Overexpression or clustering of the transmembrane form of the extracellular matrix heparan sulfate proteoglycan agrin (TM-agrin) induces the formation of highly dynamic filopodia-like processes on axons and dendrites from central and peripheral nervous system-derived neurons. Here we show that the formation of these processes is paralleled by a partitioning of TM-agrin into lipid rafts, that lipid rafts and transmembrane-agrin colocalize on the processes, that extraction of lipid rafts with methyl-β-cyclodextrin leads to a dose-dependent reduction of process formation, that inhibition of lipid raft synthesis prevents process formation, and that the continuous presence of lipid rafts is required for the maintenance of the processes. Association of TM-agrin with lipid rafts results in the phosphorylation and activation of the Src family kinase Fyn and subsequently in the phosphorylation and activation of MAPK. Inhibition of Fyn or MAPK activation inhibits process formation. These results demonstrate that the formation of filopodia-like processes by TM-agrin is the result of the activation of a complex intracellular signaling cascade, supporting the hypothesis that TM-agrin is a receptor or coreceptor on neurons.

Agrin is a proteoglycan with a molecular mass of more than 500 kDa that is expressed in many tissues (for a review, see Ref. 1). Despite its widespread expression, the function of agrin is best characterized in skeletal muscle, where it is a key organizer during formation, maintenance, and regeneration of the neuromuscular junction (2, 3). Accordingly, mice with an inactivation of the agrn gene die at birth due to nonfunctional neuromuscular junctions and subsequent respiratory failure (4).

Little is known about the role of agrin in other tissues, in particular in the central nervous system (for a review, see Ref. 5). Although neurons from mice with a targeted deletion of the agrn gene form synaptic specializations in vitro and in vivo (6, 7), the acute suppression of agrin expression or function by antisense probes or antibodies influences the function of interneuronal synapses (8, 9). Likewise, brains of agrin-deficient mice, whose perinatal death was prevented by the reexpression of agrin specifically in motor neurons, have a severely reduced number of pre- and postsynaptic specializations at excitatory synapses (10). In addition, agrin isoforms are highly expressed by central nervous system neurons before synapse formation, suggesting additional functions for agrin during axonal and dendritic elongation (11–16). Although these data are consistent with a role of agrin during CNS synaptogenesis, the precise role of agrin during CNS development remains to be clarified.

Alternative first exon usage generates either a secreted soluble agrin molecule (NtA-agrin) or a transmembrane form of agrin (TM-agrin) (17, 18). The secreted form of agrin specifically interacts with the laminin γ1-chain via its NtA-domain, resulting in a stable association with basal laminae (19, 20). In contrast, in TM-agrin, the NtA-domain is replaced by a non-cleaved signal anchor that converts agrin into a type II transmembrane protein and localizes the agrin protein in an NtA/Cex orientation in the plasma membrane (17, 18).

TM-agrin is primarily expressed in the CNS on axons and dendrites during the phase of active neurite extension (15, 21), and it has been hypothesized that neurite-associated TM-agrin might serve as a receptor or co-receptor (21). In agreement with this hypothesis, it was recently shown that either clustering or overexpression of TM-agrin in neurons during the phase of active neurite extension reorganizes the actin cytoskeleton and induces the rapid formation of numerous filopodia-like processes extending from the primary neurite (21, 22). In this study, we demonstrate that the TM-agrin-mediated formation of processes is the result of the initiation of an intracellular signaling cascade, which involves lipid rafts, the activation of the Src family kinase Fyn, and the activation of the mitogen-activated protein kinase (MAPK). These results provide essential evidence for a function of TM-agrin as a receptor or co-receptor on neurites.

**EXPERIMENTAL PROCEDURES**

**Animals**—Fertile White Leghorn (Gallus gallus domesticus) chicken eggs were purchased from a local hatchery and incubated at 38 °C in a humid atmosphere. The age of the embryos was expressed in embryonic days. All experiments were con-

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dected in accordance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany and the University of Mainz.

Materials—Methyl-β-cyclodextrin (mβC) and PDMP (dl-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propa- nol) were obtained from Sigma. The inhibitors PP1 and PD98059 (2‘-amino-3‘-methoxyflavone) were obtained from Biomol (Hamburg, Germany). PP1 and PD98059 were dis- solved in dimethyl sulfoxide to a concentration of 25 mg/ml, corresponding to 88 and 100 mM, respectively.

