α2-Chimaerin interacts with EphA4 and regulates EphA4-dependent growth cone collapse

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Edited by Eric M. Shooter, Stanford University School of Medicine, Stanford, CA, and approved August 31, 2007 (received for review July 14, 2007)

EphA4-dependent growth cone collapse requires reorganization of actin cytoskeleton through coordinated activation of Rho family GTPases. Whereas various guanine exchange factors have recently been identified to be involved in EphA4-mediated regulation of Rho GTPases and growth cone collapse, the functional roles of GTPase-activating proteins in the process are largely unknown. Here we report that EphA4 interacts with α2-chimaerin through its Src homology 2 domain. Activated EphA4 induces a rapid increase of tyrosine phosphorylation of α2-chimaerin and enhances its GTPase-activating protein activity toward Rac1. More importantly, α2-chimaerin regulates the action of EphA4 in growth cone collapse through modulation of Rac1 activity. Our findings have therefore identified a new α2-chimaerin-dependent signaling mechanism through which EphA4 transduces its signals to the actin cytoskeleton and modulates growth cone morphology.

axon guidance | ephrin | GTPase-activating protein | Rho GTPase

Axon guidance is a highly regulated process that is essential for the establishment of neural circuitry. The leading tips of growing axons known as growth cones are responsible for sensing multiple environmental guidance cues, either attractive or repulsive. These extracellular cues then transduce signals from cell surface receptors to actin cytoskeleton and regulate growth cone motility. A number of extracellular factors, including netrins, semaphorins, slits, and ephrins, have been identified as guidance cues. Among them, ephrin-A interacts with Eph receptor tyrosine kinases (Ephs) to function as negative guidance molecules and cause growth cone collapse in cultured neurons (1, 2). Activation of Eph-mediated downstream signaling leads to reorganization of actin cytoskeleton and results in cell retraction. To date, the Eph receptor family comprises the largest family of receptor tyrosine kinases. Based on the extracellular sequence similarities and binding preferences to their ephrin ligands, the Eph receptors are divided into A subclass (EphA1 to EphA10), which binds to glycosylphosphatidylinositol linked ephrin-As (ephrin-A1 to ephrin-A6), and B subclass (EphB1 to EphB3), which binds to transmembrane ephrin-Bs (ephrin-B1 to ephrin-B3), albeit with a few exceptions.

Emerging studies have identified signaling molecules downstream of Eph activation, a majority of which converge to the regulation of small Rho GTPases including Rac1, Cdc42, and RhoA. For example, activation of EphA by its ligand leads to a transient inhibition of Rac1 activity, concomitant with RhoA activation (3, 4). These small Rho GTPases act as molecular switches shuttling between an inactive GDP-bound state and an active GTP-bound state. Their activities are tightly controlled by guanine nucleotide exchange factors (GEFs), which act as positive regulators of Rho GTPases, and GTPase-activating proteins (GAPs), which act as negative regulators of Rho GTPases. Various GEFs have been identified as intermediates that link EphA receptors to small Rho GTPases at growth cones (3, 5). In particular, a Rho GEF ephexin1 is required for EphA4-mediated growth cone collapse through activation of RhoA (3, 6), whereas a Rac GEF Vav2 regulates the endocytosis of ephrin–Eph complex subsequent to Rac1 activation (5). Although it is believed that tight regulation of Rho GTPases by EphA is mediated by cooperative regulation of activities of GEFs and GAPs, the involvement of GAPs in regulating EphA-dependent signaling remains to be elucidated. It is interesting to note that α-chimaerins, a family of GAPs specific for Rac1, have been implicated in the regulation of growth cone collapse in dorsal root ganglion induced by semaphorin 3A (7).

In this study we report that α2-chimaerin is required for EphA4-dependent growth cone collapse. Prominent expression of α2-chimaerin can be detected in rat brain and cortical neurons and is enriched in postsynaptic density (PSD) fractions. Intriguingly, α2-chimaerin binds specifically to EphA4 in rat brain through its Src homology 2 (SH2) domain in a kinase-dependent manner. Ephrin-A1-stimulated activation of EphA4 receptor rapidly phosphorylates α2-chimaerin, resulting in an increase of its GAP activity toward Rac1. More importantly, our findings reveal that the SH2 interaction of α2-chimaerin with EphA4 and its GAP activity is required for the EphA4-dependent growth cone collapse.

Results

α2-Chimaerin Is Expressed in Rat Brain Throughout Development and Is Enriched in the PSD. Two isoforms of α-chimaerin (α1 and α2) have been reported to be expressed in rat brain (8). Although both α-chimaerin isoforms possess a C1 phorbol ester binding domain and a GTPase-activating domain, α2-chimaerin contains an additional SH2 domain at the amino-terminal region due to alternative splicing. To investigate whether α2-chimaerin is involved in EphA4-mediated signaling, we first examined the developmental regulation of α2-chimaerin protein in rat brain and cultured cortical neurons. Similar to the expression pattern of EphA4 in rat brain, prominent expression of α2-chimaerin could be detected in rat brain from embryonic day 16 (E16) to adult (Fig. 1*A*) and in cortical neurons from 4 to 21 days *in vitro* (DIV) (Fig. 1*B*). In contrast, the neuronal expression of α1-chimaerin was relatively low in early stages and was up-regulated later in development (Fig. 1*B*).

