RPTPα is essential for NCAM-mediated p59<sup>fyn</sup> activation and neurite elongation

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Introduction

The neural cell adhesion molecule (NCAM) forms a complex with p59<sup>fyn</sup> kinase and activates it via a mechanism that has remained unknown. We show that the NCAM140 isoform directly interacts with the intracellular domain of the receptor-like protein tyrosine phosphatase RPTPα, a known activator of p59<sup>fyn</sup>. Whereas this direct interaction is Ca<sup>2+</sup>-independent, formation of the complex is enhanced by Ca<sup>2+</sup>-dependent spectrin cytoskeleton–mediated cross-linking of NCAM and RPTPα in response to NCAM activation and is accompanied by redistribution of the complex to lipid rafts. Association between NCAM and p59<sup>fyn</sup> is lost in RPTPα-deficient brains and is disrupted by dominant-negative RPTPα mutants, demonstrating that RPTPα is a link between NCAM and p59<sup>fyn</sup>. NCAM-mediated p59<sup>fyn</sup> activation is abolished in RPTPα-deficient neurons, and disruption of the NCAM–p59<sup>fyn</sup> complex in RPTPα-deficient neurons or with dominant-negative RPTPα mutants blocks NCAM-dependent neurite outgrowth, implicating RPTPα as a major phosphatase involved in NCAM-mediated signaling.

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Abbreviations used in this paper: FGFR, FGF receptor; NCAM, neural cell adhesion molecule.

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Figure 1. Activated p59\(^{fn}\) is reduced in NCAM-deficient brain. (A) Brain homogenates from 0- to 4-d-old wild-type (NCAM\(+/+)\) and NCAM-deficient (NCAM\(-/-\)) mice were probed by Western blot with antibodies against total p59\(^{fn}\) protein. Labeling for GAPDH was included as loading control. Levels of p59\(^{fn}\) protein are increased in NCAM-deficient brains. (B) p59\(^{fn}\) immunoprecipitates from 0- to 4-d-old wild-type (NCAM\(+/+)\) and NCAM-deficient (NCAM\(-/-\)) mice were probed by Western blot with antibodies against total p59 fyn protein. Labeling for GAPDH was included as loading control. Leveling for GAPDH was included as loading control. Levels of p59 fyn protein are increased in NCAM-deficient brains. Histograms (A and B) show quantitation of the blots with OD for wild type set to 100%. Mean values \(\pm\) SEM (\(n = 6\)) are shown. *, \(P < 0.05\), paired \(t\) test.

al., 1997; Harder et al., 1998). To activate Src family tyrosine kinase, constitutively phosphorylated pTyr789 at the COOH-terminal of RPTPl binds the SH2 domain of Src family tyrosine kinase that disrupts the intra-molecular association between the SH2 and SH1 domains of the kinase. This initial binding is followed by binding between the inhibitory COOH-terminal phosphorylation site of the Src family tyrosine kinase (pTyr531 in p59\(^{fn}\)) and the D1 domain of RPTPl resulting in dephosphorylation of the inhibitory COOH-terminal phosphorylation sites in Src family tyrosine kinases (Zheng et al., 2000). These sites are hyperphosphorylated in cells lacking RPTPl, and kinase activity of pp60\(^{src}\) and p59\(^{fn}\) in RPTPl-deficient mice is reduced (Ponniah et al., 1999). Like p59\(^{fn}\) and NCAM, RPTPl is particularly abundant in the brain (Kaplan et al., 1990; Krueger et al., 1990), accumulates in growth cones (Helmke et al., 1998), and is involved in neural cell migration and neurite outgrowth (Su et al., 1996; Yang et al., 2002; Petrone et al., 2003).

Remarkably, a close homologue of RPTPl, CD45, associates with the membrane-cytoskeleton linker protein spectrin (Lokeshwar and Bourguignon, 1992; Iida et al., 1994), a binding partner of NCAM (Leshchyns’ka et al., 2003). Following this lead, we investigated the possibility that RPTPl is involved in NCAM-induced p59\(^{fn}\) activation. We show that the intracellular domains of NCAM140 and RPTPl interact directly and that this interaction is enhanced by spectrin-mediated Ca\(^{2+}\)-dependent cross-linking of NCAM and RPTPl. Levels of p59\(^{fn}\) associated with NCAM correlate with the ability of NCAM-associated RPTPl to bind to p59\(^{fn}\), and the NCAM–p59\(^{fn}\) complex is disrupted in RPTPl-deficient brains implicating RPTPl as linker molecule between NCAM and p59\(^{fn}\). RPTPl redistributes to lipid rafts in response to NCAM activation and RPTPl levels are reduced in lipid rafts from NCAM-deficient mice, suggesting that NCAM recruits RPTPl to lipid rafts to activate p59\(^{fn}\). Finally, NCAM-mediated p59\(^{fn}\) activation is abolished in RPTPl-deficient neurons and NCAM-induced neurite outgrowth is blocked in RPTPl-deficient neurons or neurons transfected with dominant-negative RPTPl mutants, demonstrating that RPTPl is a major phosphatase involved in NCAM-mediated signaling.