The following antibodies against agrin were used: rabbit anti-agrin antiserum 46 (11, 13, 23). This antiserum was generated against the C-terminal half of agrin and, thus, specifically reacts with the extracellular part of all TM-agrin and NtA-agrin iso- forms in Western blotting and immunohistochemistry. In con- trast, sheep anti-TM-agrin (generated against a peptide of the intracellular domain of TM-agrin) reacts only with TM-agrin and not with NtA-agrin (21). Throughout this study, antiserum 46 was used to induce the filopodia-like processes. Actin fila- ments were stained with Alexa488-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) (for details, see Ref. 21). Fluorescin-conjugated chola toxin β-subunit was purchased from Sigma. Polyclonal rabbit antibodies against Fyn (sc-16; FYN3; 0.2 μg/ml), which detect phosphorylated as well as non- phosphorylated Fyn (24, 25), were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). In contrast, the rabbit polyclonal antibody Src[pY418] (Invitrogen) detects specifi- cally the tyrosine phosphate at position 418 (murine) and 417 (chick) of different Src family kinases and was used according to the manufacturer’s instructions (1:1000 dilution). A rabbit antiserum against glyceraldehyde-3-phos- phate dehydrogenase was from Abcam (ab9485; Cambridge, UK) (26) (1 μg/ml). Mouse monoclonal antibodies against the transferrin receptor were from Invitrogen and used at a concentration of 0.5 μg/ml. Polyclonal antibodies against total MAPK as well as monoclonal antibodies against phos- pho-MAPK were from Cell Signaling Technologies (New England Biolabs, Frankfurt, Germany).

Cell Culture of Retinal Ganglion Cells—TM-agrin has been shown to induce filopodia-like processes on several neurons from the central and peripheral nervous system (21). Through- out the present study, retinal ganglion cells were used, because they have been shown to exclusively synthesize TM-agrin (21). Retinal ganglion cells were cultured as described previously (27) on coverslips pretreated overnight with poly-L-lysine and subsequently coated with EHS tumor-derived laminin (Sigma) at a concentration of 2 μg/cm² in phosphate-buffered saline. Cells were cultured for 1 or 2 days in vitro. Filopodia-like pro- cesses were induced by incubating with saturating amounts of the anti-agrin antiserum 46, and the number of processes was determined after the indicated time, as described previously (21). The values were expressed as the number of processes per 50 μm of neurite length.

Immunocytochemistry—Retinal neuron cultures were fixed for 10 min in 4% paraformaldehyde in 0.1% phosphate buffer, pH 7.4. Excess fixative was removed by washing the cultures twice with phosphate-buffered saline, followed by incubation with phosphate buffer containing 0.2% bovine serum albumin and 0.2% Triton X-100. Cultures were double-labeled by first incubating with a primary antibody overnight at 4 °C, followed by extensive washing in phosphate buffer. The cultures were subsequently incubated with the appropriate Alexa594- or Alexa488-conjugated secondary antibodies (donkey anti- sheep, goat anti-mouse, or goat anti-rabbit; Molecular Probes; 24 μg/ml final concentration) and with Alexa-488-conjugated phalloidin to reveal the actin cytoskeleton or with fluorescein isothiocyanate-conjugated cholera toxin, as described (21). All secondary antibodies were preabsorbed against IgG of other species, eliminating cross-reactivity. After 1.5 h, the cultures were again washed in PBST and embedded in Citifluor fluores- cent mounting medium (Plano, Wetzlar, Germany). Specimens were analyzed with a photomicroscope (Leica, DMRA; Leica, Solms, Germany) equipped with epifluorescence optics using fluorescence filters of the appropriate wavelength for the com- plete separation of the different chromophores. Pictures were acquired with a digital camera (DC200; Leica) using the Leica data acquisition software. Contrast and brightness of entire images were adjusted using Photoshop (version 7.0; Adobe, Mountain View, CA).