We next examined the subcellular distribution of α2-chimaerin in adult rat brain. Different PSD fractions were prepared from adult rat brain by using subcellular fractionation and successive

Author contributions: L.S., W.-Y.F., A.K.Y.F., and N.Y.I. designed research; L.S., W.-Y.F., and K.-W.H. performed research; C.P. and C.H. contributed new reagents/analytic tools; L.S., W.-Y.F., K.-W.H., C.H., A.K.Y.F., and N.Y.I. analyzed data; and L.S., A.K.Y.F., and N.Y.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: GEF, guanine nucleotide exchange factor; PSD, postsynaptic density; KD, kinase-dead; GAP, GTPase-activating protein; E*n*, embryonic day *n*; SFK, Src family kinase; SH2, Src homology 2; PMA, phorbol 12-myristate 13-acetate; DIV, days *in vitro*.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0706626104/DC1.

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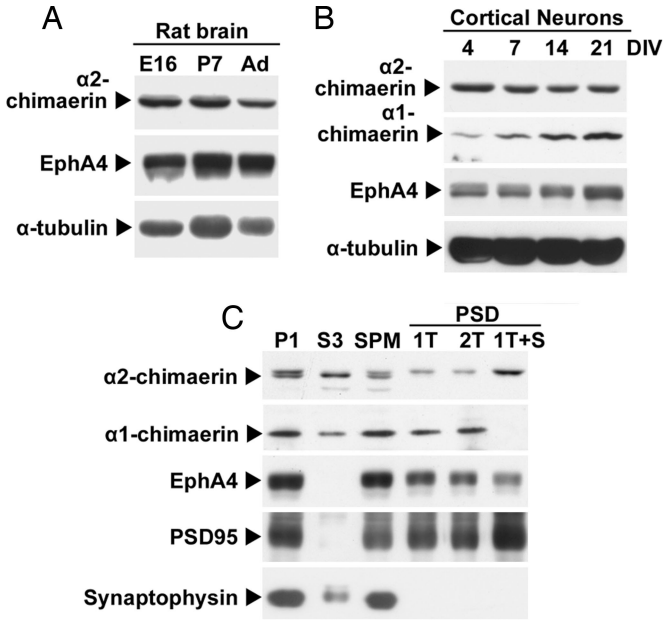

Fig. 1. α2-Chimaerin protein is expressed in rat brain during development. Lysates from rat brain [E16, postnatal day 7 (P7), and adult (Ad)] (*A*) or rat cortical neurons of 4–21 DIV (*B*) were subjected to Western blot analysis for α1-chimaerin, α2-chimaerin, and EphA4. (*C*) Lysates of adult rat brain fractions separated by differential centrifugation and extraction were subjected to Western blot analysis for α1-chimaerin, α2-chimaerin, and EphA4. PSD-95 and synaptophysin served as the PSD and presynaptic marker, respectively. P1, total brain lysates; S3, cytosolic fraction; SPM, synaptic plasma membrane. SPM was further extracted by Triton X-100 once (PSD 1T) or twice (PSD 2T) or with Triton X-100 followed by Sarkosyl (PSD 1T+S).

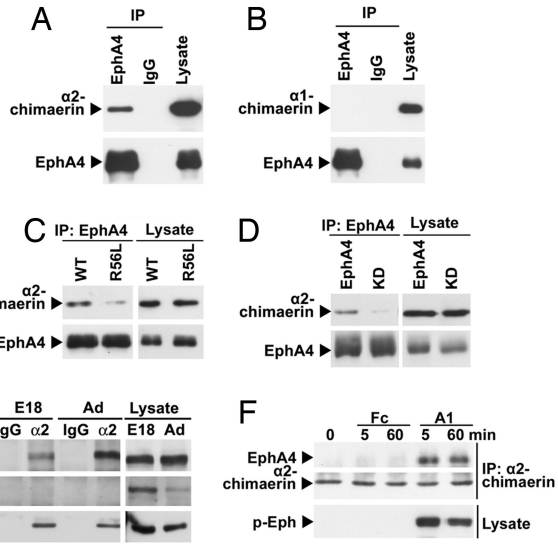

Fig. 2. EphA4 interacts with α2-chimaerin. (*A* and *B*) EphA4 associates with α2-chimaerin specifically in mammalian cells. HEK 293T cells were cotransfected with EphA4 together with HA-tagged α2-chimaerin (*A*) or α1-chimaerin (*B*) as indicated. Cell lysates were immunoprecipitated with EphA4 antibody and then immunoblotted with HA antibody specific for α-chimaerins. (*C*) The interaction of α2-chimaerin and EphA4 requires the binding of SH2 domain of α2-chimaerin with phosphotyrosine residue. (*D*) Activation of EphA4 is required for the interaction of α2-chimaerin and EphA4. (*E*) α2-Chimaerin associates with EphA4 in rat brain. Lysates of E18 or adult rat brains were immunoprecipitated with α2-chimaerin antibody and then immunoblotted with EphA4 and EphB2 antibodies. (*F*) Ephrin-A1 increases the association between EphA4 and α2-chimaerin. Cultured cortical neurons (6 DIV) were treated with ephrin-A1 for 0–60 min as indicated. Cell lysates were immunoprecipitated with α2-chimaerin antibody followed by immunoblotting using EphA4 antibody. EphA4 activation was analyzed by using antibody against phosphotyrosine residues (Tyr-596/602) of EphA4 (p-Eph).

detergent extraction. Western blot analysis revealed that α2-chimaerin remained enriched in the PSD fraction even after extraction with a harsh detergent, *N*-lauroyl sarcosinate, which extracts a significant portion of core PSD proteins including NMDA receptor subunits, whereas α1-chimaerin could be extracted with a milder nonionic detergent, Triton X-100. This observation suggests that α2-chimaerin is tightly associated with the PSD fractions, likely as a core PSD protein (Fig. 1*C*).