Results

Activation of p59\(^{fn}\) is impaired in NCAM-deficient brains

Cross-linking of NCAM at the cell surface results in a rapid activation of p59\(^{fn}\) kinase (Beggs et al., 1997; Niethammer et al., 2002) via an unknown mechanism. To analyze whether or not NCAM deficiency may affect the activation status of p59\(^{fn}\), we compared levels of activated p59\(^{fn}\) characterized by dephosphorylation at Tyr-531 and phosphorylation at Tyr-420 in the brains of wild-type and NCAM-deficient mice. Whereas the level of p59\(^{fn}\) protein was higher in brain homogenates of NCAM-deficient mice (Fig. 1 A), labeling with antibodies recognizing only p59\(^{fn}\) dephosphorylated at Tyr-531 or with antibodies recognizing only p59\(^{fn}\) phosphorylated at Tyr-420 was reduced in brain homogenates of NCAM-deficient mice (Fig. 1 B), indicating that activation of p59\(^{fn}\) is inhibited in NCAM-deficient brains and suggesting that NCAM is involved in the regulation of p59\(^{fn}\) function.
NCAM forms a complex with RPTPα

The intracellular domain of NCAM does not contain sequences known to induce p59Fyn activation. Thus, NCAM may form a complex with a protein, possibly a protein tyrosine phosphatase, to activate p59Fyn. One possible candidate is the RPTPα that dephosphorylates Tyr-531 of p59Fyn (Bhandari et al., 1998) and is highly enriched in neurons and growth cones (Helmke et al., 1998). Remarkably, in RPTPα-deficient cells, both dephosphorylation of the COOH-terminal tyrosine residue and autophosphorylation of the tyrosine residue within the activation loop of pp60c-src is reduced (von Wichert et al., 2003), resembling the phenotype of NCAM-deficient mice. Furthermore, a close homologue of RPTPα, CD45, associates with the membrane-cytoskeleton linker protein spectrin (Lokeshwar and Bourguignon, 1992; Iida et al., 1994), a binding partner of NCAM (Leshchyns’ka et al., 2003). To investigate if NCAM interacts with RPTPα, we analyzed the distribution of both proteins in cultured hippocampal neurons. NCAM and RPTPα partially colocalized along neurites, and both proteins accumulated in growth cones where clusters of NCAM partially overlapped with accumulations of RPTPα (Fig. 2 A). To verify whether or not NCAM interacts with RPTPα, we induced clustering of NCAM at the cell surface of live hippocampal neurons by incubation with antibodies against NCAM. Clustering of NCAM enhanced overlap between NCAM and RPTPα localization (mean correlation between NCAM and RPTPα localization being 0.3 ± 0.05 and 0.6 ± 0.03 in neurons treated with nonspecific IgG or NCAM antibodies, respectively; Fig. 2 B), indicating that RPTPα partially redistributed to NCAM clusters and suggesting that NCAM and RPTPα form a complex. Because antibodies against RPTPα were directed against its intracellular domain, RPTPα contained in intracellular organelles could have been recognized as colocalizing with NCAM that associates with intracellular organelles of trans-Golgi network origin (Sytnyk et al., 2002). Thus, the redistribution of RPTPα to NCAM clusters may represent redistribution of intracellular carriers containing RPTPα. To analyze whether or not NCAM associates with RPTPα in the plasma membrane, we transfected neurons with RPTPα containing the HA tag in the extracellular domain and induced clustering of NCAM and HA-RPTPα with antibodies against NCAM and the HA tag. HA-RPTPα partially redistributed to NCAM clusters (Fig. 2 C), indicating that both proteins form a complex at the cell surface.

Finally, we examined the association between NCAM and RPTPα in the brain by coimmunoprecipitation. RPTPα communoprecipitated with NCAM from brain homogenates (Fig. 2 D), confirming that NCAM associates with RPTPα. Interestingly, we found that the level of RPTPα was approximately two times higher in the brain of NCAM-deficient mice when compared with wild-type mice (Fig. 2 D), indicating a functional relationship between NCAM and RPTPα.

NCAM140 is the most potent RPTPα-binding NCAM isoform

To identify the NCAM isoform interacting with RPTPα, we expressed NCAM120, NCAM140, and NCAM180 in CHO cells and analyzed their association with RPTPα by coimmu-

Figure 2. NCAM forms a complex with RPTPα. (A) High magnification image of a growth cone of a hippocampal neuron labeled with antibodies against NCAM and RPTPα. Note that clusters of NCAM overlap with accumulations of RPTPα. (B) Live hippocampal neurons were treated with nonspecific IgG or with antibodies against NCAM. Note that antibodies against NCAM induced clustering of NCAM at the cell surface. Labeling with antibodies against RPTPα showed that RPTPα partially redistributed to NCAM clusters (arrows). The corresponding profiles show NCAM and RPTPα labeling intensities along neurites. Note increased overlap of NCAM and RPTPα clusters in neurons treated with NCAM antibodies. (C) Hippocampal neurons transfected with wild-type RPTPα containing an HA tag extracellularly were incubated live with antibodies against the HA tag and NCAM. Cell surface RPTPα partially redistributed to NCAM clusters (arrows). Bars, 10 μm. (D) Brain homogenates of wild-type (NCAM+/−) and NCAM-deficient (NCAM−/−) mice (total brain) and NCAM immunoprecipitates (IP, NCAM) were probed with antibodies against RPTPα by Western blot. Labeling for GAPDH was included as loading control. RPTPα coimmunoprecipitates with NCAM. Note increased expression of RPTPα in NCAM-deficient brains. Histogram shows quantitation of the RPTPα level in wild type (+/+) and NCAM-deficient (−/−) brains. OD for wild type was set to 100%. Mean values ± SEM (n = 6) are shown. *, P < 0.05, paired t test.
Intracellular domain of NCAM140 directly interacts with the intracellular domain of RPTPα.