Isolation of Lipid Rafts—Lipid rafts were prepared as described by Simons and Ikonen (28) with minor modifications. Retinal ganglion cells either untreated or treated with anti-agrin antibodies were washed once in phosphate-buffered saline at 4 °C and then lysed for 1 h on ice in lysis buffer (50 mM Tris-base, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100 containing protease inhibitors (Complete EDTA-free; Roche Applied Science) as well as phosphatase inhibitors (Phospa- tase Inhibitor Mixture 1 and 2; Sigma) according to the manufac- turer’s instructions. Cells were homogenized through a 27-gauge needle and subsequently through a 22-gauge needle three times each. The lysate was then adjusted to a concentration of 40% using the OptiPrep density gradient medium according to the manufacturer’s instructions (Sigma), loaded at the bottom of a STLS55 centrifuge tube (Beckmann Coulter, Krefeld, Germany), and overlaid with 1.2 ml of 30% OptiPrep in lysis buffer and 0.2 ml of 5% OptiPrep. The samples were centrifuged at 55,000 rpm for 3 h at 4 °C. After centrifugation, eight fractions (0.25 ml each) were collected from top to bottom and designated as fractions 1–8. Fractions 1 and 2 (corresponding to ~15% OptiPrep) contained Fyn and are referred to as lipid raft fractions. To concentrate the proteins, the fractions were diluted with water containing protease inhibitors and centrifuged for 1 h at 4 °C and 55,000 rpm in a TLA 55 rotor. The protein pellet was then dissolved in 60 μl of SDS buffer (100 mM Tris-Cl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mer- captoethanol, 0.01% bromphenol blue) and subjected to SDS- PAGE and Western blotting.

SDS-PAGE and Immunoblotting—Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoroide membranes (Millipore, Schwalbach, Germany) by electroblotting. The membranes were blocked with 4% nonfat milk in Tris- buffered saline containing 0.1% Tween 20. The dilutions of the antibody were as described above. Horseradish peroxidase- conjugated secondary antibodies were from Jackson Immuno- research (West Grove, PA) and were used at dilutions of 1:5,000. Peroxidase-associated signals were detected using the enhanced chemiluminescence method (SuperSignal West Pico;
Pierce, Bonn, Germany), followed by exposure to BioMax HyperFilm ECL (Amersham Biosciences).

Protein bands in Western blots were quantified, and densitometric analysis was performed using the AIDA Image Analyzer software (version 3.28; Raytest; Straubenhardt, Germany) according to the manufacturer’s instructions. Values of phosphoprotein bands were normalized to the values of the corresponding total proteins to account for loading variations and random variability in the different lanes. Mean normalized values from the indicated number of experiments were plotted and compared statistically as previously described (29). Data are expressed as the mean ± S.E. for n ≥ 3.

RESULTS

TM-agrin and Cholesterol-rich Lipid Rafts Colocalize on Neurites—Clustering of TM-agrin by polyclonal antibodies or overexpression of TM-agrin cDNA is sufficient to induce the formation of highly dynamic filopodia-like processes on axons and dendrites from central or peripheral nervous system neurons (21, 22). Since similar processes can also be induced in nonneuronal cells from several species (22), the intracellular signaling mechanism appears not to be restricted to neurons. Lipid rafts are glycosphingolipid- and cholesterol-enriched microdomains within cell membranes that serve as signaling platforms in many different cell types of many different species (30). To determine if lipid rafts are involved in TM-agrin-induced process formation, processes were induced on retinal ganglion cell axons and stained with fluorescein isothiocyanate-conjugated ganglion cell axons and stained with fluorescein isothiocyanate (FITC)-conjugated second antibody and visualized by confocal microscopy (18, 19). Treatment of RGCs with anti-agrin antibodies (antiserum 46) resulted in a concentration of TM-agrin (specifically detected by the sheep anti-TM-agrin antiserum) in the raft fractions (lanes 1 and 2, characterized by the presence of Fyn) as well as in the nonraft fractions (lanes 6–8; Fig. 2, left). In contrast, separation of membranes prepared 5 min after TM-agrin clustering

FIGURE 1. Lipid rafts codistribute with TM-agrin on retinal ganglion cell axons and on TM-agrin induced processes. Lipid rafts labeled with fluorescein isothiocyanate-conjugated β-subunit of cholera toxin (CTx) were present on untreated retinal ganglion cell axons (A) and on filopodia-like processes induced on retinal ganglion cells by anti-agrin antiserum 46 (arrows in B), indicating that lipid rafts are found on TM-agrin-induced processes. C–H, high magnification views of untreated axons (C–E) and of axons on which processes had been induced by anti-agrin antiserum 46. Specimens were double-labeled with antibodies specifically detecting TM-agrin (D and G) as well as with fluorescein isothiocyanate-conjugated CTx (C and F). Overlay of both immunoreactivities demonstrates the overlapping staining pattern on untreated axons as well as on filopodia-like processes (arrows in F–H), indicating that TM-agrin is associated with lipid rafts in the presence and absence of antibodies. Scale bar, 10 µm.

