Local control of AMPA receptor trafficking at the postsynaptic terminal by a small GTPase of the Rab family

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Running title: Rab8 controls AMPA receptor synaptic trafficking

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SUMMARY

The delivery of neurotransmitter receptors into the synaptic membrane is essential for synaptic function and plasticity. However, the molecular mechanisms of these specialized trafficking events and their integration with the intracellular membrane transport machinery are virtually unknown. Here, we have investigated the role of the Rab family of membrane sorting proteins in the late stages of receptor trafficking into the postsynaptic membrane. We have identified Rab8, a vesicular transport protein associated to trans-Golgi network membranes, as a critical component of the cellular machinery that delivers AMPA-type glutamate receptors into synapses. Using electron microscopic techniques, we have found that Rab8 is localized in close proximity to the synaptic membrane, including the postsynaptic density. Electrophysiological studies indicated that Rab8 is necessary for the synaptic delivery of AMPA receptors during plasticity (long-term potentiation) and during constitutive receptor cycling. In addition, Rab8 is required for AMPA receptor delivery into the spine surface, but not for receptor transport from the dendritic shaft into the spine compartment or for delivery into the dendritic surface. Therefore, Rab8 drives specifically the local delivery of AMPA receptors into synapses. These results demonstrate a new role for the cellular secretory machinery in the control of synaptic function and plasticity directly at the postsynaptic membrane.
INTRODUCTION

Synaptic plasticity is critical for the establishment and maturation of functional neuronal circuits in the brain and is widely thought of as the cellular process responsible for learning and memory. An important mechanism controlling synaptic maturation and remodeling is the targeting and delivery of neurotransmitter receptors into synapses. However, very little is known of the exocytic processes that sort neurotransmitter receptors into the postsynaptic membrane. In addition, it remains to be determined how these specialized trafficking events are integrated with the intracellular membrane transport machinery. In fact, most of our current knowledge on membrane trafficking at the synapse derives from studies on neurotransmitter vesicle fusion at the presynaptic terminal (reviewed in (1,2)).

The AMPA-type glutamatergic receptors (AMPARs) are highly dynamic components of excitatory synapses. They can traffic in and out of the synaptic membrane constitutively or in an activity-dependent manner, and this regulated trafficking is known to contribute to synaptic plasticity during brain development and in adulthood (3-6). AMPARs are hetero-oligomeric molecules composed of different combinations of GluR1 to GluR4 subunits (7). In hippocampus, AMPARs containing GluR1 or GluR4 subunits are delivered to synapses in an activity-dependent manner upon NMDA receptor activation, leading to long-lasting synaptic potentiation (8-11). In contrast, AMPARs containing only GluR2 and GluR3 subunits cycle continuously in and out of synapses in a manner largely independent from synaptic activity (10,12), but dependent on NSF (13-15) and Hsp90 (16) function. These two distinct trafficking routes have been coined as regulated and constitutive pathways, respectively (17). However, the cellular and molecular mechanisms mediating membrane transport in these pathways are largely unknown.
Rab8 controls AMPA receptor synaptic trafficking

Members of the Rab family of small GTPases are important regulators of intracellular membrane sorting in eukaryotes. In particular, they are proposed to mediate membrane transport specificity (18-20). Rab proteins are likely to be important for neuronal function, since alterations in Rab protein regulation can lead to mental retardation in humans (21). Rab3 is a presynaptic member of this family (22). It controls the fusion of neurotransmitter vesicles with the plasma membrane (23,24) and mediates some presynaptic forms of synaptic plasticity (25,26). In contrast, the potential role of Rab proteins in neurotransmitter receptor targeting and synaptic function at the postsynaptic terminal has never been tested.

Besides Rab3, three members of the Rab family are involved in exocytic delivery into the plasma membrane: Rab4, Rab8 and Rab11. In neurons, Rab8 has an exclusive somatodendritic distribution (27). In epithelial (28,29) and photoreceptor (30,31) cells, Rab8 mediates the transport of trans-Golgi network-derived membranes into specific compartments of the plasma membrane. Rab4 is involved in the recycling pathway from early endosomes back into the plasma membrane, whereas Rab11 mediates membrane transport from late endosomes and trans-Golgi network (32-39). These three Rab proteins act on late stages of the exocytic trafficking into the plasma membrane, and therefore, are attractive candidates to mediate receptor targeting into the postsynaptic membrane.

In this study, we have explored the role of these exocytic Rab proteins in synaptic function and plasticity, and in particular, in the late trafficking events that deliver AMPARs into synapses. Using a combination of molecular biology, electrophysiology and imaging techniques on organotypic hippocampal slice cultures, we have found that Rab8, but not Rab4 or Rab11, are required for the late stages of AMPAR synaptic delivery into the postsynaptic membrane.
EXPERIMENTAL PROCEDURES