(A) Lysates and NCAM immunoprecipitates (IP: NCAM) from CHO cells transfected with NCAM120, NCAM140, NCAM180, or GFP were probed by Western blot with antibodies against NCAM and RPTPα. Note that NCAM isoforms were expressed in approximately equal amounts whereas RPTPα immunoprecipitated only with NCAM140 but not NCAM180 or NCAM120. Labeling for GAPDH was included as loading control. (B) Intracellular domains of NCAM140, NCAM180, or CHL1 were bound to Ni-NTA agarose beads. The gel was stained with Coomassie blue and shows that approximately equal amounts whereas RPTPα intracellular domains of NCAM140, NCAM180, or CHL1 were bound to plastic and assayed by ELISA for the ability to bind increasing concentrations of RPTPα in a concentration-dependent manner, with the intracellular domain of NCAM140 binding with a higher affinity than the intracellular domain of NCAM180 (Fig. 3 C). No binding with the intracellular domain of CHL1 was observed (Fig. 3 C). We conclude that NCAM binds directly to RPTPα via the intracellular domain, with NCAM140 being the most potent RPTPα-binding NCAM isoform.

RPTPα binds NCAM140 via the D2 domain and links NCAM140 to p59fyn

To identify the part of the intracellular domain of RPTPα responsible for the interaction with NCAM140, we coexpressed, in CHO cells, NCAM140 together with the wild-type form of RPTPα (wtRPTPα), RPTPα lacking the D2 domain (RPTPαΔD2), or catalytically inactive form of RPTPα containing a mutation within the D1 catalytic domain (RPTPαC433S) and analyzed binding of NCAM140 to these RPTPα mutants by coimmunoprecipitation. All transfected CHO cells endogenously express RPTPα that was detected with RPTPα antibodies as a band with a molecular mass identical to RPTPα detected in brain homogenates (unpublished data). Although transfected CHO cells expressed NCAM120, NCAM140, and NCAM180 in similar amounts, RPTPα coimmunoprecipitated only with NCAM140 (Fig. 3 A). However, after prolonged exposure of the film we could also detect RPTPα in NCAM180 immunoprecipitates (unpublished data). RPTPα did not coimmunoprecipitate with NCAM120. We conclude that RPTPα associates predominantly with NCAM140 and to a lesser extent with NCAM180.

Inability of NCAM120, the GPI-linked NCAM isoform without the intracellular domain, to bind RPTPα suggested that the intracellular domain of NCAM is involved in the formation of a complex between NCAM and RPTPα. Furthermore, the extracellular domain of NCAM (NCAM-Fc) did not bind to RPTPα in brain lysates, confirming that the extracellular domain of NCAM does not bind to RPTPα (unpublished data). To verify that the NCAM intracellular domain interacts directly with the intracellular domain of RPTPα, we analyzed binding of the recombinant intracellular domain of RPTPα to the intracellular domain of NCAM180 or NCAM140 in a pull-down assay. For comparison, the intracellular domain of CHL1, another adhesion molecule of the immunoglobulin superfamily, was used. The intracellular domain of RPTPα bound to the intracellular domain of NCAM180 or NCAM140 but not to the intracellular domain of CHL1 (Fig. 3 B). Interaction between the intracellular domains of RPTPα and NCAM140 was severalfold stronger than between the intracellular domains of RPTPα and NCAM180 (Fig. 3 B). To confirm this finding, we examined the direct interaction between the intracellular domains of RPTPα and NCAM180 or NCAM140 by ELISA. Intracellular domain of RPTPα bound to the intracellular domains of NCAM180 or NCAM140 in a concentration-dependent manner, with the intracellular domain of NCAM140 binding with a higher affinity than the intracellular domain of NCAM180 (Fig. 3 C).
RPTPα constructs contained the HA tag to distinguish them from endogenous RPTPα. As seen for endogenous RPTPα, transfected wtRPTPα coimmunoprecipitated with NCAM140 (Fig. 4 A). Similar amounts of RPTPαC433S coimmunoprecipitated with NCAM140, whereas RPTPαΔ2 did not coimmunoprecipitate (Fig. 4 A), indicating that the D2 domain is required for the interaction between RPTPα and NCAM140.

