 homodimer but not heterodimer interaction.

A fraction of both RACK1 and Gβ is found in the synaptosomal fraction. Therefore, our data suggest that Gβ associates RACK1 at the membrane to stabilize the trimolecular complex of NR2B/RACK1/Fyn at the synapse (Fig. 6α). This correlates with observations made by Chen and colleagues (22), suggesting that Gβ recruits RACK1 to the membrane in COS-7 cells. We propose that the formation of the Gβ/RACK1 heterodimer enables the cross-talk between a GPCR and the NMDAR. In particular, treatment of hippocampal slices with PACAP(1–38) releases RACK1 from the tri-molecular complex (3). PACAP(1–38) activates its receptor, causing a dissociation of Ga from Gβγ. Free Gβγ has a reduced affinity for RACK1, and this destabilizes the trimolecular complex (Fig. 6b). Taken together, our data correlate with in vitro observations that the binding affinity of the RACK1 dimer is greater for the G protein holoenzyme than for Gβγ alone (2). However, a recent study from the same group suggests that in COS-7 cells, RACK1 binds free Gβγ (22). This discrepancy may be explained by the multifunctional nature of RACK1. It is not unlikely that differential binding of RACK1 with its various partners occurs in different cellular environments. Indeed, for example, the scaffolding of NR2B and Fyn by RACK1 is observed in the hippocampus but not in the cortex, indicating brain region specificity for RACK1 interaction with its binding partners (24).

Homo- and heterodimerization have previously been shown to play important roles in the function of other adaptor and scaffolding proteins. For example, 14-3-3 adaptor proteins are important regulators of diverse functions mediated via the binding of 14-3-3 to serine-phosphorylated proteins (25). The 14-3-3 proteins exist as dimers, and each half of the dimer is capable of binding phosphoserine residues, allowing for the multiplicity of function (25). Another example is the Homer family of scaffolding proteins. The coiled-coil structure of Homer mediates the formation of both homo- and heteromultimers (26). By forming multimers, Homer proteins are capable of connecting the metabotropic glutamate receptors mGluR1 and mGluR5 to both the intracellular storage sites by interacting with the inositol 1,4,5-trisphosphate or ryanodine receptor and indirectly to the NMDAR complex via an interaction with yet another dimer-forming protein Shank (26).

Furthermore, the ability of RACK1 to heterodimerize with Gβ opens up the possibility of further interactions with other WD40 motif-containing proteins. WD40 domains are found in a number of proteins involved in a wide variety of processes such as RNA processing, cell cycle control, and signal transduction (6). RACK1 has many and varied functions within the cell, dependent on both its binding partners and its subcellular localization (5). Investigation of WD40 motif-mediated binding may begin to explain the unique flexibility in function of RACK1. It is interesting that both Gβ and RACK1 interact with Pleckstrin homology domain-containing proteins (27, 28), and RACK1 was recently pulled out in a yeast two-hybrid screen as a binding partner of the first PSD95/Disc-large/ZO-1 (PDZ) domain of the human Na+/H+ exchanger regulatory factor (29). Many Pleckstrin homology and PDZ domain-containing proteins are found in the postsynaptic density and are very important for the regulation of neuronal functions. This raises the possibility that RACK1 is a core protein for a multicomplex protein scaffold at the postsynaptic density.

Formation of signaling complexes that connect GPCRs and ion channels is gaining increasing attention as an important means for regulation of and cross-talk between, both types of receptors. For example, the PDZ domains containing Drosophila melanogaster protein InaD binds the rhodopsin GPCR and the calcium ion channel TRP (30). InaD also interacts with components of the protein kinase C signaling pathways, allowing the regulation of the channel via the activation of the GPCR (30). The formation of a signaling complex was also suggested by Davare et al., who reported that the β-adrenergic receptor 2 interacts directly with the CaV1.2 calcium channel (31). The complex also contains G proteins and proteins from the PKA signaling pathways, allowing the rapid and precise regulation of the activity of the channel via the GPCR (31).

Finally, elucidation of the consequences of direct interaction between NMDAR and functionally diverse membrane-bound receptors is in its relatively early stages and adds another layer of regulation to NMDAR-mediated channel activity. For example, NR1 was found to bind to the EphB receptors in response to activation of EphB by its ligand ephrinB, resulting in a clustering of NMDARs at the postsynaptic site (32). This causes increased NMDAR-mediated calcium influx and gene expression (33). EphB is a receptor tyrosine kinase, and acti-
vation by ephrinB results in recruitment of Src to the EphB receptor and increased phosphorylation of NR2B (33). In addition, Dopamine D1 GPCR and D5 have been shown to bind to NMDAR (34). It is interesting that D1 receptor binding is 2-fold, taking place on two different NMDA subunits, NR1 and NR2A. This results in distinct outcomes (i.e. suppression of NMDA receptor-mediated channel activity and inhibition of NMDA receptor-mediated cell death) depending on the interaction (34). As well as modulating channel activity, these interactions can exert an effect on the regulation of intracellular signaling. In contrast with studies on the action of D1 receptors on NMDAR, Pei and colleagues have shown recently that activation of NMDA receptors can recruit D1 receptors to the plasma membrane and thus increase D1 receptor-mediated accumulation of cAMP within the cell (35). This may explain the transduction of signals such as the phosphorylation via homo- and hetero-dimerization of RACK1 are highly important for the transduction of signals such as the phosphorylation and activation of the NMDAR.

Acknowledgments—We thank Dr. Steven Dowdy (University of California San Diego) for the kind gift of the pTAT-HA plasmid and Dr. Daria Mochly-Rosen (Stanford University) for the GST-RACK1 construct. We thank Drs. Antonello Bonci, Rachel Jurd, and Jennifer Whistler for critical reading of the manuscript.

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Spatial and Temporal Regulation of RACK1 Function and N-methyl-D-aspartate Receptor Activity through WD40 Motif-mediated Dimerization
Claire Thornton, Ka-Choi Tang, Khanhky Phamluong, Ken Luong, Alicia Vagts, Donna Nikanjam, Rami Yaka and Dorit Ron

J. Biol. Chem. 2004, 279:31357-31364.
doi: 10.1074/jbc.M402316200 originally published online May 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402316200

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