Constructs of recombinant proteins and expression. Rab8a, Rab4a and Rab11a coding sequences were cloned by PCR from a commercial rat brain cDNA preparation (Clontech, cat. # 7150). The rat Rab8a sequence that we cloned has 96% and 97% identity to the mouse and human sequences, respectively. The reported Rab8a rat sequence in the NCBI database (accession numbers M83675 and NM_053998) has a significant N-terminal truncation (83 amino acids) with respect to the mouse and human sequences and with respect to the rat sequence we cloned. This is probably due to a sequencing error in the rat sequence submitted originally. Our cloned Rab4a sequence was identical to the reported rat Rab4a sequence in the NCBI database (accession number P05714) with the exception of two differences: Q47S and T75R. These two amino acids in our sequence (47S) and (75R) are conserved in the mouse and human sequences reported in the NCBI database (accession numbers NP_033029 and NP_004569, respectively). Therefore, these two differences probably correspond to sequencing errors in the previously reported rat sequence. Our cloned Rab11a sequence was identical to the reported one in the NCBI database (accession number NM_031152). Rab8a, Rab4a and Rab11a coding sequences were cloned as fusion proteins downstream from EGFP using the pEGFP-C1 plasmid (Clontech, cat. # 6084). The dominant negative mutants (GDP-bound form) were generated by PCR introducing a single-amino acid substitution previously described (T22N for Rab8dn, S22N for Rab4dn and S25N for Rab11dn). The RFP fusion construct of Rab8dn was generated with a red fluorescence protein variant (tdimer2(12); (40)) generously provided by Dr. Roger Tsien (UCSD). All constructs were recloned into pSinRep5 for Sindbis virus preparation (41). Hippocampal slices are prepared from young rats (postnatal day 5 to 7) and placed in culture on
Rab8 controls AMPA receptor synaptic trafficking

semiporous membranes (42). After 4-7 days in culture, the recombinant gene is delivered into the slices. For the experiments shown in Fig. 3A and B, Fig. 4, Fig. 5B and C and Fig. 7, we used the biolistic delivery method (43), which allowed us to co-express several proteins with plasmids bearing the CMV promoter. For the rest of the experiments we use the Sindbis virus expression system (44). This is a replication-deficient, low-toxicity, neurotropic virus, allowing the expression of recombinant proteins exclusively in neurons by injecting the viral solution extracellularly in the desired area of a hippocampal slice (41). Expression of the recombinant proteins was for 36 hours when AMPA receptor subunits were expressed (Fig. 3A and B, Fig. 4 and Fig. 7), or for 15 hours in the rest of the cases. Neurons remain morphologically and electrophysiologically intact during these expression times. All biosafety procedures and animal care protocols were approved by the University of Michigan.

**Electrophysiology.** Simultaneous double whole-cell recordings were obtained from nearby pairs of infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. The recording chamber was perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl₂, 4 mM MgCl₂, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 11 mM glucose, 0.1 mM picrotoxin, 10 µM bicuculline and 2 µM 2-chloroadenosine, at pH 7.4, gassed with 5% CO₂/95% O₂. Patch recording pipettes (3-6 MΩ) were filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₃GTP, 10 mM sodium phosphocreatine and 0.6 mM EGTA at pH 7.25. In the rectification experiments, i.e. Fig 7, 0.1 mM spermine was added. Voltage-clamp whole-cell recordings were carried out with multiclamp 700A amplifiers (Axon Instruments, Union City, California, USA). Synaptic responses were evoked with two bipolar electrodes with single voltage pulses (200 µs,
Rab8 controls AMPA receptor synaptic trafficking

up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 μm and 500 μm from the recorded cells. Since only CA1, but not CA3 cells are infected, this configuration ensures that recombinant proteins are always expressed exclusively in postsynaptic cells. Synaptic AMPA receptor-mediated responses were measured at –60 mV and NMDA receptor-mediated responses at +40 mV and were averaged over 50-100 trials. In the rectification experiment (Fig 7), NMDA receptor-mediated responses were blocked pharmacologically using 0.1 mM DL-APV. Synaptic AMPA receptor-mediated responses were measured at –60 mV and +40 mV and their ratio was used as an index of rectification. LTP experiments were carried out as previously described (16), by pairing 0 mV postsynaptic depolarization with 3 Hz presynaptic stimulation (300 pulses).

Biochemistry. Hippocampal extracts were prepared in homogenization buffer containing protease inhibitors (10 mM HEPES, 500 mM NaCl, 10 mM EDTA, 4 mM EGTA, 0.1 mM phenylmethysulfonyl fluoride, 2 μg/ml chymostatin, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml pepstatin and 1% triton X-100) as previously described (8). Expression of Rab proteins was analyzed by Western blot with anti-Rab4 (BD Biosciences), anti-Rab8 (Pharmingen) and anti-Rab11 (Zymed Laboratories) antibodies. Phosphorylation of αCaMKII at Thr286 was analyzed with phospho-specific and regular anti-αCaMKII antibodies (Upstate) using the homogenization buffer described above supplemented with phosphatase inhibitors (10 mM NaF, 1 μM microcystin LR and 0.5 μM calyculin A).