Remarkably, among the major NCAM isoforms, only NCAM140 forms a complex with p59fyn (Beggs et al., 1997) that we found to correlate with its ability to bind RPTPα (see the previous section). RPTPα directly interacts with p59fyn (Bhandari et al., 1998). Accordingly, p59fyn coimmunoprecipitated with wtRPTPα from transfected CHO cells (Fig. 4 A). Approximately the same amount of p59fyn coimmunoprecipitated with RPTPαΔ2 (Fig. 4 A), indicating that this truncated construct also binds p59fyn probably via the D1 domain. In accordance with previous reports, p59fyn showed reduced ability to bind RPTPαC433S, a catalytically inactive mutant of RPTPα (Fig. 4 A; Zheng et al., 2000).

To analyze the role of RPTPα in NCAM140–p59fyn complex formation, we coimmunoprecipitated p59fyn with NCAM140 in the presence of RPTPα mutants. In CHO cells cotransfected with NCAM140 and wtRPTPα, p59fyn coimmunoprecipitated with NCAM140 (Fig. 4 A). The amount of p59fyn coimmunoprecipitated with NCAM140 was reduced in cells cotransfected with RPTPαC433S (Fig. 4 A), correlating with the reduced ability of this catalytically inactive RPTPα mutant to bind p59fyn (see previous paragraph; Zheng et al., 2000). When NCAM140 was cotransfected with RPTPαΔ2, p59fyn no longer coimmunoprecipitated with NCAM140 (Fig. 4 A). Because RPTPαΔ2 binds p59fyn (Fig. 4 A), it is conceivable that this mutant, which does not bind NCAM140, competes with endogenous RPTPα for binding to p59fyn and thus inhibits NCAM140–p59fyn complex formation.

To extend this analysis to neurons, we transfected hippocampal neurons with GFP alone or cotransfected with GFP and RPTPαΔ2 or RPTPαC433S and analyzed the redistribution of p59fyn to NCAM clusters after cross-linking NCAM with NCAM antibodies (Fig. 4 B). In neurons transfected with RPTPαΔ2 or RPTPαC433S, the level of p59fyn in NCAM clusters was reduced by ~30% when compared with GFP only transfected cells, suggesting that RPTPαΔ2 or...
Figure 5. **NCAM–p59<sup>yn</sup> complex formation and NCAM-mediated p59<sup>yn</sup> activation are abolished in RPTPα-deficient neurons.** (A) p59<sup>yn</sup> immunoprecipitates from 4-d-old wild-type (RPTPα<sup>+/+</sup>) and RPTPα-deficient (RPTPα<sup>−/−</sup>) brain homogenates were probed by Western blot with antibodies against total p59<sup>yn</sup> protein, p59<sup>yn</sup> dephosphorylated at Tyr-531, or p59<sup>yn</sup> phosphorylated at Tyr-420. Levels of p59<sup>yn</sup> dephosphorylated at Tyr-531 and p59<sup>yn</sup> phosphorylated at Tyr-420 are reduced in RPTPα-deficient brains. Histograms show quantitation of the blots with OD for wild type set to 100%. Mean values ± SEM (n = 6) are shown. *, P < 0.05, paired t test. (B) NCAM immunoprecipitates (IP: NCAM) from wild-type (RPTPα<sup>+/+</sup>) and RPTPα-deficient (RPTPα<sup>−/−</sup>) brain homogenates were probed with antibodies against NCAM and p59fyn by Western blot. Note that p59fyn coimmunoprecipitates with NCAM in wild-type but not in RPTPα-deficient brains. (C) Wild-type and RPTPα-deficient hippocampal neurons were incubated live with NCAM antibodies to cluster NCAM. Cells were fixed and labeled with antibodies against p59<sup>yn</sup>. Note that redistribution of p59<sup>yn</sup> to NCAM clusters was reduced in RPTPα-deficient neurons. Bar, 10 μm. The corresponding profiles show NCAM and p59<sup>yn</sup> labeling intensities along neurites. The histogram shows mean labeling intensity of p59<sup>yn</sup> in NCAM clusters. Mean values ± SEM (n = 20 neurons) are shown in arbitrary units (AU). *, P < 0.05, t test. (D) Wild-type and RPTPα-deficient hippocampal neurons were incubated live with nonspecific IgG or NCAM antibodies, and fixed and labeled with antibodies against p59<sup>yn</sup> dephosphorylated at Tyr-531. Immunofluorescence signals were inverted to accentuate the difference in immunolabeling intensities between groups. Bar, 10 μm. Note that application of NCAM antibodies increased levels of p59<sup>yn</sup> dephosphorylated at Tyr-531 in wild type, but not in RPTPα-deficient neurons. The histogram shows mean labeling intensity of p59<sup>yn</sup> dephosphorylated at Tyr-531 along neurites. Mean values ± SEM (n > 20 neurons) are shown in arbitrary units (AU). *, P < 0.05, t test.
RPTPαC433S inhibit NCAM–p59Fyn complex formation by competing with endogenous RPTPα. The combined observations indicate that NCAM140–p59Fyn complex formation correlates with the ability of NCAM140-associated RPTPα to bind to p59Fyn, implicating RPTPα as a linker between NCAM140 and p59Fyn.