α2-Chimaerin Interacts with EphA4. To explore the possibility that α2-chimaerin exists as a signaling complex with EphA4, we examined whether α2-chimaerin interacts with EphA4 in mammalian cells. When overexpressed in HEK 293T cells, EphA4 was found to be constitutively active (data not shown and ref. 9). Specific association of EphA4 with α2-chimaerin, but not α1-chimaerin, could be detected in cells coexpressing these constructs, suggesting that EphA4 interacts with α2-chimaerin through its isoform-specific SH2 domain (Fig. 2 *A* and *B*). Consistent with this observation, EphA4 interacted only weakly with the R56L mutant of α2-chimaerin, a mutation generated at Arg-56 in the SH2 domain of α2-chimaerin that inhibits the binding of α2-chimaerin to phosphotyrosine residue(s) (10) (Fig. 2*C*). Because phosphotyrosine residues provide docking sites for SH2 domains of cytoplasmic signaling proteins, we next asked whether the interaction between α2-chimaerin and EphA4 depends on EphA4 tyrosine phosphorylation. When compared with WT EphA4, interaction between kinase-dead (KD) mutant of EphA4 (KD EphA4) and α2-chimaerin was substantially reduced, suggesting that the association required active EphA4 (Fig. 2*D*). We then examined whether endogenous α2-chimaerin associates with EphA4 specifically in rat brain. Interestingly, α2-chimaerin coimmunoprecipitated with EphA4 but not with EphB2 in E18 and adult brains (Fig. 2*E*), indicating that α2-chimaerin is present in the EphA4-signaling complex *in vivo*. Whereas interaction between α2-chimaerin and EphA4 was barely detected in cultured cortical neurons, a significant enhancement of their association was observed upon ephrin-A1 treatment (Fig. 2*F*). Ligand-dependent interaction between α2-chimaerin and EphA4 is relatively stable and could be detected for up to 60 min after ephrin-A1 stimulation.

EphA4 Activation Induces Tyrosine Phosphorylation of α2-Chimaerin in Cultured Neurons. To investigate whether α2-chimaerin is a downstream target of EphA4, we examined whether α2-chimaerin is phosphorylated by EphA4. HA-tagged α1- or α2-chimaerin together with WT or KD EphA4 constructs were coexpressed in HEK 293T cells. We found that tyrosine phosphorylation of α2-chimaerin could be observed in the presence of WT EphA4 but not KD EphA4 and that only α2- but not α1-chimaerin was phosphorylated by activated EphA4 in mammalian cells (Fig. 3*A*). These findings suggest that EphA4 phosphorylates the SH2 domain of α2-chimaerin. Importantly, the phosphorylation of R56L α2-chimaerin mutant by EphA4 was greatly attenuated when compared with that of WT (Fig. 3*B*), suggesting that tyrosine phosphorylation of α2-chimaerin by EphA4 requires the interaction of these two proteins.

To further examine whether ephrin-A1-induced EphA4 activation stimulates tyrosine phosphorylation of α2-chimaerin *in vivo*, cultured rat cortical neurons at 14 DIV were treated with clustered ephrin-A1. We found that ephrin-A1 stimulation induces a rapid tyrosine phosphorylation of α2-chimaerin within 5 min (Fig. 3*C*), which was sustained for at least 60 min. Interestingly, the tyrosine phosphorylation of α2-chimaerin is