Fluorescence immunohistochemistry. Immunohistochemical detection of recombinant AMPA receptors was carried out with anti-GFP mouse antibody (Roche). Fluorescence labeling was
Rab8 controls AMPA receptor synaptic trafficking

achieved with anti-mouse biotinylated secondary antibody (Sigma) and streptavidin tagged with AlexaFlour 594 (Molecular Probes; Fig. 3) or with Cy5 (Amersham Biosciences; Fig. 4). Detergents were omitted in all incubations to evaluate surface expression. Images were taken using Olympus FV 500 confocal microscopy. A 60 X lens with water immersion interface was used. FluoView software was used for acquiring the images. ImageJ was used for 3D reconstruction and quantification of fluorescence intensities. Analysis of surface immunostaining at spines and dendrites was carried out as follows. Line plots of fluorescence intensity were generated across spine heads and the adjacent dendritic shafts. Fluorescence intensity at each compartment was quantified from the peaks corresponding to the spine and the dendrite after background subtraction (Fig. 4B). Then, surface ratios are calculated as the ratio between the GFP signal (total receptor) and the Cy5 signal (surface receptor).

**Immunogold electron microscopy.** Hippocampal slices were fixed and processed for osmium-free post-embedding immunogold labeling, essentially as previously described (45). Rab8 was labeled with anti-Rab8 antibody (Pharmingen) and an anti-mouse antibody coupled to 6 nm gold particles (Electron Microscopy Sciences). Electron micrographs were obtained with a Philips CM-100 transmission electron microscope and a Kodak 1.6 Megaplus digital camera.
RESULTS

Role of Rab4, Rab8 and Rab11 in AMPA receptor-mediated synaptic transmission

As a first step to evaluate the role of these three exocytic Rab proteins in synaptic transmission, we blocked their function individually by expressing the corresponding dominant negative (Rab-dn) forms as GFP-fusion proteins in organotypic hippocampal slice cultures (see Experimental Procedures). Point mutations known to confer dominant negative (GDP-bound) phenotypes to these proteins have been employed in multiple occasions: Rab4(S22N) (46-48), Rab8(T22N) (31,49-51), Rab11(S25N) (36-39,52-55). Also, GFP-Rab fusions have been used successfully in numerous studies to study membrane trafficking in living cells (31,33,56-58). Rab4dn-, Rab8dn- and Rab11dn-GFP were efficiently expressed in hippocampal slice cultures, as assayed by Western blot analysis (see left insets in Fig. 1).

The effect of these dominant negative proteins on AMPA and NMDA receptor function was evaluated by recording simultaneously from nearby infected and uninfected CA1 neurons, allowing direct comparison of synaptic responses evoked by stimulating the Schaffer collateral pathway from the CA3 region. Importantly, only CA1 but not CA3 cells express the recombinant dominant negative protein, and therefore, the site of action of these proteins when monitoring CA3-to-CA1 synaptic transmission is necessarily postsynaptic. As shown in Fig. 1A, Rab8dn significantly depressed AMPAR-mediated currents, without affecting NMDAR-mediated currents. Other electrophysiological parameters were not significantly different between uninfected and Rab8dn-infected neurons (input resistance: 168±23 MΩ -uninfected-, 192±25 MΩ -infected-; holding current: -68±13 pA –uninfected-, -57±9 pA –infected-). This result suggests that Rab8 function is required for AMPAR delivery into synapses. In contrast, neither
Rab8 controls AMPA receptor synaptic trafficking

Rab4dn (Fig. 1B) nor Rab11dn (Fig. 1C) changed significantly AMPAR or NMDAR-mediated synaptic responses. Furthermore, a dominant negative form of the endocytic Rab5 protein (S34N) did not alter AMPA or NMDA transmission either (unpublished observations). These results serve to verify that the depression of AMPAR responses by Rab8dn was not due to virus infection or non-specific sequestration of regulatory proteins, like GDP/GTP exchange factors (GEFs) or GDP dissociation inhibitors (GDIs).

To determine whether Rab8 is a rate-limiting factor for AMPAR delivery, we overexpressed Rab8wt (Fig. 1D, left inset) and compared AMPA and NMDA R-mediated responses from control and infected nearby neurons. As shown in Fig. 1D, overexpression of Rab8wt did not produce any significant effect on AMPA or NMDA receptor-mediated synaptic transmission. Since Rab8wt overexpression did not increase AMPA currents, this result suggests that Rab8 is not rate-limiting for AMPAR delivery into synapses.

Expression and ultrastructural localization of Rab8 in hippocampal neurons.

The electrophysiological experiments shown above suggest a role for Rab8 in AMPA receptor synaptic trafficking. To further investigate this possibility, we determined the ultrastructural localization of Rab8 in hippocampal neurons from brain tissue. Although previous studies have localized Rab8 at the somatodendritic compartment in dissociated neuronal cultures (27), its localization relative to synaptic contacts has not been addressed before. To this end, we used post-embedding anti-Rab8 immunogold labeling on the synaptic region of the CA1 hippocampus (stratum radiatum; see Experimental Procedures). Rab8 was abundant in postsynaptic terminals (Fig. 2A, arrows; Fig. 2B, “Postsynaptic”), whereas the presynaptic labeling was marginal (Fig. 2B, “Pre-”). Quantification of the immunogold particles in the
postsynaptic terminal indicated that Rab8 accumulates in intracellular compartments in close proximity to the synapse (Fig. 2B, “Intra”), and in some cases directly at the postsynaptic density (Fig. 2B, “PSD”). This result supports a potential role for Rab8 in the local transport of membrane proteins into synapses.