FIGURE 2. Partitioning of TM-agrin into lipid rafts after process induction. Triton X-100-insoluble fractions from antibody-treated and -untreated cultures of retinal ganglion cells were subject to discontinuous OptiPrep density gradient centrifugation. Eight fractions were collected from top to bottom (lanes 1–8) and subjected to Western blot analysis using the indicated antibodies. Left, distribution of TM-agrin (detected with the TM-agrin-specific sheep anti-peptide antiserum) within the fractions from nontreated retinal ganglion cells. Untreated RGCs contained TM-agrin in the raft fractions (lanes 1 and 2, characterized by the presence of Fyn) as well as in the nonraft fractions (lanes 6–8, characterized by the presence of the transferrin receptor (TIR) and of glycerinaldehyde-3-phosphate dehydrogenase (GAPDH)). Right, 5-min incubation of RGCs with anti-agrin antibodies (antiserum 46) resulted in a concentration of TM-agrin (specifically detected by the sheep anti-TM-agrin antiserum) in the raft fractions and a depletion of TM-agrin from the nonraft fractions.

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and subsequent process formation (21) showed that agrin was highly concentrated in the low density lipid raft fractions, and little immunoreactivity remained in the high density nonraft fractions (Fig. 2, right). A similar result was obtained when lipid rafts were analyzed 3 h after antibody treatment (not shown), a time point where the number of processes has reached a maximum (21). In contrast, the transferrin receptor as well as glyceraldehyde-3-phosphate dehydrogenase remained associated exclusively with the nonraft fractions (Fig. 2, right). Although the large size of agrin and its migration as a broad smear in Western blots precluded a precise quantification, these results are consistent with the idea that TM-agrin is associated with lipid rafts and with nonraft fractions in nontreated neurons and that formation of an actin-rich filopodia-like process by clustering of TM-agrin is paralleled by the partitioning of the majority of TM-agrin into lipid rafts.

To determine if lipid rafts are required for TM-agrin-mediated process induction, retinal ganglion cells were preincubated for 1 h with mβC, followed by clustering of TM-agrin to induce process formation. Methyl-β-cyclodextrin is a watersoluble cyclic oligomer that sequesters cholesterol from plasma membranes within its hydrophobic core and, thus, acutely disperses lipid rafts (30, 37, 38). Methyl-β-cyclodextrin treatment for 4 h had no effect on the growth of the neurites or on growth cone morphology (data not shown), indicating that mβC does not affect retinal ganglion cell viability under these conditions. As shown in Fig. 3A, incubation of retinal ganglion cells with mβC resulted in a dose-dependent reduction of the number of TM-agrin-induced processes, and at a concentration of 2 mM, the number of processes was reduced to control values. These results demonstrate that extraction of cholesterol from lipid rafts using mβC inhibits TM-agrin-dependent process formation.

To obtain additional evidence for a role of lipid rafts in TM-agrin-dependent process formation, the synthesis of lipid rafts was inhibited using threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). PDMP inhibits the biosynthesis of glycosphingolipids, key components of lipid rafts, and thus reduces the number of lipid rafts in the plasma membrane by a different mechanism compared with mβC (39, 40). Preincubation of retinal ganglion cells with PDMP for 24 h did not influence their viability or growth cone morphology (data not shown). However, as shown in Fig. 3B, treatment of retinal ganglion cells with PDMP reduced the number of TM-agrin-induced processes. This reduction was dose-dependent, and values similar to nontreated control cultures were reached at a concentration of 40 μM PDMP. In summary, our results show that TM-agrin is associated with lipid rafts and that lipid rafts are required for the TM-agrin-dependent formation of filopodia-like processes.