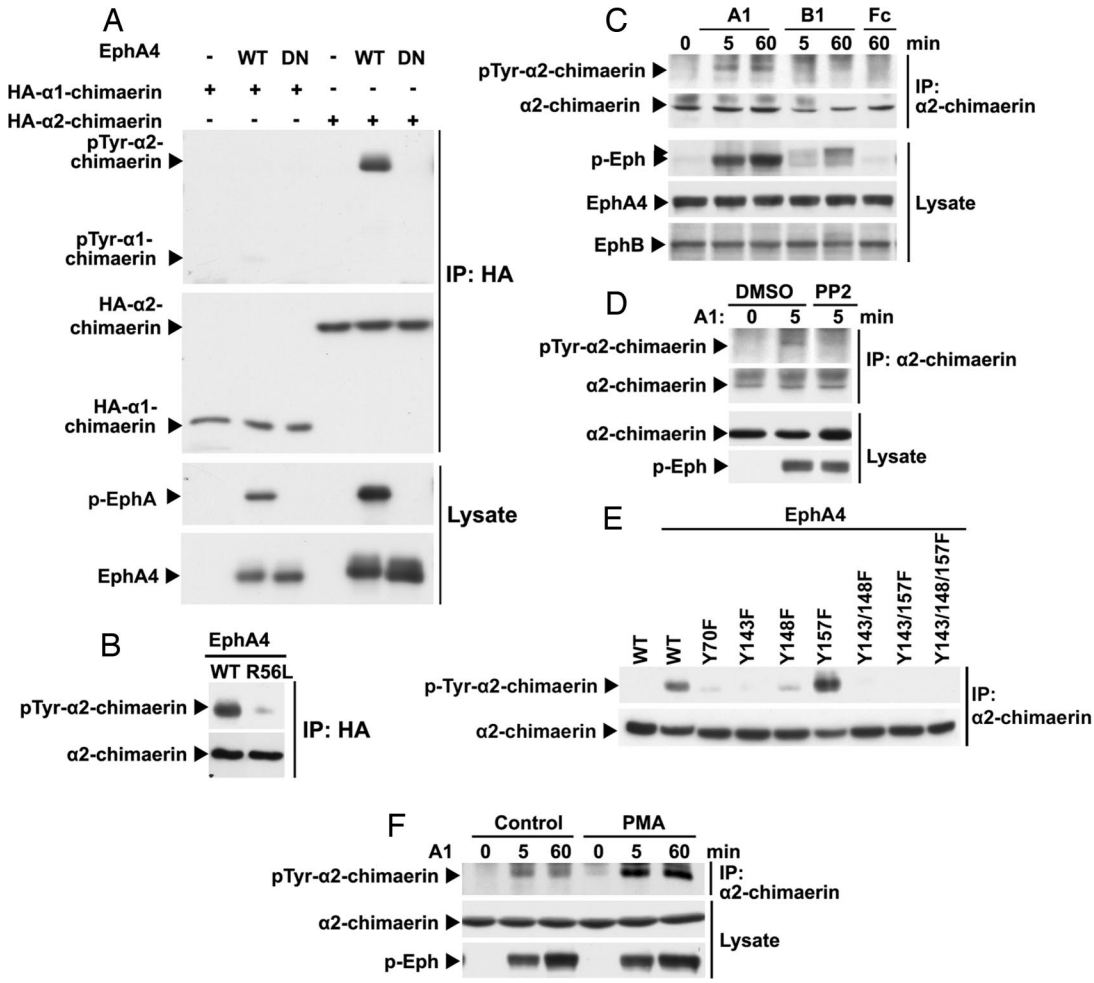

Fig. 3. EphA4 activation induces tyrosine phosphorylation of α2-chimaerin. (*A*) HEK 293T cells were cotransfected with WT or KD EphA4 in combination with HA-tagged α1-chimaerin or α2-chimaerin. Cell lysates were immunoprecipitated with HA antibody followed by immunoblotting with phosphotyrosine antibody (4G10). (*B*) Association of α2-chimaerin with EphA4 is required for the tyrosine phosphorylation of α2-chimaerin by EphA4. (*C*) Ephrin-A1 treatment stimulates tyrosine phosphorylation of α2-chimaerin in cortical neurons. Rat cortical neurons (14 DIV) were treated with clustered ephrin-A1–Fc, ephrin-B1–Fc, or Fc alone, and cell lysates were collected at indicated time points (5 and 60 min). The lysates were immunoprecipitated with α2-chimaerin antibody and immunoblotted with 4G10 antibody. Only ephrin-A1 treatment induced the tyrosine phosphorylation of α2-chimaerin. (*D*) Inhibition of SFK activity in neurons attenuates ephrin-A1-stimulated tyrosine phosphorylation of α2-chimaerin. Pretreatment of cultured cortical neurons with a specific Src inhibitor, PP2, for 30 min abolished the ephrin-A1-stimulated tyrosine phosphorylation of α2-chimaerin. (*E*) Phosphorylation of α2-chimaerin at multiple tyrosine residues by EphA4 activation. HEK 293T cells were transfected with expression constructs encoding α2-chimaerin (WT), its mutants, or EphA4 as indicated. Total cell lysates were immunoprecipitated by using α2-chimaerin antibody followed by immunoblotting using 4G10 antibody. (*F*) Treatment of cultured cortical neurons with PMA, a DAG analog, for 30 min augments the ephrin-A1-stimulated tyrosine phosphorylation of α2-chimaerin.

specific for ephrin-A stimulation because tyrosine phosphorylation of α2-chimaerin was not observed when the neurons were treated with ephrin-B1 or Fc alone (Fig. 3*C*). These findings corroborate the notion that α2-chimaerin is a downstream target of EphA4-mediated signaling.

We then investigated whether α2-chimaerin is directly phosphorylated by EphA4 or by its downstream tyrosine kinases, i.e., Src family kinases (SFKs). We found that the ephrin-stimulated tyrosine phosphorylation of α2-chimaerin was substantially reduced in neurons after pretreatment with a specific Src inhibitor PP2 (Fig. 3*D*). These results indicate that the ephrin-A1-induced tyrosine phosphorylation of α2-chimaerin likely depends on the activity of SFKs. To identify the potential tyrosine phosphorylation sites on α2-chimaerin by SFKs, we generated different tyrosine phosphorylation mutants in the amino-terminal region of α2-chimaerin (mutating the tyrosine to phenylalanine at individual and/or multiple sites as indicated) (Fig. 3*E*). Intriguingly, we found that the α2-chimaerin mutants with mutations at Tyr-70, Tyr-143, and/or Tyr-148 were not tyrosine phosphorylated when coexpressed with activated EphA4, whereas mutating the Tyr-157 site on α2-chimaerin did not significantly affect its EphA4-dependent phosphorylation (Fig. 3*E*). Taken together, these findings suggest that SFKs phosphorylate α2-chimaerin at multiple tyrosine sites. However, further studies will be required to confirm whether these tyrosine sites of α2-chimaerin are phosphorylated by SFKs *in vivo*.