We also examined Rab8wt-GFP distribution in CA1 neurons from hippocampal slices by imaging GFP fluorescence with confocal microscopy. Rab8wt-GFP was broadly expressed in the cell (Fig. 2C, left), including distal dendrites and spines (Fig. 2C, right). Although these results do not show specific targeting of Rab8-GFP to spines, they do suggest that the recombinant protein has access to membrane compartments in close proximity to synapses.

Rab8 is not required for the large-scale transport of AMPA receptors along dendrites.

Early studies using antisense oligonucleotides in dissociated neuronal cultures suggested that Rab8 may be involved in the export of membrane proteins from the neuronal cell body into dendrites (27). Therefore, it is conceivable that Rab8dn may depress AMPAR-mediated transmission by interfering with their transport into dendrites. We tested this possibility by coexpressing GFP-tagged GluR2 and Rab8dn using biolistic gene delivery (the same constructs and transfection method used for the electrophysiological experiments shown in Fig. 7A; see below). The surface distribution of the recombinant receptor was monitored by immunostaining using an anti-GFP antibody in non-permeabilized conditions (the GFP tag is placed at the N-terminus of GluR2, and it is therefore exposed to the extracellular side of the plasma membrane). As shown in Fig. 3B, Rab8dn did not qualitatively alter the export of GluR2-GFP into the dendritic surface, including secondary and tertiary distal dendrites (similar to the immunostaining distribution obtained for GluR2-GFP expressed alone –Fig. 3A) (see Fig. 4,
below, for a quantitative analysis of surface receptor delivery at spines and dendrites). As control, neurons expressing Rab8dn-GFP alone did not show any surface immunostaining (Fig. 3C), consistent with the intracellular distribution of Rab8. These results suggest that Rab8dn depresses AMPA transmission not by globally altering AMPAR transport into dendrites, but by interfering with a local trafficking step, possibly the synaptic delivery of the receptor.

Rab8 is required for local AMPA receptor delivery into the spine surface.

To test whether Rab8 is involved in a local step of AMPAR trafficking, we quantitatively evaluated the surface delivery of AMPARs at dendritic spines and their adjacent dendritic shafts. For this experiment we substituted the GFP tag on Rab8dn with a red fluorescence protein (Rab8dn-RFP, see Experimental Procedures), so its fluorescence can be separated from the coexpressed GluR2-GFP (the functionality of Rab8dn-RFP was confirmed electrophysiologically by coexpressing it with GluR2(R607Q)-GFP and monitoring receptor delivery into synapses using a rectification assay; see below in description for Fig. 7). Surface immunostaining was then carried out with an anti-GFP antibody under non-permeabilized conditions, as described above. In this case the labeling was visualized with an infrared fluorophore (Cy5). Therefore, this experimental design allows us to monitor the total amount of receptor (GFP channel), the fraction exposed to the cell surface (Cy5 channel) and the presence of the coexpressed Rab8dn (RFP channel) (see Fig. 4A as an example).

Line plots of fluorescence intensity were generated across the spine head and the adjacent dendritic shaft. Surface ratios for spine and dendrites were then calculated as the ratio between the corresponding peaks in the Cy5 channel (surface receptor) and the GFP channel (total receptor) after background subtraction (see Fig. 4B). Spines are solely selected from their GFP
Rab8 controls AMPA receptor synaptic trafficking

image, precluding any bias with respect to their surface immunostaining. The results of this analysis are shown in Fig. 4C. When expressing GluR2-GFP alone, surface ratios were similar in spine and dendrites. In contrast, surface ratio was significantly lower in the spines as compared to their adjacent dendrites when Rab8dn was coexpressed. Importantly, absolute surface ratio at dendrites was not altered by Rab8dn (Cy5/GFP ratios were 0.31±0.05 for GluR2 alone versus 0.31±0.04 for GluR2 plus Rab8dn). These data indicate that Rab8dn specifically impairs the delivery of AMPARs to the surface of the spine. Interestingly, Rab8dn did not decrease the total amount of receptor (GFP channel) in the spine compared to the dendrite (Fig 4D) nor total receptor abundance at dendrites (absolute GFP values were 50±4 for GluR2 alone versus 54±3 for GluR2 plus Rab8dn). These results support the interpretation that Rab8 is involved in a local trafficking step from an intracellular membrane compartment inside the spine to the postsynaptic plasma membrane.

Rab8 is necessary for both the constitutive and regulated delivery of AMPA receptors into synapses.

As described above, AMPARs may reach synapses through a constitutive pathway, in an activity-independent manner, and through a regulated pathway, triggered by NMDA receptor opening and CaMKII activation (17). In order to test the role of Rab8 in the constitutive pathway, we expressed Rab8 dominant negative in slices kept in conditions of reduced neuronal activity (12 mM Mg$^{2+}$ was added to the culture medium immediately after infecting with the virus expressing Rab8dn; recordings were carried out in the presence of the standard 4 mM Mg$^{2+}$). As shown in Fig. 5A, Rab8dn depresses AMPAR-mediated responses in conditions of
Reduced neuronal activity, suggesting that Rab8 is necessary for the constitutive (activity-independent) delivery of AMPARs into synapses.

