

# RPTP $\alpha$ is essential for NCAM-mediated p59<sup>fyn</sup> activation and neurite elongation

Vsevolod Bodrikov,<sup>1</sup> Iryna Leshchyns'ka,<sup>1</sup> Vladimir Sytnyk,<sup>1</sup> John Overvoorde,<sup>2</sup> Jeroen den Hertog,<sup>2</sup> and Melitta Schachner<sup>1</sup>

<sup>1</sup>Zentrum für Molekulare Neurobiologie, Universität Hamburg, 20246 Hamburg, Germany

<sup>2</sup>Hubrecht Laboratory, Netherlands Institute for Developmental Biology, 3584 CT Utrecht, Netherlands

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 RPTP $\alpha$ , 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 RPTP $\alpha$  in response to NCAM activation and is ac-

companied by redistribution of the complex to lipid rafts. Association between NCAM and p59<sup>fyn</sup> is lost in RPTP $\alpha$ -deficient brains and is disrupted by dominant-negative RPTP $\alpha$  mutants, demonstrating that RPTP $\alpha$  is a link between NCAM and p59<sup>fyn</sup>. NCAM-mediated p59<sup>fyn</sup> activation is abolished in RPTP $\alpha$ -deficient neurons, and disruption of the NCAM-p59<sup>fyn</sup> complex in RPTP $\alpha$ -deficient neurons or with dominant-negative RPTP $\alpha$  mutants blocks NCAM-dependent neurite outgrowth, implicating RPTP $\alpha$  as a major phosphatase involved in NCAM-mediated signaling.

## Introduction

The neural cell adhesion molecule (NCAM) is involved in several morphogenetic events, such as neuronal migration and differentiation, neurite outgrowth, and axon fasciculation. NCAM-induced morphogenetic effects depend on activation of Src family tyrosine kinases and, in particular, p59<sup>fyn</sup> kinase (Schmid et al., 1999). NCAM-dependent neurite outgrowth is impaired in neurons from p59<sup>fyn</sup>-deficient mice (Beggs et al., 1994) and is abolished by inhibitors of Src kinase family members (Crossin and Krushel, 2000; Kolkova et al., 2000; Cavallaro et al., 2001). The NCAM140 isoform has been observed in a complex with p59<sup>fyn</sup>, whereas p59<sup>fyn</sup> does not associate significantly with NCAM180 or glycosylphosphatidylinositol-linked NCAM120 (Beggs et al., 1997). However in oligodendrocytes, p59<sup>fyn</sup> is also associated with NCAM120 in isolated lipid rafts (Kramer et al., 1999), whereas in tumor cells NCAM is also associated with pp60<sup>c-src</sup> (Cavallaro et al., 2001), suggesting that additional molecular mechanisms may define NCAM's specificity of interactions with Src kinase family members. Several lines of evidence suggest that NCAM's association with lipid rafts is critical for p59<sup>fyn</sup> activation. NCAM not only

colocalizes with p59<sup>fyn</sup> in lipid rafts (He and Meiri, 2002) but disruption of NCAM140 association with lipid rafts either by mutation of NCAM140 palmitoylation sites or by lipid raft destruction attenuates activation of the p59<sup>fyn</sup> kinase pathway, completely blocking neurite outgrowth (Niethammer et al., 2002). However, in spite of compelling evidence that NCAM can activate Src family tyrosine kinases, the mechanism of this activation has remained unclear.

The activity of Src family tyrosine kinases is regulated by phosphorylation (Brown and Cooper, 1996; Thomas and Brugge, 1997; Bhandari et al., 1998; Hubbard, 1999; Petrone and Sap, 2000). The two best-characterized tyrosine phosphorylation sites in Src family tyrosine kinases perform opposing regulatory functions. The site within the enzyme's activation loop (Tyr-420 in p59<sup>fyn</sup>) undergoes autophosphorylation, which is crucial for achieving full kinase activity. In contrast, phosphorylation of the COOH-terminal site (Tyr-531 in p59<sup>fyn</sup>) inhibits kinase activity through intramolecular interaction between phosphorylated Tyr-531 and the SH2 domain in p59<sup>fyn</sup>, which stabilizes a noncatalytic conformation.