It has been well established that activity of chimaerins is regulated through its binding to phorbol esters or DAG analogues via their C1 domain (11). Phorbol esters or DAG analogues are able to translocate chimaerins from cytosolic to membrane fractions, which leads to the modulation of its Rac–GAP activity and allows transduction of signals to the actin cytoskeleton. To examine whether phorbol ester or DAG-dependent signaling is involved in the ephrin-A1-induced tyrosine phosphorylation of α2-chimaerin, we took advantage of the DAG analogue phorbol 12-myristate 13-acetate (PMA).

Intriguingly, pretreatment with PMA markedly enhanced the ephrin-A1-induced tyrosine phosphorylation of α2-chimaerin (Fig. 3*F*), suggesting that enhanced recruitment of α2-chimaerin to plasma membrane would facilitate the phosphorylation of α2-chimaerin by EphA4. It was recently reported that phospholipase Cγ, an enzyme that generates DAG by hydrolyzing PIP2, is activated by EphA4-mediated signaling (12). Thus, it is tempting to speculate that activation of phospholipase Cγ is involved in regulating the ephrin-A1-mediated tyrosine phosphorylation of α2-chimaerin.

EphA4 Promotes the GAP Activity of α2-Chimaerin Toward Rac1. To determine how EphA4 activation regulates the GAP activity of α2-chimaerin toward Rac1, we evaluated the level of active Rac1 in COS-7 cells after transfection with α2-chimaerin together with EphA4 or its KD mutant (Fig. 4*A*). A pull-down assay using the GST fusion protein containing Rac1-binding domain of Pak (GST-PBD), which binds only to active Rac1 (the GTP-bound form), was performed. The level of GTP-bound Rac1 for each transfection condition was examined by Western blot analysis using Rac1 antibody (Fig. 4*A*). Whereas expression of EphA4 could not down-regulate the Rac1 activity in COS-7 cells, expression of α2-chimaerin alone led to a moderate decrease of Rac1 activity. Intriguingly, when coexpressed with EphA4, α2-chimaerin caused a significant inhibition of Rac1 activity. On the other hand, inhibition of the kinase activity of either EphA4 or SFKs abrogated the EphA4-dependent down-regulation of Rac1 activity, suggesting that tyrosine phosphorylation of α2-chimaerin is required for its GAP activity (Fig. 4*A*).

SH2 Domain Interaction and GAP Activity of α2-Chimaerin Are Required for EphA4-Dependent Downstream Signaling and EphA4 Clustering. Because ephrin-A-induced down-regulation of Rac1 activity has been reported to inhibit Pak phosphorylation (3), we examined whether knockdown of α2-chimaerin in cultured neurons regulates the ephrin-A1-stimulated inhibition of Pak phosphorylation. Whereas Pak phosphorylation was significantly reduced in control neurons after ephrin-A1 treatment, similar reduction of Pak phosphorylation was not observed in α2-chimaerin knocked-down neurons upon ephrin-A1 treatment (Fig. 4*B*). These results suggest that α2-chimaerin is involved in the inhibition of EphA-dependent Rac1/Pak signaling. Proper clustering of EphA4 receptors, which is required in eliciting the maximal activation of EphA4 signaling, has been demonstrated to be important for growth cone collapse (2). We therefore examined whether α2-chimaerin regulates the extent of ephrin-A-stimulated EphA4 clustering. Consistent with our previous finding (13), the number of EphA4 clusters in cultured hippocampal neurons was increased in response to ephrin-A stimulation. Although the number of EphA4 clusters in hippocampal neurons transfected with GAP mutant or SH2 mutant of α2-chimaerin was relatively unchanged, the size of EphA4 clusters in the neurons overexpressing these mutants was significantly reduced (Fig. 4*C*). Because EphA4 clustering was suggested to be important for recruiting or activating cytoplasmic effectors such as ephexin1, it is tempting to speculate that the SH2 interaction and GAP activity of α2-chimaerin contribute, at least in part, to the regulation of EphA4-dependent signaling.

α2-Chimaerin Is Required for the EphA4-Dependent Growth Cone Collapse. Whereas inhibition of Rac1 activity is observed during ephrin-A-stimulated growth cone collapse, the mechanism by which Rac1 activity is down-regulated is largely unclear. Nonetheless, it is interesting to note that Rac–GAP activity of α2-chimaerin is necessary for growth cone collapse induced by semaphorin 3A in dorsal root ganglions (7). To examine whether ephrin-A elicits its effect on growth cone collapse through α2-chimaerin, expression of α2-chimaerin was knocked down in neurons by using an RNAi approach. Cultured rat hippocampal neurons (0 DIV) were transfected with pSUPER-α2-chimaerin RNAi and EGFP construct and then treated with ephrin-A1 at 3 DIV. The efficiency of knockdown of endogenous α2-chimaerin was confirmed by Western blot analysis (Fig. 5*B*).