The role of Rab8 in the activity-dependent delivery of AMPARs was tested with a truncated form of αCaMKII (t-CaMKII) that is constitutively active (8,59). Expression of t-CaMKII potentiated AMPAR-mediated responses (Fig. 5B), as previously described (8,59). However, this potentiation was fully blocked when Rab8dn was co-expressed with t-CaMKII (Fig. 5C). This result is consistent with Rab8 being also necessary for the regulated delivery of AMPARs triggered by CaMKII. To test whether the absence of potentiation is due to the interference of Rab8dn with CaMKII activity, we quantified the autophosphorylation levels of αCaMKII at Thr\(^{286}\) in control and Rab8dn-expressing neurons. As shown in Fig 5D, phospho-CaMKII levels from CA1 dissected regions were similar between infected and uninfected slices.

**Rab8 is necessary for synaptic plasticity.**

Long term potentiation (LTP) in the CA1 hippocampus is one of the most thoroughly studied forms of synaptic plasticity, and it is accompanied by the synaptic delivery of GluR1-containing AMPARs (8). To test whether Rab8 is necessary for the synaptic delivery of AMPARs during synaptic plasticity, we examined pairing-induced LTP in CA1 neurons infected with Rab8dn or Rab8wt. Consistent with its effect on CaMKII-induced AMPAR delivery, Rab8dn blocked LTP expression (Fig. 6). Unexpectedly, Rab8dn led to synaptic depression. This late depression upon LTP induction has been previously reported in situations when AMPAR delivery was impaired (8). Importantly, LTP was normal in cells expressing Rab8wt, as compared with uninfected cells (Fig. 6). These results indicate that Rab8 function is necessary for LTP.
Rab8 is necessary for the constitutive delivery of GluR2 and for the CaMKII-triggered delivery of GluR1 into synapses.

In order to test directly the role of Rab8 in the pathway-specific delivery of different AMPAR populations into synapses, we co-expressed Rab8dn with individual recombinant AMPAR subunits and monitored their delivery taking advantage of their rectification properties (electrophysiological tagging (8-11)). The constitutive delivery of GluR2 can be assessed using the inwardly rectifying GluR2 mutant, GluR2 (R607Q), which does not conduct outward current at positive membrane potentials. Expression of this recombinant receptor produced inward rectification (Fig 7A; compare uninfected to GluR2(Q)), as previously described (10), indicating the delivery of the homomeric receptor. Co-expression of Rab8dn with GluR2 (R607Q) blocked this rectification (Fig. 7A), indicating that Rab8 is necessary for the constitutive delivery of GluR2 (similar rectification values were obtained with Rab8dn-GFP – 2.1±0.3, n=12 – and with Rab8dn-RFP – 2.3±0.2, n=10). As a control, co-expression of Rab8wt with GluR2 (R607Q) did produce rectification. This control indicates that the effect of Rab8dn was not due to insufficient co-expression of two recombinant proteins (see also Rab8wt control in Fig. 7B). In addition, Rab8dn did not produce any change in rectification if no recombinant AMPAR subunit was co-expressed (Fig. 7A).

The activity-dependent delivery of GluR1-containing AMPARs can be evaluated by using recombinant GluR1 and t-CaMKII to trigger its delivery. Co-expression of GluR1 and t-CaMKII produced inward rectification (Fig. 7B), as previously described (8), revealing the delivery of homomeric GluR1. Co-expression of Rab8dn with GluR1 and t-CaMKII blocked rectification (Fig. 7B), indicating that Rab8 is necessary for the regulated delivery of GluR1-containing AMPARs.
Rab8 controls AMPA receptor synaptic trafficking

Rab8 is necessary for the delivery of AMPARs induced by PSD95.

It has been recently described that overexpression of PSD95 leads to potentiation of AMPAR-mediated responses (60,61) in a process that is accompanied by the synaptic delivery of GluR1-containing receptors (62). Although the mechanisms are not completely understood, it has been proposed that this synaptic delivery is mediated by stargazin, which binds both PSD95 and AMPARs (63,64). We wished to determine whether PSD95-induced delivery of AMPARs also requires Rab8. To this end we co-expressed recombinant GluR1, PSD95 and Rab8dn. As previously reported (62), co-expression of PSD95 and GluR1 leads to the delivery of recombinant, homomeric GluR1 receptors, producing inward rectification (Fig. 7B). However, when Rab8dn was co-expressed with GluR1 and PSD95, the increase in rectification was blocked, indicating that Rab8 is also necessary for this AMPAR delivery pathway. As control, co-expression of GluR1 with PSD95 and Rab8wt did show rectification (Fig. 7B), indicating that the absence of rectification observed with the Rab8dn is not due to inefficient co-expression of three proteins.
DISCUSSION

The Rab family of small GTPases is known to confer directionality to intracellular membrane trafficking in several cell types (reviewed in (20)). However, it is surprising that very few studies have addressed the role of Rab proteins in neuronal function, where polarized membrane traffic is essential to maintain presynaptic and postsynaptic function. The notable exception is Rab3, which is known to regulate neurotransmitter vesicle fusion and short-term plasticity at the presynaptic terminal (26), although the precise mechanisms underlying this regulation are still being elucidated (65,66).