A well known activator of Src family tyrosine kinases is the receptor protein tyrosine phosphatase RPTP $\alpha$  (Zheng et al., 1992, 2000; den Hertog et al., 1993; Su et al., 1996; Ponniah et al., 1999). It contains two cytoplasmic catalytic domains, D1 and D2, of which only D1 is significantly active in vitro and in vivo (Wang and Pallen, 1991; den Hertog et al., 1993; Wu et

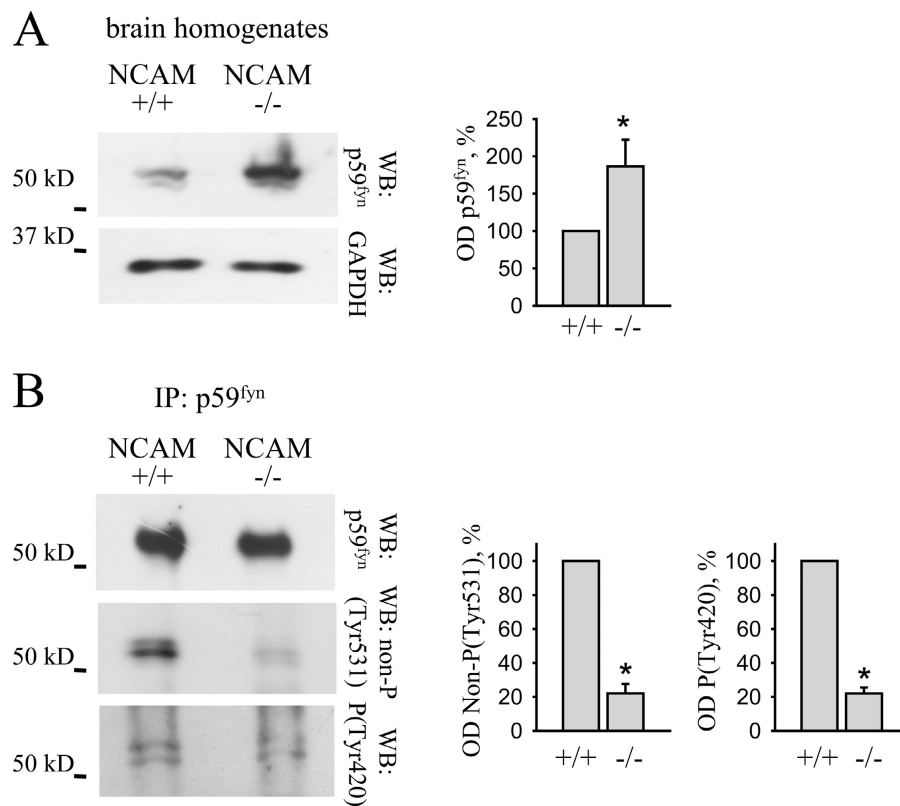
V. Bodrikov, I. Leshchyns'ka, and V. Sytnyk contributed equally to this paper.

The online version of this article includes supplemental material.

Correspondence to Melitta Schachner: melitta.schachner@zmnh.uni-hamburg.de

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

**Figure 1. Activated p59<sup>fyn</sup> is reduced in NCAM-deficient brain.** (A) Brain homogenates from 0- to 4-d-old wild-type (NCAM<sup>+/+</sup>) and NCAM-deficient (NCAM<sup>-/-</sup>) mice were probed by Western blot with antibodies against total p59<sup>fyn</sup> protein. Labeling for GAPDH was included as loading control. Levels of p59<sup>fyn</sup> protein are increased in NCAM-deficient brains. (B) p59<sup>fyn</sup> immunoprecipitates from 0- to 4-d-old wild-type (NCAM<sup>+/+</sup>) and NCAM-deficient (NCAM<sup>-/-</sup>) mice were probed by Western blot with antibodies against total p59<sup>fyn</sup> protein, p59<sup>fyn</sup> dephosphorylated at Tyr-531, or p59<sup>fyn</sup> phosphorylated at Tyr-420. Activated p59<sup>fyn</sup> is reduced 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 RPTP $\alpha$  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<sup>fyn</sup>) and the D1 domain of RPTP $\alpha$  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 RPTP $\alpha$ , and kinase activity of pp60<sup>c-src</sup> and p59<sup>fyn</sup> in RPTP $\alpha$ -deficient mice is reduced (Ponniah et al., 1999). Like p59<sup>fyn</sup> and NCAM, RPTP $\alpha$  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).