**Association between NCAM and p59Fyn and NCAM-mediated p59Fyn activation are abolished in RPTPα-deficient neurons**

To substantiate further our finding that RPTPα is a linker protein between NCAM and p59Fyn, we analyzed p59Fyn activation and association of p59Fyn with NCAM in RPTPα-deficient brains. As for NCAM-deficient brains, levels of p59Fyn dephosphorylated at Tyr-531 and levels of p59Fyn phosphorylated at Tyr-420 were reduced in brain homogenates of RPTPα-deficient mice (Fig. 5 A), further suggesting that RPTPα plays a role in NCAM-mediated p59Fyn activation in the brain. To analyze the role of RPTPα in the formation of the complex between NCAM and p59Fyn, we immunoprecipitated NCAM from wild-type and RPTPα-deficient brains and probed immunoprecipitates with antibodies against p59Fyn. Whereas p59Fyn coimmunoprecipitated with NCAM from wild-type brains, p59Fyn did not coimmunoprecipitate with NCAM from RPTPα-deficient brains (Fig. 5 B). Furthermore, when NCAM was clustered at the surface of wild-type and RPTPα-deficient cultured hippocampal neurons, levels of p59Fyn were significantly reduced in NCAM clusters in RPTPα-deficient neurons when compared with wild-type cells (Fig. 5 C), indicating that RPTPα is required for complex formation between NCAM and p59Fyn.

NCAM clustering at the cell surface induces rapid p59Fyn activation (Beggs et al., 1997). To analyze whether or not RPTPα is required for NCAM-induced p59Fyn activation, we treated live hippocampal neurons from wild-type and RPTPα-deficient mice with NCAM antibodies and analyzed levels of p59Fyn dephosphorylated at Tyr-531 along neurites of the stimulated neurons. Clustering of NCAM increased levels of Tyr-531–dephosphorylated p59Fyn along neurites of wild-type neu-
rons by ~60% (Fig. 5 D). However, NCAM-mediated p59^{tyrosine} activation was completely abolished in RPTPα-deficient neurons (Fig. 5 D), demonstrating that RPTPα is required for NCAM-mediated p59^{tyrosine} activation.

Formation of the complex between RPTPα and NCAM is enhanced by Ca^{2+}

Coimmunoprecipitation experiments were performed either in the presence of Ca^{2+} or with 2 mM EDTA, a Ca^{2+}-sequestering agent. Whereas RPTPα coimmunoprecipitated with NCAM from brain homogenates under both conditions, coimmunoprecipitated complexes were reduced by ~60% in the presence of EDTA (Fig. 6 A), suggesting that Ca^{2+} promotes formation of the NCAM–RPTPα complex. These results are in accordance with findings of Zeng et al. (1999), who found that NCAM and RPTPα did not coimmunoprecipitate in the presence of EDTA. To analyze if the direct interaction between NCAM and RPTPα is Ca^{2+} dependent, we assayed binding of the intracellular domain of NCAM140 to the intracellular domain of RPTPα by ELISA in the presence or absence of Ca^{2+} (Fig. 6 B), showing that the direct interaction is Ca^{2+}-independent and suggesting that additional binding partners of NCAM and/or RPTPα may enhance complex formation in a Ca^{2+}-dependent manner. Spectrin, which directly interacts with the intracellular domain of NCAM (Leshchyns’ka et al., 2003) and contains a Ca^{2+} binding domain (De Matteis and Morrow, 2000), is one of the possible candidates. Indeed, RPTPα coimmunoprecipitated with spectrin from brain homogenates (Fig. 6 C). In the presence of 2 mM EDTA, RPTPα coimmunoprecipitating with spectrin was reduced by ~80% (Fig. 6 C), whereas coimmunoprecipitation of NCAM with spectrin did not depend on Ca^{2+} (Fig. 6 C). We conclude that RPTPα directly interacts with NCAM in a Ca^{2+}-independent manner. However, formation of the complex is enhanced by Ca^{2+}-dependent cross-linking of NCAM140 and RPTPα via spectrin.
The NCAM-RPTPα complex redistributes to lipid rafts after NCAM activation

Whereas p59<sup>yn</sup> is mainly associated with lipid rafts (van’t Hof and Resh, 1997; Niethammer et al., 2002; Filipp et al., 2003), only 4–8% of all RPTPα molecules were found in lipid rafts of brain (unpublished data). In hippocampal neurons extracted with cold 1% Triton X-100 to isolate lipid rafts (Niethammer et al., 2002; Leshchyns’ka et al., 2003), detergent-insoluble clusters of RPTPα only partially overlapped with the lipid raft marker ganglioside GM1 (Fig. 7 A), further confirming that RPTPα and p59<sup>yn</sup> are segregated at the subcellular level. Because activation of NCAM results in its redistribution to lipid rafts (Leshchyns’ka et al., 2003), it may also promote redistribution of NCAM-associated RPTPα to lipid rafts and thus activate raft-associated p59<sup>yn</sup>. To verify this hypothesis, we studied association of NCAM and RPTPα with lipid rafts in hippocampal neurons activated or not activated with NCAM-Fc or NCAM antibodies. In accordance with previous results (Leshchyns’ka et al., 2003), application of NCAM-Fc or NCAM antibodies increased GM1 levels in detergent-insoluble clusters of NCAM, indicating that NCAM redistributed to lipid rafts (Fig. 7, A–C). Application of NCAM-Fc or NCAM antibodies also increased the level of RPTPα in NCAM clusters, indicating that NCAM activation promoted NCAM–RPTPα complex formation (Fig. 7, A–C).