We have now tested the role of the other three exocytic Rab proteins, namely Rab4, Rab8 and Rab11, at the postsynaptic terminal. These studies have identified Rab8 as a critical player in postsynaptic membrane trafficking and plasticity. Rab8 was proposed to mediate dendritic membrane trafficking and neurite outgrowth nearly a decade ago (27,67). These early studies, using neuronal dissociated cultures, suggested the involvement of Rab8 in early stages of the secretory pathway. Since then, no reports have tested whether Rab8 plays a more direct role in synaptic function. Here we have shown that Rab8 is a necessary mediator of the local delivery of AMPARs into excitatory synapses. Using quantitative surface immunostaining, we show that Rab8 is required for the delivery of AMPARs into the spine surface, but not for their transport from the dendritic shaft into the spine compartment or for their delivery into the dendritic plasma membrane. In addition, by monitoring electrophysiologically the synaptic targeting of endogenous and recombinant AMPARs, we demonstrate that Rab8 is necessary for both the constitutive cycling of AMPARs and their regulated synaptic delivery, as triggered by CaMKII activation, PSD95 overexpression or LTP induction. Therefore, we propose that Rab8 drives the
local transport of AMPARs from an intracellular membrane compartment, possibly in the dendritic spine, to the synaptic membrane.

Rab8 is a known mediator of exocytic transport from the trans-Golgi network into the plasma membrane. As such, it is possibly involved in the surface delivery of a variety of membrane proteins in neurons. However, an unexpected result of the present study is that Rab8 drives the local targeting of AMPARs into the highly specialized compartment that constitutes the postsynaptic membrane. Several lines of evidence support this role of Rab8 in the short-range synaptic delivery of AMPARs. Firstly, Rab8, besides being present in the dendritic shaft, is localized in very close proximity to the synapse, including the postsynaptic density. Secondly, Rab8 is necessary for the continuous synaptic cycling of GluR2/3-containing AMPARs. Although the molecular details of this activity-independent pathway are not well understood, its fast kinetics (13,15) suggests that it occurs in close proximity to the synapse. And thirdly, quantitative immunohistochemical analysis showed that blockade of endogenous Rab8 function by expression of a Rab8 dominant negative does not affect the transport of AMPARs in dendrites, but it does impair receptor delivery into the spine surface. This result is in contrast with a previous publication showing that Rab8 was necessary for the dendritic transport of a recombinant viral glycoprotein (27). Although we do not know the reason for this discrepancy, it is possible that Rab8 plays a more general role in membrane trafficking in developing neurons, such as those in dissociated primary cultures, as compared with the more differentiated neurons present in our organotypic slice cultures. This interpretation is consistent with the proposed role of Rab8 in neurite outgrowth before axons and dendrites are differentiated (67).

As mentioned above, Rab8 mediates exocytic transport from the trans-Golgi network. Interestingly, it has been recently described that distal dendrites contain intracellular
Rab8 controls AMPA receptor synaptic trafficking

compartments belonging to the trans-Golgi network (68,69). Our functional results fit very well with these morphological observations, and suggest that AMPARs are driven locally from distal trans-Golgi network outposts into synapses via a Rab8-mediated process. Exocytic delivery of membrane proteins from trans-Golgi network can proceed directly into the plasma membrane or through intermediate recycling endosomes (29,70). Rab8 is present in both trans-Golgi network membranes and recycling endosomes (29), and to date, a possible role of Rab8 in controlling membrane sorting from both compartments has not been ruled out. Interestingly, we have found that Rab8 mediates both the continuous cycling of GluR2/3 receptors, and the transient, activity-dependent delivery of GluR1/2 receptors. At this point, we cannot resolve whether these two populations of receptors are delivered from different intracellular compartments, or whether a single but sub-compartmentalized storage place is the source for both types of exocytic events. However, biochemical membrane fractionations (71) and imaging experiments (10,72) suggest that GluR1/2 and GluR2/3 receptors have different subcellular distributions. Therefore, we propose that the synaptic delivery of GluR1/2 receptors during synaptic plasticity is regulated from the trans-Golgi network, whereas the activity-independent cycling of GluR2/3 receptors occurs from a specialized recycling compartment in close proximity to the synapse (see Fig. 8).

In this sense, it is important to note that neither Rab4 nor Rab11 seem to be involved in the continuous cycling of AMPARs (the corresponding dominant negative mutants did not affect basal AMPAR-mediated synaptic transmission). This observation is somehow surprising, since these Rab proteins are known to mediate the trafficking of various populations of recycling endosomes (32-36,52). Our results, then, support the interpretation that GluR2/3 synaptic delivery occurs from a specialized membrane compartment, whose cycling is controlled by Rab8 but not by Rab4 or Rab11. This interpretation does not rule out a role for Rab4 and/or Rab11 in
other steps of AMPAR trafficking. For instance, it has been reported that AMPARs that return to the plasma membrane after activity-dependent internalization colocalize with Rab4-positive compartments (73,74). However, our results indicate that Rab8 is the Rab family member that directly mediates the late stages of membrane transport that result in the synaptic delivery of AMPARs both during constitutive receptor cycling and during activity-dependent delivery (LTP).