To analyze the role of lipid rafts in the maintenance of TM-agrin-induced processes, neurites were pretreated with anti-
agrin antibodies for 3 h to induce the maximal number of filopodia-like processes and then incubated for 1.5 h with increasing concentrations of mβC to disperse lipid rafts. In the absence of mβC but continuous presence of anti-agrin antibodies, the number of processes was not changed compared with the initial number of processes (bar ab + 0 mM in Fig. 3C), in agreement with the previous observation that extended incubation times with antibodies does not lead to the formation of more processes (21). Likewise, removal of the antibodies in the absence of mβC did not affect the number or stability of the process (bar ab in Fig. 3C; see also Ref. 21). However, incubation of process-containing retinal ganglion cells with either 2 or 5 mM mβC for 1.5 h in the presence of anti-agrin antibodies resulted in a dose-dependent reduction of the number of processes (Fig. 3C). These data show that extraction of cholesterol from lipid rafts eliminates existing TM-agrin-induced processes, demonstrating that rafts are not only required for the initial formation of TM-agrin-induced processes but also for their long term stability and maintenance.

**Process Formation Requires the Phosphorylation and Activation of the Src Family Kinase Fyn**—Many signaling cascades initiated from lipid rafts involve phosphorylation of tyrosine residues. Accordingly, tyrosine phosphate immunoreactivity was associated with TM-agrin-induced processes (not shown). Moreover, Western blotting of total proteins from retinal ganglion cells with and without prior TM-agrin clustering using tyrosine phosphate-specific antibodies showed an increase in the phosphorylation of at least two specific proteins migrating at an apparent molecular mass of 60 and 45 kDa, respectively (not shown). The conspicuous molecular mass of 60 kDa together with the presence of Fyn in lipid rafts (see Fig. 2), suggested that the Src family kinase Fyn might become phosphorylated in response to TM-agrin clustering. To test this hypothesis, Western blots were reacted with an antibody detecting total Fyn as well as with an antibody reacting specifically with the phosphorylated tyrosine residue at position 417 (corresponding to residue 418 in murine Fyn) within the SH1 domain of several chick Src family kinases (41). Fig. 4A shows a representative Western blot of proteins isolated from retinal ganglion cells that were left either untreated (–ab) or incubated with anti-agrin antiserum 46 for 5 min, 30 min, or 3 h to induce the formation of processes. The blot was reacted with an Src family-specific anti-phosphotyrosine antibody (lane pY418 in Fig. 4A) and a panspecific anti-Fyn antibody (lane Fyn in Fig. 4A). Reprobing after stripping of the phosphotyrosine blots with Fyn-specific antibodies confirmed that the phosphotyrosine-reactive band corresponds to Fyn and not to another Src family kinase (data not shown). The level of Fyn phosphorylation was high already 5 min after the process induction. However, the values were rather heterogeneous, particularly at early time points. The results were therefore quantified as described in detail under “Experimental Procedures” by determining the ratio of phosphorylated Fyn compared with total Fyn (Fig. 4B). The blots were normalized to control values (untreated cultures), which were set to 1 (dashed line in Fig. 4B). Total Fyn as well as phosphorylated Fyn was also analyzed in chick brain homogenate (lane cb in Fig. 4A) to control for antibody specificity. Although treatment with preimmune serum (lane pre in Fig. 4A) does not lead to the formation of more processes (Fig. 3C), the presence of anti-agrin antibodies for 1.5 h with increasing concentrations of mβC to disperse lipid rafts results in a dose-dependent reduction of the number of processes (Fig. 3C).