Furthermore, NCAM activation also increased GM1 levels in detergent-insoluble clusters of RPTPα (Fig. 7 D), confirming that NCAM-associated RPTPα also redistributed to lipid rafts and suggesting that NCAM recruits RPTPα to lipid rafts. To further analyze this possibility, we compared levels of RPTPα in lipid rafts in brains of wild-type and NCAM-deficient mice. Indeed, RPTPα was reduced by ~60% in lipid rafts isolated from NCAM-deficient brains (Fig. 7 E), confirming that NCAM plays a role in RPTPα targeting to lipid rafts. The levels of p59<sup>yn</sup> were increased in NCAM-deficient lipid rafts (100% and 124 ± 7.6% in wild-type and NCAM deficient rafts, respectively) probably reflecting increased levels of p59<sup>yn</sup> in NCAM-deficient brains. Levels of GM1 were not different in lipid rafts from wild-type and NCAM-deficient brains (100% and 103.3 ± 6.8% in wild-type and NCAM-deficient rafts, respectively), showing that lipid rafts were isolated with the same efficacy from wild-type and NCAM-deficient brains (Fig. 7 E).

NCAM-mediated recruitment of RPTPα to lipid rafts is enhanced by NCAM-induced FGF receptor (FGFR)-dependent increase in intracellular Ca<sup>2+</sup>.

NCAM activation increases intracellular Ca<sup>2+</sup> concentrations via a FGFR-dependent mechanism (Walsh and Doherty, 1997; Kamiguchi and Lemmon, 2000; Juliano, 2002). This increase in intracellular Ca<sup>2+</sup> may account for the enhanced association between NCAM and RPTPα after NCAM activation (Fig. 7, A–D) because of spectrin-mediated cross-linking of NCAM140 and RPTPα (Fig. 6). Interestingly, NCAM activation also induces redistribution of NCAM-associated spectrin to lipid rafts (Leshchyns’ka et al., 2003). To analyze the role of FGFR and Ca<sup>2+</sup> in the recruitment of RPTPα to an NCAM complex, we estimated levels of RPTPα associated with NCAM following NCAM activation in control neurons and neurons incubated with BAPTA-AM, a membrane-permeable Ca<sup>2+</sup> chelator (Williams et al., 1992; Cavallaro et al., 2003).

Figure 8. NCAM-mediated neurite outgrowth depends on RPTPα activation. (A) Hippocampal neurons were incubated with NCAM-Fc alone or with NCAM-Fc together with vanadate, and lengths of the longest neurites were measured. NCAM-Fc increased neurite length when compared with control neurons. Vanadate decreased neurite outgrowth in the NCAM-Fc-stimulated group to the control group level but did not affect basal neurite outgrowth over poly-L-lysine. (B) Hippocampal neurons transfected with GFP alone or cotransfected with GFP and RPTPαΔD2 or RPTPαC433S were incubated with NCAM-Fc after transfection and lengths of the longest neurites were measured. NCAM-Fc increased neurite lengths in GFP-transfected neurons. NCAM-Fc-stimulated neurite outgrowth was blocked in the group cotransfected with RPTPαC433S or RPTPαΔD2. (C) Lengths of the longest neurites were measured in wild-type (+/+) and RPTPα-deficient (−/-) neurons not treated or treated with NCAM-Fc. NCAM-Fc increased neurite length in wild-type but not in RPTPα-deficient neurons. For A–C, mean values ± SEM are shown (n > 150 neurons; * P < 0.05, t test). Experiments were performed two times with the same effect.
2001), or a specific FGFR inhibitor (Niethammer et al., 2002; Leshchyns’ka et al., 2003). Whereas NCAM activation increased levels of RPTPα and GM1 in NCAM clusters (Fig. 7 F), treatment with BAPTA-AM or FGFR inhibitor abolished recruitment of RPTPα to NCAM clusters in response to NCAM activation (Fig. 7 F). In accordance with previous findings (Leshchyns’ka et al., 2003), NCAM redistribution to lipid rafts was not affected by the FGFR inhibitor or BAPTA-AM (Fig. 7 F). BAPTA-AM or FGFR inhibitor did not affect the level of RPTPα associated with NCAM under nonactivated conditions (Fig. 7 F). We conclude that, whereas at resting conditions Ca2+ does not play a major role in the interaction between NCAM and RPTPα, NCAM-induced FGFR-dependent elevations of intracellular Ca2+ levels strengthen the interactions between NCAM and RPTPα in response to NCAM activation, most likely via spectrin (see the section Formation of the complex between RPTPα and NCAM is enhanced by Ca2+).