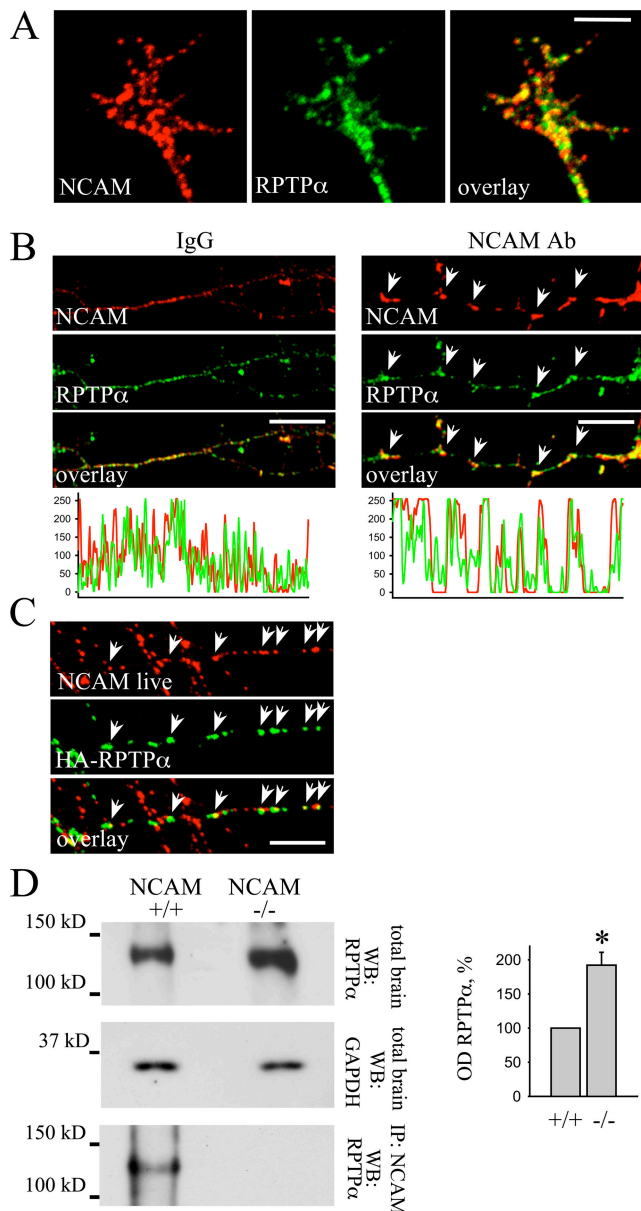
Remarkably, a close homologue of RPTP $\alpha$ , CD45, associates with the membrane-cytoskeleton linker protein spectrin (Lokeshwar and Bourguignon, 1992; Iida et al., 1994), a binding partner of NCAM (Leshchyn'ska et al., 2003). Following this lead, we investigated the possibility that RPTP $\alpha$  is involved in NCAM-induced p59<sup>fyn</sup> activation. We show that the intracellular domains of NCAM140 and RPTP $\alpha$  interact directly and that this interaction is enhanced by spectrin-mediated Ca<sup>2+</sup>-dependent cross-linking of NCAM and RPTP $\alpha$ . Levels of p59<sup>fyn</sup> associated with NCAM correlate with the ability of NCAM-associated RPTP $\alpha$  to bind to p59<sup>fyn</sup>, and the NCAM-p59<sup>fyn</sup> complex is disrupted in RPTP $\alpha$ -deficient brains

implicating RPTP $\alpha$  as linker molecule between NCAM and p59<sup>fyn</sup>. RPTP $\alpha$  redistributes to lipid rafts in response to NCAM activation and RPTP $\alpha$  levels are reduced in lipid rafts from NCAM-deficient mice, suggesting that NCAM recruits RPTP $\alpha$  to lipid rafts to activate p59<sup>fyn</sup>. Finally, NCAM-mediated p59<sup>fyn</sup> activation is abolished in RPTP $\alpha$ -deficient neurons and NCAM-induced neurite outgrowth is blocked in RPTP $\alpha$ -deficient neurons or neurons transfected with dominant-negative RPTP $\alpha$  mutants, demonstrating that RPTP $\alpha$  is a major phosphatase involved in NCAM-mediated signaling.

## Results

### Activation of p59<sup>fyn</sup> is impaired in NCAM-deficient brains

Cross-linking of NCAM at the cell surface results in a rapid activation of p59<sup>fyn</sup> 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<sup>fyn</sup>, we compared levels of activated p59<sup>fyn</sup> 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<sup>fyn</sup> protein was higher in brain homogenates of NCAM-deficient mice (Fig. 1 A), labeling with antibodies recognizing only p59<sup>fyn</sup> dephosphorylated at Tyr-531 or with antibodies recognizing only p59<sup>fyn</sup> phosphorylated at Tyr-420 was reduced in brain homogenates of NCAM-deficient mice (Fig. 1 B), indicating that activation of p59<sup>fyn</sup> is inhibited in NCAM-deficient brains and suggesting that NCAM is involved in the regulation of p59<sup>fyn</sup> function.