**FIGURE 4. Phosphorylation of Fyn is required for process formation.** Cell lysates were prepared from retinal ganglion cells that were either untreated (lane –ab in A) or treated for 5 min, 30 min, or 3 h with anti-agrin antiserum 46 or with preimmune serum (lane pre in A) or pretreated for 1 h with PP1 and subsequently incubated with anti-agrin antiserum (lane PP1 in A). Total chick brain immunoreactivity (lane cb in A) was used to control the specificity of the antibodies. Western blots were then quantified to determine total Fyn as well as phosphorylated Fyn in the lysates using the Tyr(P)417 antibody, A, representative Western blot; B, densitometric analysis of three independent experiments. The values of tyrosine-phosphorylated Fyn in untreated control cultures (similar to lane –ab in A) were set to 1 (dashed line in B), and the relative increase or decrease of the ratio of phosphorylated Fyn compared with total Fyn was determined. An 4-fold increase in Tyr(P)417 phosphorylation was detected after 5 min of treatment with anti-agrin antibodies. Elevated levels of Tyr(P)417 phosphorylation remained present 30 min and 3 h after process induction by anti-agrin antibodies. Incubation of ganglion cells with the inhibitor PP1 reduced Fyn phosphorylation below control levels (bar 5 min PP1 in B). C, dose-dependent inhibition of process formation by the Src family kinase inhibitor PP1. A concentration of PP1 of 10 μM was sufficient to reduce the formation of processes to control levels. The bars in B and C represent the mean ± S.E. with n = 3; *, p < 0.05; ***, p < 0.001 (unpaired Student’s t test).
Fig. 4A) had no influence on Fyn phosphorylation, clustering of TM-agrin with anti-agrin antibodies resulted in a more than 4-fold increase in tyrosine 417-phosphorylated Fyn within 5 min (Fig. 4B). This increase in tyrosine phosphorylation was subsequently reduced to ~2.5-fold but remained rather constant 30 min and 3 h after antisera-induced clustering of TM-agrin. Pretreatment of the neurons for 1 h with the Src family kinase inhibitor PP1 (10 μM) (42) and subsequent clustering of TM-agrin for 5 min resulted in the reduction of tyrosine-phosphorylated Fyn below control levels. These results indicate that the Src family kinase Fyn becomes specifically tyrosine-phosphorylated at residue 417 within minutes after TM-agrin clustering.

To investigate if phosphorylation of Fyn is necessary for TM-agrin-induced process formation, retinal ganglion cell cultures were preincubated for 1 h with increasing concentrations of the inhibitor PP1 before processes were induced. PP1 had no influence on the growth of RGCs in culture and on growth cone morphology. However, the number of processes that could be induced by anti-agrin antibodies in the presence of PP1 was significantly reduced, and this reduction was dose-dependent. Representative axon segments stained with fluorescein-conjugated phalloidin are shown in Fig. S1, and the quantification of three independent experiments is shown in Fig. 4C. Although PP1 also inhibits other tyrosine kinases in addition to Fyn (43), these results are consistent with the hypothesis that Fyn becomes tyrosine-phosphorylated after TM-agrin clustering and that this activation of Fyn is required for TM-agrin-dependent process formation.

Process Formation Requires the Phosphorylation and Activation of the Mitogen-activated Protein Kinase—We next investigated the hypothesis that MAPK might be involved in TM-agrin-mediated process outgrowth. To this end, we analyzed the influence of TM-agrin clustering on tyrosine phosphorylation of MAPK by phosphotyrosine-specific antibodies (phosphorylated MAPK) using Western blotting. The ratio of phosphorylated MAPK/total MAPK was determined by quantitative Western blot analysis. As shown in Fig. 5, clustering of TM-agrin resulted in a small but significant increase in the phosphorylated form of the MAPK already 5 min after the addition of the antibodies. After 30 min, the phosphorylated form of MAPK was increased 5-fold compared with untreated control cultures (dashed line in Fig. 5B). The level of phosphorylated MAPK declined thereafter and was at control levels 3 h after antibody treatment. Preincubation of the cultures for 30 min with the MAPK-specific inhibitor PD98059 (44), and the subsequent addition of anti-agrin antibodies for 30 min resulted in a decrease of phosphorylated MAPK below control levels (lane PD in Fig. 5A and lane 30 min + PD, respectively, in Fig. 5B). A Western blot of total chick brain homogenate with antibodies against total MAPK as well as with antibodies against phospho-MAPK was used to control for the specificity of the antibodies (lane cb in Fig. 5A). These results demonstrate that clustering of TM-agrin on RGC neurites induces tyrosine phosphorylation of MAPK.

To test if MAPK phosphorylation is involved in TM-agrin-induced process formation, TM-agrin was clustered on RGCs in the presence of increasing concentrations of PD98059. Inhibition of MAPK phosphorylation by PD98059 resulted in a dose-dependent decrease in the number of processes that could be induced by anti-agrin antibodies (Fig. 5C). We were, however, unable to reduce the number of processes to control levels, since concentrations of PD98059 above 10 μM resulted in significant changes in growth cone morphology and in a retardation of neurite extension (not shown). These results demonstrate that the MAPK is specifically phosphorylated at tyrosine residues in response to clustering TM-agrin and that this phosphorylation is necessary for TM-agrin-induced process formation of RGC neurites.