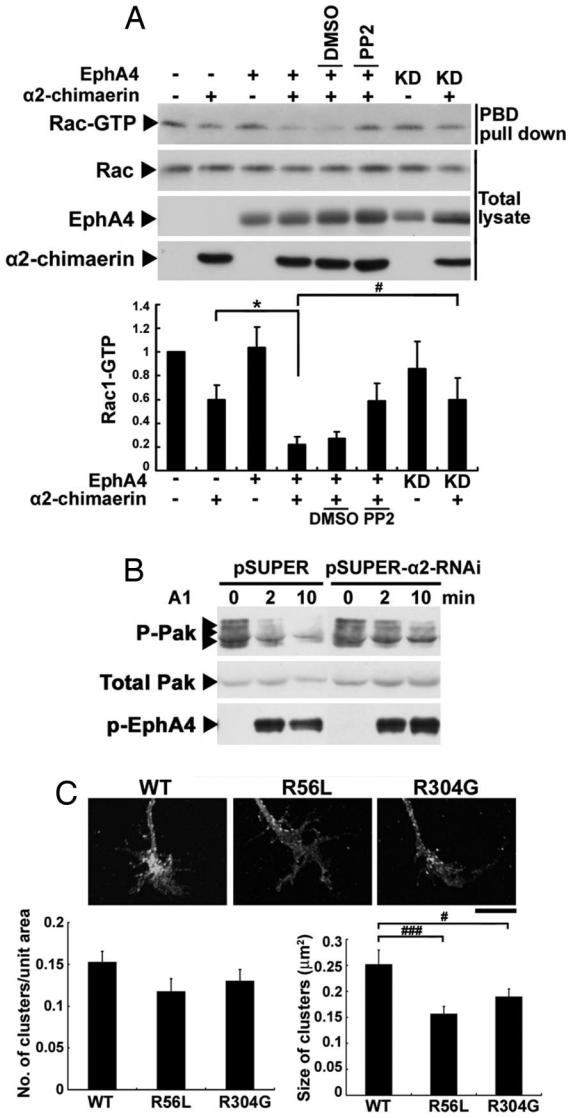

Fig. 4. α2-Chimaerin regulates ephrin-A1-stimulated downstream signaling. (*A*) EphA4 promotes GAP activity of α2-chimaerin toward Rac1. COS-7 cells were cotransfected with WT or KD EphA4 in combination with α2-chimaerin and treated with PP2 as indicated. Cell lysates were collected and incubated with GST–Rac1-binding domain of Pak (PBD) for pull-down of GTP-bound Rac1 (GTP–Rac1). Shown is a quantification of fold change of Rac1 activity ($n = 3$); data represent mean ± SEM. *, $P < 0.05$ (expression of EphA4 and α2-chimaerin versus α2-chimaerin alone); #, $P < 0.05$ [expression of α2-chimaerin and EphA4 (WT) versus α2-chimaerin and KD] (*Lower*). (*B*) Knockdown of α2-chimaerin abolishes the ephrin-A1-stimulated inhibition of Pak phosphorylation. Dissociated cortical neurons were transfected with pSUPER-α2-chimaerin RNAi (pSUPER-α2-RNAi), and then treated with clustered ephrin-A1 for the indicated time points. The lysates were immunoblotted with phospho-Pak antibody. (*C*) SH2 domain interaction and GAP activity of α2-chimaerin contribute to the EphA4 clustering upon ephrin-A1 treatment. Confocal images show growth cones of cultured hippocampal neurons after transfection with mutants of α2-chimaerin and treatment with ephrin-A1 at 3 DIV for 5 min. (Scale bar: 10 μm.) Shown is a quantification of the cluster density and average size of EphA4 clusters (*Lower*). Data represent mean ± SEM of at least three experiments. ###, $P < 0.005$; #, $P < 0.05$ (mutants versus WT after ephrin-A1 treatment).