**NCAM-induced neurite outgrowth depends on NCAM association with RPTPα**

NCAM-induced neurite outgrowth depends on p59fn activation (Kolkova et al., 2000), suggesting that NCAM association with RPTPα may be involved. To analyze the role of protein tyrosine phosphatases in NCAM-induced neurite outgrowth, we incubated cultured hippocampal neurons with 100 μM vanadate, an inhibitor of these phosphatases (Helmke et al., 1998). NCAM-Fc–enhanced neurite outgrowth was abolished by vanadate, indicating that activation of protein tyrosine phosphatases is required for NCAM-mediated neurite outgrowth. Vanadate did not affect neurite outgrowth in nonstimulated neurons, indicating that vanadate does not lead to nonspecific impairments (Fig. 8 A). To directly assess the role of RPTPα in NCAM-induced neurite outgrowth, we transfected hippocampal neurons with the dominant-negative mutants of RPTPα. Both, RPTPαD2, which does not bind NCAM but associates with p59fn, and catalytically inactive RPTPαC433S, which associates with NCAM but binds p59fn with a lower efficiency than endogenous RPTPα, inhibited association of NCAM with p59fn by competing with endogenous RPTPα (Fig. 4). In neurons transfected with GFP only, stimulation with NCAM-Fc significantly enhanced neurite length when compared with control nonstimulated neurons (Fig. 8 B). However, neurons transfected with RPTPαD2 or RPTPαC433S remained unresponsive to NCAM-Fc stimulation (Fig. 8 B), indicating that RPTPα plays a major role in NCAM-induced neurite outgrowth. To confirm this finding, we analyzed NCAM-mediated neurite outgrowth in hippocampal neurons from RPTPα-deficient mice. Whereas NCAM-Fc–enhanced neurite outgrowth in neurons from RPTPα wild-type littermates by ~100%, NCAM-Fc–induced neurite outgrowth was completely abolished in RPTPα-deficient neurons (Fig. 8 C), further confirming that RPTPα is required for NCAM-mediated neurite outgrowth.
Discussion

It is by now well established that in response to homophilic or heterophilic binding cell adhesion molecules of the immunoglobulin superfamily, such as NCAM, L1, or CHL1, activate Src family tyrosine kinases, and in particular p69fyn or p59shc, resulting in morphogenetic events, such as cell migration and neurite outgrowth. However, the mechanisms of Src family tyrosine kinase activation in these paradigms have remained unresolved. Here, we identify a cognate activator of p59fyn, the receptor protein tyrosine phosphatase RPTPα, as a novel binding partner of NCAM. Activation of p59fyn is reduced in NCAM-deficient mice and interaction between NCAM and p59fyn is abolished in RPTPα-deficient brains. Interestingly, we found that the levels of p59fyn and RPTPα are increased in NCAM-deficient brains, possibly reflecting a compensatory reaction to the decreased activity of these enzymes in the mutant and further indicating a tight functional relationship between NCAM, RPTPα, and p59fyn. NCAM-induced neurite outgrowth is completely abrogated in RPTPα-deficient neurons or in neurons transfected with dominant-negative RPTPα mutants, indicating that RPTPα links NCAM to p59fyn both physically and functionally.

Role of Ca²⁺ in NCAM–RPTPα–p59fyn complex formation

Interactions between NCAM and RPTPα and NCAM–RPTPα–p59fyn complex formation leading to neurite outgrowth are tightly regulated (Fig. 9). First, whereas direct interaction between NCAM and RPTPα is Ca²⁺ independent, NCAM–RPTPα complex formation is enhanced by Ca²⁺-dependent cross-linking via spectrin. Remarkably, NCAM activation results in an increase in intracellular Ca²⁺ concentration via influx through Ca²⁺ channels or release from intracellular stores, and may thus provide a positive feedback loop between NCAM activation and NCAM–RPTPα complex formation involving spectrin. RPTPα binding to spectrin may also elevate RPTPα enzymatic dephosphorylation activity (Lokeshwar and Bourguignon, 1992). Interestingly, NCAM activation also induces activation of PKC (Kolkova et al., 2000; Leshchyns’ka et al., 2003), which is known to phosphorylate RPTPα and stimulate its activity (den Hertog et al., 1995; Tracy et al., 1995; Zheng et al., 2002). Thus, a network of activated intracellular signaling molecules may underlie the induction and maintenance of NCAM-mediated neurite outgrowth. It is interesting in this respect that the NCAM140 isoform predominates in these interactions: it interacts more efficiently with p59fyn via RPTPα and enhances neurite outgrowth more vigorously than NCAM180 (Niethammer et al., 2002). The structural dispositions of NCAM140 for this preference will remain to be established.