Activation of Fyn Precedes Activation of MAPK—The previous results had shown that the phosphorylation of Fyn was
 maximal ∼5 min after TM-agrin clustering, whereas phosphorylation of MAPK had its maximum around 30 min after TM-agrin clustering. To analyze if both kinases belong to the same or to a different signaling cascade and to determine if the activation of one kinase precedes the activation of the other, we preincubated RGCs with either kinase inhibitor (PP1 or PD98059, respectively) for 1 h, followed by incubation with anti-agrin antibodies for 5 min (Fig. 6). Total Fyn and MAPK as well as their respective phosphorylated forms (as detected by anti-agrin antibodies for 5 min (Fig. 6). Total Fyn and MAPK as well as their activated forms (Tyr(P)417 (pY417)) and phospho-MAPK (pMAPK), respectively) were analyzed using Western blots and specific antibodies. Incubation with PP1 inhibits phosphorylation of Fyn as well as of MAPK, whereas incubation with PD98059 only inhibits phosphorylation of MAPK, demonstrating that activation of MAPK is downstream of the phosphorylation and activation of Fyn.

DISCUSSION

Overexpression or clustering of TM-agrin results in a reorganization of the actin cytoskeleton and the subsequent formation of numerous highly motile filopodia-like processes on axons and dendrites of neurons from the central- and the peripheral nervous system (21, 22). The main conclusion from this study is that the processes form in response to the activation of a complex intracellular signaling cascade. Several lines of evidence indicate that the initial event critical for TM-agrin-induced process formation involves lipid rafts. Lipid rafts are microdomains within the plane of the plasma membrane that have an altered lipid composition, act as signal transduction platforms for many receptors, and have links to the cytoskeleton. However, their distribution and physiological role is still controversial (45). We show that lipid rafts are associated with the filopodia-like processes, that TM-agrin codistributes with lipid rafts and is concentrated in low density lipid raft fractions, and finally that extraction of lipid rafts and inhibition of lipid raft biosynthesis inhibit process formation. We cannot distinguish if processes do not form due to a disruption of a direct link between lipid rafts and the actin cytoskeleton (46) or due to interference with lipid raft-dependent signaling cascades. However, since lipid rafts are almost ubiquitously present signaling platforms, the involvement of lipid rafts might explain the TM-agrin-mediated formation of processes in several different neurons from the central and peripheral nervous system as well as in nonneuronal cells from different species (21, 22, 47).

Previous studies have shown a critical role for cholesterol-rich lipid raft membrane microdomains in the agrin-dependent formation and maintenance of the neuromuscular junction (48–51). Moreover, agrin has been detected in lipid raft fractions from lymphocytes at immunological synapses (52) and from Schwann cells (53), although it was not determined if this was the transmembrane form. Thus, it is possible that lipid raft association of TM-agrin might be a common mechanism of agrin signaling initiation in different cell types.

Lipid rafts are important in the developing CNS for the formation of glutamatergic synapses (for a review, see Ref. 45). For example, depletion of cholesterol/sphingolipids leads to instability of surface AMPA receptors and gradual loss of synapses (both inhibitory and excitatory) and of dendritic spines (36). In addition, several ligand-gated ion channels, including the neuronal acetylcholine receptor, the GABA receptor, and the AMPA-type glutamate receptor, have been shown to be part of lipid raft microdomains (for a review, see Ref. 45). Moreover, dendritic filopodia-like protrusions actively initiate synapse formation by being precursors for spines (e.g. see Refs. 54–56). Our results suggest that translocation of TM-agrin into lipid rafts is an essential step in the formation of highly dynamic filopodia-like processes. These TM-agrin-induced processes have a complex cytoskeleton (21) and a length and a time course of appearing and disappearing that is similar to that described for spine precursors in vivo and in vitro (54, 57, 58). Together, these results therefore open the possibility that TM-agrin partitioning into lipid rafts might be the initial event leading to the activation of an intracellular signaling cascade, which culminates in the formation of filopodia-like processes that subsequently mature into synapses. In agreement with this hypothesis, agrin-deficient mice with a selective reexpression of agrin in motor neurons have a strongly reduced number of presynaptic and postsynaptic specializations in their cortex and a decrease in the frequency of miniature postsynaptic currents (10).