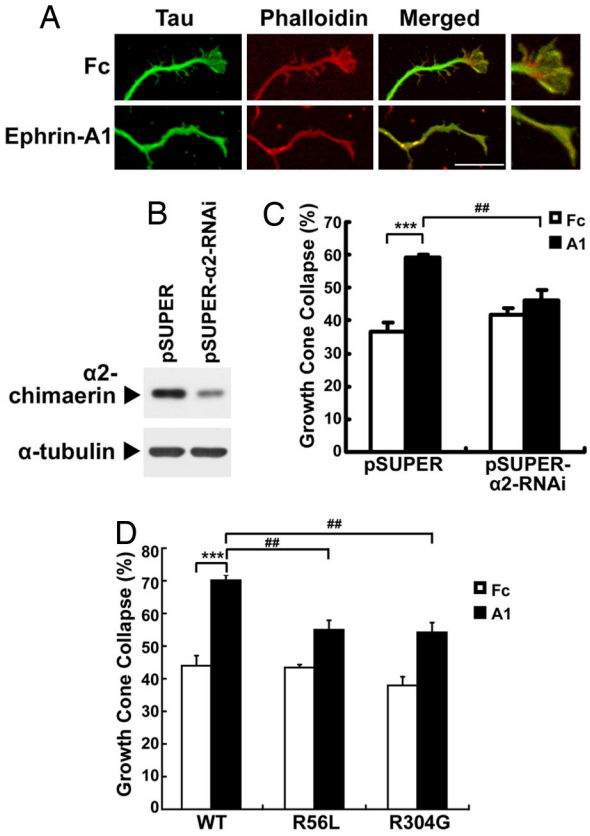

Fig. 5. α2-Chimaerin is required for the EphA4-mediated growth cone collapse. (*A*) Ephrin-A1 induces growth cone collapse in hippocampal neurons. E18 hippocampal neurons (3 DIV) were treated with clustered ephrin-A1 for 1 h and stained with Tau-1 antibody (green) and Alexa Fluor 555-conjugated phalloidin (red). Representative images depict the typical morphology of a well spread growth cone (*Upper*) and a typical example of collapsed growth cone stimulated by ephrin-A1 (*Lower*). (*B*) Knockdown of α2-chimaerin expression by pSUPER-α2-RNAi. pSUPER-α2-chimaerin or pSUPER vector was delivered into cortical neurons by nucleofection, and cell lysates were collected 3 days after transfection for Western blot analysis. (*C*) Knockdown of α2-chimaerin expression by pSUPER-α2-RNAi impairs the ephrin-A1-induced growth cone collapse. Hippocampal neurons transfected with pSUPER-α2-RNAi and GFP plasmid were treated with ephrin-A1 for 60 min. Growth cone collapse was scored in a blinded manner. Data represent mean ± SEM of at least three experiments. ***, $P < 0.005$ (ephrin-A1 versus Fc treatment); ##, $P < 0.01$ (pSUPER-α2-RNAi versus pSUPER in the ephrin-A1-treated neurons). (*D*) SH2 domain interaction of α2-chimaerin and its GAP activity attribute to the ephrin-A1-stimulated growth cone collapse. Hippocampal neurons transfected with GFP and α2-chimaerin (WT) or its mutants (R56L and R304G) were treated with ephrin-A1. The collapsed growth cones were quantified. Data represent mean ± SEM of at least three experiments. ***, $P < 0.005$ (ephrin-A1 versus Fc treatment); ##, $P < 0.01$ (mutants versus WT in the ephrin-A1-treated condition).

Whereas ephrin-A1 treatment induced growth cone collapse (≈60%) in pSUPER vector-transfected neurons, knockdown of α2-chimaerin abolished the effect of ephrin-A1 (Fig. 5*C*). To confirm that this effect of ephrin-A1 was mediated by EphA4 activation, we silenced the EphA4 expression in hippocampal neurons using pSUPER-EphA4 RNAi and found that knockdown of EphA4 abolished the ephrin-A1-stimulated growth cone collapse (data not shown).

To determine whether the interaction of α2-chimaerin with EphA4 or its GAP activity is involved in EphA4-dependent growth cone collapse, cultured hippocampal neurons were transfected with the SH2 and GAP mutants of α2-chimaerin (R56L and R304G), respectively (10). Mutation of Arg-56 at the SH2 domain of α2-chimaerin abolished its interaction with the phosphotyrosine residues without affecting its GAP activity, whereas mutation of Arg-304 at the GAP domain of α2-chimaerin inhibits its GAP activity. Consistent with the involvement of reduced Rac1 activity, expression of WT α2-chimaerin in hippocampal neurons increased the percentage of growth cone collapse under basal conditions (from ≈35% to 45%). Stimulation of these neurons with ephrin-A1 induced a further increase in growth cone collapse in α2-chimaerin-expressing neurons compared with that in control neurons (≈70%). Interestingly, expression of either SH2 mutant or GAP mutant of α2-chimaerin significantly inhibited the ephrin-A1-stimulated growth cone collapse, suggesting that both SH2 interaction and GAP activity of α2-chimaerin contribute in part to the EphA4-dependent signaling during growth cone collapse (Fig. 5*D*). Taken together, these findings strongly support the notion that α2-chimaerin is required for ephrin-A-stimulated growth cone collapse.

Discussion

EphA receptors and their ephrin ligands play critical roles in axon guidance and growth cone collapse through concerted regulation of small Rho GTPases. Ephrin-mediated EphA activation leads to enhanced RhoA activity and reduced Rac1 activity in neurons during growth cone collapse. Here we demonstrate that α2-chimaerin, a Rac–GAP that down-regulates Rac1 activity, plays an important role in EphA4-dependent growth cone collapse. α2-Chimaerin interacts with tyrosine-phosphorylated EphA4 through its amino-terminal SH2 domain. Stimulation of neurons by ephrin-A increases the tyrosine phosphorylation of α2-chimaerin and subsequently regulates its GAP activity. More importantly, the Rac–GAP activity of α2-chimaerin is required for the ephrin-A1-induced growth cone collapse. These findings not only demonstrate an essential role of α2-chimaerin in EphA4-dependent growth cone collapse, but also provide evidence that inhibition of Rac1 activity is critical for growth cone collapse.

Rho GTPases have been suggested to be the primary regulators for actin dynamics at growth cones (14). The precise regulation of Rac1 activity is thought to control the formation and retraction of lamellipodia and filopodia at the leading edges of growth cones through actin reorganization (15, 16). Although Rac1 activation has been suggested to play an important role in the EphA4-mediated forward signaling through modulating the endocytosis of ephrin–EphA complex (5), ephrin-A-stimulated growth cone collapse is preceded by a transient reduction of Rac1 activity (4). Whether the reduction of Rac1 activity regulates EphA4-dependent F-actin cytoskeletal reorganization or the endocytosis of ephrin–EphA complex awaits further studies. However, identification of α2-chimaerin as a signaling integrator in EphA4-dependent growth cone collapse in this study may provide insights into the molecular mechanism underlying the precise temporal control of Rac1 activity during ephrin-A-induced growth cone collapse.