The role of lipid rafts

Additional regulation of RPTPα-mediated p59fyn activation is achieved by segregation of RPTPα and p59fyn to different subdomains in the plasma membrane (Fig. 9). Approximately 90% of all RPTPα molecules in the brain are located in a lipid raft-free environment and are thereby segregated from lipid raft-associated p59fyn under nonstimulated conditions. Whereas segregation of receptor protein tyrosine phosphatases from their potential substrates due to targeting to different plasma membrane domains has been suggested as a general mechanism of the regulation of receptor protein tyrosine phosphatase function (Petrone and Sap, 2000), the mechanisms that target receptor protein tyrosine phosphatases to lipid rafts have remained unclear. We show that levels of raft-associated RPTPα in the NCAM-deficient brain are reduced, and NCAM redistribution to lipid rafts in response to NCAM activation also induces redistribution of RPTPα to lipid rafts via its NCAM association. The combined observations indicate that NCAM plays a role in recruiting NCAM-associated RPTPα to lipid rafts via NCAM palmitoylation (Niethammer et al., 2002; Fig. 9) or via NCAM interaction with GPI-anchored components of lipid rafts, such as the GPI-linked GDNF receptor (Paratcha et al., 2003). Investigations on the regulatory mechanisms underlying palmitoylation will be required to understand the subcellular compartment-specific distribution of the NCAM–RPTPα–p59fyn complex. Furthermore, the localization of adhesion molecules and receptors will have to be elucidated in view of lipid rafts heterogeneities.

Potential role of protein tyrosine phosphatases in signaling mediated by other cell adhesion molecules

Besides NCAM, activation of L1 and CHL1, other cell adhesion molecules of the immunoglobulin superfamily, also results in the activation of Src family tyrosine kinases (Schmid et al., 2000; Buhusi et al., 2003). As for NCAM, the intracellular domains of L1 and CHL1 do not possess structural motif for protein tyrosine phosphatase activity, suggesting that yet unidentified protein tyrosine phosphatases may be involved. Identification of protein tyrosine phosphatases associated with other cell adhesion molecules of the immunoglobulin superfamily and conjunctions with RPTPα-activated integrins (Zeng et al., 2003) will be an important next step in the elucidation of the mechanisms that cell adhesion molecules use differentially to guide cell migration and neurite outgrowth in the developing nervous system.

Materials and methods

Antibodies and toxins

Rabbit polyclonal antibodies against NCAM (Niethammer et al., 2002) were used in immunoprecipitation, immunoblotting, and immunocytochemical experiments; and rat mAbs H28 against mouse NCAM (Gennarini et al., 1984) were used in immunocytochemical experiments. Both antibodies recognize the extracellular domain of all NCAM isoforms. Hybridoma clone H28 was a gift of C. Goridis (Centre National de la Recherche Scientifique UMR 8542, Paris, France). Rabbit antibodies against RPTPα were a gift of C.J. Pallen (University of British Columbia, Vancouver, Canada) or were generated as described previously (den Hertog et al., 1994). Rabbit polyclonal antibodies against human erythrocyte spectrin, rabbit polyclonal antibodies against the HA tag, nonspecific rabbit immunoglobulins, and cholera toxin B subunit tagged with fluorescein to label GM1 were obtained from Sigma-Aldrich. Mouse mAbs against the HA tag (clone 12CA5) were obtained from Roche Diagnostics. Rabbit polyclonal antibodies and mouse mAbs against p59fyn protein were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies against Tyr-527–Dephosphorylated or
The plasmid encoding the intracellular domain of RPTP alpha and membrane-distal tyrosine-phosphatase domains of various RPTPs. 

Detergent extraction of cultured neurons

Colocalization analysis

Colocalization quantification was performed as described previously (den Hertog and Hunter, 1996). Within the outlined areas the mean intensities of NCAM, RPTP alpha and membrane-distal tyrosine-phosphatase domains of various RPTPs. 

Data analysis

We thank Achim Dallmann and Eva Kronberg for genotyping and animal care, Dr. Patricia Maness and Elisabeth Bock for NCAM cDNAs, Dr. Cathie Iida, N. B. Lokeshwar, and L. Y. Bourguignon. 1994. Mapping the fodrin binding domain of N-CAM to mouse chromosome 3. EMBO J. 13:3016–3027.

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Animals

To compare wild-type and NCAM-deficient mice, C57Bl/6J mice and NCAM-deficient mice (Cremer et al., 1994) inbred for at least nine generations onto the C57Bl/6J background were used. NCAM-deficient mice were a gift of H. Cremer (Developmental Biology Institute of Marseille, Marseille, France). To compare wild-type and RPTPalpha-deficient mice, RPTPalpha-negative and negative littermates obtained from heterozygous breeding were used (see online supplemental material).

Image acquisition and manipulation

Coverslips were embedded in Aqua-Poly/Mount (Polysciences, Inc.). Images were acquired at RT using a confocal laser scanning microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.), LSM510 software (version 3; Carl Zeiss MicroImaging, Inc.), and oil Plan-Neofluar 40× objective (NA 1.3; Carl Zeiss MicroImaging, Inc.) at 3× digital zoom. Contrast and brightness of the images were further adjusted in Photo-Paint 9 (Corel Corporation).

Online supplemental material

Details on cultures and transfection of hippocampal neurons and CHO cells, immunofluorescence labeling, ELISA and pull-down assay, communoprecipitation, isolation of lipid enriched microdomains, gel electrophoresis, immunoblotting, and generation of RPTPalpha-deficient mice are given in online supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200404037/DC1.

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