In conclusion, these results expand our view of the cellular functions accomplished by the Rab protein-driven exocytic machinery in neurons, and shed light into the membrane trafficking events that control the dynamic organization of the postsynaptic terminal.
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ABBREVIATIONS

AMPA R, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors; NMDA R, N-methyl-D-aspartate receptor; GluR1-4, glutamate receptor subunit 1-4; CaMKII, Ca$^{2+}$/calmodulin dependent protein kinase II; LTP, long term potentiation.
REFERENCES


Rab8 controls AMPA receptor synaptic trafficking


Rab8 controls AMPA receptor synaptic trafficking

FIGURE LEGENDS:

Figure 1. Rab8 is necessary for AMPA receptor delivery into synapses. (A-D) Insets, Western blot analysis of the expression of recombinant Rab-GFP in hippocampal slices using the Sindbis virus method. Big arrowheads indicate recombinant Rab-GFP proteins (about 52 KDa); small arrows indicate endogenous Rab proteins (about 25 KDa). Sample trace of evoked AMPA- and NMDA-receptor mediated synaptic responses recorded at –60 mV and +40 mV, respectively, from uninfected and infected cells. Scale bars, 20 pA and 40 ms. Left, average AMPAR-mediated current amplitude (i.e. the peak of the response recorded at –60 mV) from infected (inf) neurons expressing Rab8dn (A), Rab4dn (B), Rab11dn (C) or Rab8wt (D) and control neighboring cells not expressing the recombinant protein (uninf). n represents the number of pathways from cell pairs; p is the probability value according to the Wilcoxon test. Middle, average NMDA R-mediated current amplitude (recorded at +40 mV at a latency when AMPA responses are fully decayed, 60 ms) from uninfected and infected cells (n also represents the number of pathways from cell pairs). Right, average AMPA/NMDA ratios for uninfected and infected cells (n represents the number of pathways).

Figure 2. Localization of Rab8 at postsynaptic terminals. (A) Ultrastructural localization of Rab8 at CA1 hippocampal synapses by electron microscopy. Rab8 immunogold particles (arrows) were found at the postsynaptic terminal, including the postsynaptic density. Labeling was also detected in dendritic shafts (not shown). Presynaptic terminals are labeled with asterisks. Scale bar, 100 nm. (B) Quantification of immunogold labeling. Most of the gold particles were found postsynaptically, with marginal presence presynaptically (Pre), indicating
specificity for the immunogold labeling. The majority of the gold particles were found intracellularly (Intra). Gold particles were also found at the postsynaptic density (PSD) as well as extrasynaptic membranes (Extra). For this quantification, only gold particles within 600 nm of the synapse were included. (C) Rab8-GFP is present at dendritic spines. Representative confocal images showing the distribution of the recombinant Rab8-GFP in hippocampal neurons from slices. Rab8 is present in cell body and dendrites (left), as well as in dendritic spines (right).

Figure 3. Immunohistochemical localization of surface AMPA receptors in slices expressing Rab8dn. (A) Confocal imaging of GluR2-GFP transfected neuron. GFP fluorescence signal (top) and surface anti-GFP immunoreactivity of the same neuron in non-permeabilized conditions (bottom). (B) Confocal imaging for a neuron cotransfected with GluR2-GFP and Rab8dn-GFP. GFP signal and surface anti-GFP immunoreactivity, as in A. (C) Control neurons expressing Rab8dn alone. Negative surface immunostaining (bottom) is expected given the intracellular distribution of Rab8-GFP.

Fig. 4. Rab8dn decreases AMPAR surface delivery locally at spines. (A) Representative confocal image of a neuron cotransfected with GluR2-GFP and Rab8dn-RFP. Left: GFP fluorescence signal showing total GluR2 receptor distribution. Middle: surface GluR2-GFP receptors assayed by anti-GFP immunoreactivity of the same neuron in non-permeabilized conditions. Right: RFP fluorescence signal from the same neuron indicating expression of the cotransfected Rab8dn-RFP (neurons transfected only with GluR2-GFP do not show any RFP signal above background; not shown). Scale bar: 30 μm. (B) Representative line plot analysis of total (left; GFP signal) and surface (right; Cy5 signal) receptor across a spine and the adjacent
Rab8 controls AMPA receptor synaptic trafficking

dendritic shaft of GluR2 transfected or GluR2 plus Rab8dn cotransfected neurons, as indicated. Values for surface and total receptors were taken from the fluorescence intensity peaks after background subtraction (dashed line). Scale bar: 2 μm. (C) Surface ratio from pairs of spines and dendrites in GluR2 transfected neurons (left) or in GluR2 and Rab8dn cotransfected neurons (middle). Surface ratios for spines and dendrites were calculated by dividing the corresponding background-subtracted Cy5 and GFP values. Surface ratios are normalized by the mean dendrite value (0.31±0.05 for GluR2 alone and 0.31±0.04 for GluR2 plus Rab8dn). Cumulative probabilities (right) of spine/dendrite ratios show a significant difference between the surface ratio distribution of GluR2 and GluR2 plus Rab8dn transfected neurons. For comparison, dashed lines indicate that 50% of the spine-dendrite pairs have less surface ratio in the spine than in the dendrite for cells transfected with GluR2 alone (black line: cumulative probability=0.5 for spine/dendrite=1). In contrast, 75% of the spine-dendrite pairs have less surface ratio in the spine than in the dendrite for cells cotransfected with Rab8dn (red line: cumulative probability=0.75 for spine/dendrite=1). (D) Total receptor amount (GFP signal) in spines and dendrites are calculated in GluR2 transfected neurons (left) and in GluR2 plus Rab8dn cotransfected neurons (middle). Values are normalized by the mean obtained at dendrites (50±4 for GluR2 alone and 54±3 for GluR2 plus Rab8dn). The cumulative probability (right) indicates that Rab8dn does not significantly change the total receptor distribution in spines versus dendrites.