Although we demonstrate that EphA4 and α2-chimaerin exist as a signaling complex in neurons, whether α2-chimaerin interacts directly with EphA4 remains to be elucidated. Nonetheless, tyrosine phosphorylation of α2-chimaerin, which is critical for the EphA4-dependent stimulation of its GAP activity, is preceded by the interaction of α2-chimaerin with activated EphA4. This finding strongly suggests that recruitment of α2-chimaerin to activated EphA4 is an important event before its activation. Because EphA4 is known to recruit several signaling proteins including SFKs and Vav2 through the SH2 interaction, it is conceivable that the mechanism by which these signaling proteins interact with EphA4 constitutes an essential molecular control of EphA4-mediated signaling (5, 17, 18). Indeed, we found that SFKs such as Src and Fyn are present in the

EphA4/α2-chimaerin signaling complex (unpublished observation and ref. 18). Further studies are required to elucidate the precise mechanism by which SFKs control the tyrosine phosphorylation of α2-chimaerin upon EphA4 activation. The ability of PMA pretreatment in enhancing the phosphorylation of α2-chimaerin upon ephrin-A1 activation suggests that the translocation of α2-chimaerin to plasma membrane by PMA can facilitate the tyrosine phosphorylation by EphA4. Indeed, a recent study reported that EphA4 mediates its signaling through activation of phospholipase Cγ (12), an enzyme that cleaves PIP2 into DAG and second messenger IP3. These findings raise an interesting possibility that EphA4 might regulate the activity of α2-chimaerin indirectly by generating more DAG and increasing the susceptibility of α2-chimaerin to tyrosine phosphorylation by EphA4.

Although the involvement of Rho GTPases in EphA4-dependent growth cone collapse has been well elucidated, signal transducers linking the receptor to Rho GTPases are just beginning to be unraveled. For example, the GEF ephexin1 has been identified to be an essential mediator for EphA-dependent growth cone collapse through activation of RhoA and inhibition of Rac1 (3, 6). Here we propose a new mechanism by which signals are transduced from EphA4 to Rac1 through α2-chimaerin. It is envisaged that α2-chimaerin regulation is tightly coupled with that of ephexin1 during EphA4-dependent growth cone collapse. In light of the fact that EphA4-mediated regulation of ephexin1 depends on the activation of various kinases including SFKs and Cdk5, it is interesting to note that α2-chimaerin is also a substrate of the SFKs (unpublished observation) and has been reported to exist as a signaling complex with Cdk5 in brain (19). It will be important to examine how the phosphorylation of ephexin1 and α2-chimaerin by SFKs and Cdk5 regulates their actions in EphA4-mediated signaling pathways. Moreover, enrichment of α2-chimaerin in PSD fractions, together with the involvement of EphA4-mediated signaling in dendritic spine retraction, implies that α2-chimaerin is also likely to be involved in EphA4-dependent synapse development and plasticity.

Materials and Methods

For details, see supporting information (SI) *Materials and Methods*.

Chemicals, Constructs, and Antibodies. Expression constructs encoding HA-tagged α1-chimaerin, α2-chimaerin, and different mutants of α2-chimaerin (R56L and R304G) were generated as described (7), whereas the tyrosine phosphorylation mutants of α2-chimaerin were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The pSUPER-α2-chimaerin was designed as described (13). For details, see *SI Materials and Methods*.

Cell Cultures and Transfection. HEK 293T cells were cultured and transfected by using LipofectAMINE PLUS reagents (Invitrogen, Carlsbad, CA). Primary cortical and hippocampal neurons were prepared and cultured from E18 to E19 rat embryos as previously described (13). For details, see *SI Materials and Methods*.

Rac GTPase Activation Assay. The levels of GTP-bound Rac1 in COS-7 cells expressing combinations of constructs were measured by using GST–Rac1-binding domain of Pak as previously described (13). For details, see *SI Materials and Methods*.

Growth Cone Collapse Assay and Quantification of EphA4 Clustering. Dissociated hippocampal neurons were transfected at 0 DIV with different plasmids plus EGFP by using the rat neuron Nucleofector kit (Amaxa Biosystems, Gaithersburg, MD) (20). Ephrin-A1 was treated at 3 DIV. Percentage of collapsed growth cones and the number and size of EphA4 clusters were then analyzed. For details, see *SI Materials and Methods*.

We are grateful to R. Z. Qi and Jerry Wang (Hong Kong University of Science and Technology) for the α1-chimaerin cDNA construct and Busma Butt, Ke Kong, and Cara Kwong for expert technical assistance. This work was supported in part by the Research Grants Council of Hong Kong (Grants 6119/04M, 6421/05M, and HKUST 3/03C), the Area of Excellence Scheme of the University Grants Committee (Grant AoE/B-15/01), and the Hong Kong Jockey Club. N.Y.I. was the recipient of a Croucher Foundation Senior Research Fellowship.

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