R. Ramseger, R. White, and S. Kröger, unpublished observation.
TM-agrin-induced Signaling Cascade

Clustering of TM-agrin induces the phosphorylation of the Src family kinase Fyn, and this activation is necessary for the formation of the filopodia-like processes. One possibility is that clustering of TM-agrin and partitioning of TM-agrin into lipid rafts results in the initiation of a direct interaction of TM-agrin and Fyn within the lipid raft microdomains. The intracellular domain of TM-agrin from human, rat, mouse, and chick contains the consensus sequence RXPR (where R represents a hydrophobic amino acid and X can be any amino acid) for Fyn binding (59, 60), making a direct interaction between TM-agrin and Fyn possible. However, deleting the intracellular domain of TM-agrin does not affect process formation in neurons and nonneuronal cells (22), strongly arguing against a direct interaction of the intracellular part of TM-agrin with Fyn. The activation of Fyn by TM-agrin might therefore involve additional upstream proteins.

As a downstream event of Fyn activation, we detected the temporally delayed phosphorylation of MAPK. Similar to the activation of Fyn, the phosphorylation of MAPK was necessary for process formation. It remains to be analyzed, however, if the activation of MAPK is a direct effect of Fyn (e.g. by physical interaction of Fyn and MAPK) (61, 62) or if additional signaling molecules are needed to mediate this effect. The requirement of TM-agrin-induced process formation for extracellular calcium (21) and the known calcium sensitivity of MAPK make a Fyn-independent activation of MAPK via calcium possible. An activation of MAPK by agrin has previously been observed in cultures of retinal ganglion cells (63) and hippocampal neurons (64) and in cortical neurons (65). Similar to our study, Src family kinases were necessary for the activation of MAPK in the case of cortical neurons (65). In these studies, however, process outgrowth was not reported, and the activation of MAPK was the response to soluble Nta-agrin. This might indicate that the intracellular signaling cascades activated by Nta-agrin and TM-agrin share a common intracellular pathway. The observations that neurons from agrin-deficient brains form less glutamatergic synapses and also have a defective MAPK pathway (10) further support the hypothesis that TM-agrin is involved in CNS synaptogenesis via the formation of filopodia-like processes. Since the MAPK signal transduction pathway regulates gene transcription, our results as well as previous studies (10, 14, 66) also open the possibility that in addition to the immediate formation of filopodia-like processes, activation of TM-agrin might lead to changes in gene transcription possibly involving, for example, the activation of CREB by MAPK (64).

It has previously been shown that activation of the Rho family GTPase Cdc42 correlates with TM-agrin-mediated process formation (22), but at which stage of the signaling cascade Cdc42 exerts its effect is unknown. The intracellular domain of TM-agrin does not contain a consensus sequence for Cdc42 binding and is not required for process formation (22), making a direct binding between TM-agrin and Cdc42 unlikely as the cause for the formation of the processes. However, an interaction between MAPK and Cdc42 has been reported (67–69), suggesting that activation of MAPK could cause the downstream activation of Cdc42, which then leads to the reorganization of the actin cytoskeleton and the subsequent formation of the processes.

The identification of a transmembrane form of agrin and its expression during the phase of active neurite extension as well as the formation of filopodia-like processes by overexpression or clustering of TM-agrin has led to the hypothesis that TM-agrin might serve as receptor or co-receptor (21) on neurons. This would require the initiation of a signaling cascade in response to ligand binding. Here we show that clustering of TM-agrin by anti-agrin antibodies induces the formation of processes, that this is paralleled by the partitioning of TM-agrin into lipid rafts, and that lipid rafts are required for the formation and maintenance of the processes. Several other membrane antigens that serve as receptors or coreceptors have been shown to reversibly partition into lipid rafts (30, 70). For example, the transmembrane heparan sulfate proteoglycan syndecan-4 is associated with lipid rafts (71), and this association can be mediated either by clustering with polyclonal antibodies (72) or by ligand binding (73). Likewise, TrkB (74), cRet (37), and ErbB4 (38), once stimulated by their respective ligand, translocate into lipid rafts, and this translocation is necessary for the initiation of downstream intracellular signaling events. In this study, we show that the formation of filopodia-like processes is the result of the initiation of a complex intracellular signaling cascade, providing essential evidence in support of the hypothesis that TM-agrin is a receptor or coreceptor on growing neurites.

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