Figure 5. Rab8 is necessary for both the constitutive and regulated delivery of AMPA receptors into synapses. (A-C) Inset, sample trace of evoked AMPA receptor mediated synaptic responses recorded at –60 mV from uninfected/non-transfected and infected/transfected cells. Scale bars, 20 pA and 10 ms. (A) Rab8dn significantly reduced AMPA transmission in the
presence of high Mg\(^{2+}\) (12 mM MgCl\(_2\)). Average current amplitude at \(-60\) mV from uninfected and infected cells was 46.9±9.9 pA and 26.7±4.5 pA, respectively. \(n\) represents the number of pathways from cell pairs. \(p = 0.03\), according to the Wilcoxon test. (B) Expression of t-CaMKII significantly increases AMPAR-mediated transmission. Non-transfected: 32.5±6.1 pA; transfected with t-CaMKII: 81.5±14.4 pA. \(p = 0.001\), according to the Wilcoxon test; \(n\) represents the number of the pathways from cell pairs. (C) Rab8dn blocked t-CaMKII-induced potentiation of AMPA responses. Non-transfected: 25.0±4.2 pA; transfected with t-CaMKII and Rab8dn: 19.7±3.8 pA. (D) Rab8dn does not change the phosphorylation levels of CaMKII. Left, Western blot analysis showing the P-Thr\(^{286}\) αCaMKII / total αCaMKII ratios in Rab8dn-infected slices and uninfected slices. Each lane in the western blot is the result of pooling together extracts from three dissected CA1 regions. Right, Quantification by densitometric scanning of four independent experiments as the one shown in the left.

**Figure 6. Rab8 is necessary for LTP.** Organotypic slice cultures were infected with virus expressing either Rab8wt or Rab8dn. Whole-cell recordings were established from neurons expressing the recombinant proteins or uninfected cells, and LTP was induced by pairing 3 Hz presynaptic stimulation with 0 mV postsynaptic depolarization, as previously described (8). (A) Pairing significantly increased AMPA receptor-mediated responses in uninfected \((p = 0.002,\) paired t-test) and Rab8wt-infected \((p = 0.001,\) paired t-test) neurons. However, pairing significantly decreased synaptic responses in Rab8dn expressing neurons \((p = 0.001,\) paired t-test). Inset, sample trace of evoked AMPA receptor-mediated synaptic responses recorded at \(-60\) mV before pairing (thin line) and 30 min after pairing (thick line) from control (uninfected) neurons or infected cells, as indicated. Scale bars, 20 pA and 10 ms. (B) Normalized steady-state
AMPAR response amplitudes in paired (LTP induction) and control (Unpaired) pathways in cells expressing Rab8wt-GFP, Rab8dn-GFP and uninfected control cells. AMPAR responses from control pathways were not statistically different from baseline responses.

**Figure 7. Rab8 is necessary for the constitutive delivery of GluR2 and for the delivery of GluR1 triggered by t-CaMKII or by PSD95 into synapses.** Rectification values were calculated as the ratio between the amplitude of the synaptic response at -60 mV over the amplitude at +40 mV. Endogenous receptors conduct current at -60 mV and +40 mV, whereas recombinant receptors conduct only at negative membrane potentials. Therefore, delivery of the recombinant homomeric receptors is accompanied by an increase in the rectification value (a decrease in the outward current, depicted by the arrow head in A, with respect to the inward current). (A) Average rectification values were: control (uninfected), 1.9±0.2; GluR2(R586Q), 3.3±0.2; GluR2(R586Q) plus Rab8dn, 2.1±0.3; GluR2(R586Q) plus Rab8wt, 3.3±0.2; Rab8dn, 2.3±0.3. (B) Average rectification values were: GluR1 plus t-CaMKII, 3.9±0.7; GluR1 plus t-CaMKII and Rab8dn, 1.8±0.2; GluR1 plus PSD95, 3.5±0.5; GluR1 plus PSD95 and Rab8dn, 2.0±0.2; GluR1 plus PSD95 and Rab8wt, 3.5±0.4. n represents the number of pathways, p is the probability value according to the Student’s t-test. Inset, sample traces of evoked AMPA receptor mediated synaptic responses recorded at –60 mV and +40 mV from control or transfected cells as indicated. Scale bars, 20 pA and 20 ms.

**Figure 8. Membrane sorting model for the control of AMPAR synaptic delivery by Rab8.** The regulated (activity-dependent) delivery of GluR1/2 receptors would be mediated by Rab8 from a membrane compartment belonging to the trans-Golgi network (TGN). In contrast, the
continuous (activity-independent) delivery of GluR2/3 receptors would occur from specialized recycling endosomes (RE) whose sorting is also controlled by Rab8.
Fig. 1
Fig. 2
Fig. 3
A. GluR2-GFP + Rab8dn-RFP

B. Spine and dendrite fluorescence analysis

C. Surface ratio (Cy5/GFP)

D. Total receptor (GFP)

Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Local control of AMPA receptor trafficking at the postsynaptic terminal by a small GTPase of the Rab family
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