**Figure 2. NCAM forms a complex with RPTP $\alpha$ .** (A) High magnification image of a growth cone of a hippocampal neuron labeled with antibodies against NCAM and RPTP $\alpha$ . Note that clusters of NCAM overlap with accumulations of RPTP $\alpha$ . (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 RPTP $\alpha$  showed that RPTP $\alpha$  partially redistributed to NCAM clusters (arrows). The corresponding profiles show NCAM and RPTP $\alpha$  labeling intensities along neurites. Note increased overlap of NCAM and RPTP $\alpha$  clusters in neurons treated with NCAM antibodies. (C) Hippocampal neurons transfected with wild-type RPTP $\alpha$  containing an HA tag extracellularly were incubated live with antibodies against the HA tag and NCAM. Cell surface RPTP $\alpha$  partially redistributed to NCAM clusters (arrows). Bars, 10  $\mu$ m. (D) Brain homogenates of wild-type (NCAM $^{+/+}$ ) and NCAM-deficient (NCAM $^{-/-}$ ) mice (total brain) and NCAM immunoprecipitates (IP: NCAM) were probed with antibodies against RPTP $\alpha$  by Western blot. Labeling for GAPDH was included as loading control. RPTP $\alpha$  coimmunoprecipitates with NCAM. Note increased expression of RPTP $\alpha$  in NCAM-deficient brains. Histogram shows quantitation of the RPTP $\alpha$  level in wild type ( $+/+$ ) and NCAM-deficient ( $-/-$ ) brains. OD for wild type was set to 100%. Mean values  $\pm$  SEM ( $n = 6$ ) are shown. \*,  $P < 0.05$ , paired  $t$  test.

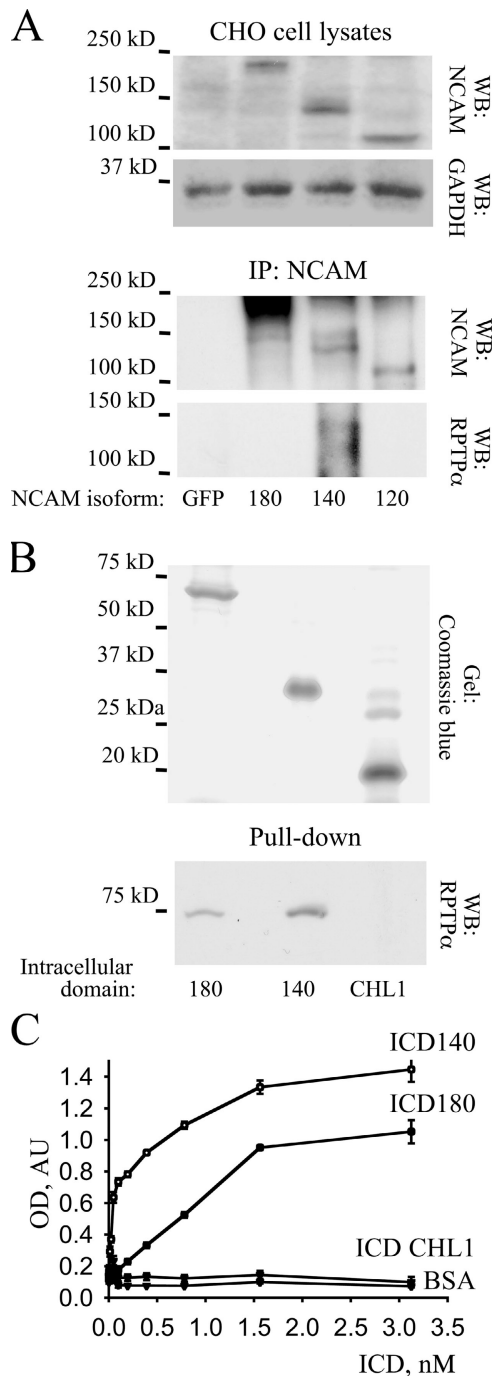
### NCAM forms a complex with RPTP $\alpha$

The intracellular domain of NCAM does not contain sequences known to induce p59<sup>fyn</sup> activation. Thus, NCAM may form a complex with a protein, possibly a protein tyrosine phosphatase, to activate p59<sup>fyn</sup>. One possible candidate is the RPTP $\alpha$  that dephosphorylates Tyr-531 of p59<sup>fyn</sup> (Bhandari et al., 1998) and is highly enriched in neurons and growth cones (Helmke et al., 1998). Remarkably, in RPTP $\alpha$ -deficient cells, both dephosphorylation of the COOH-terminal tyrosine residue and autophosphorylation of the tyrosine residue within the activation loop of pp60<sup>c-src</sup> is reduced (von Wichert et al., 2003), resembling the phenotype of NCAM-deficient mice. Furthermore, a close homologue of RPTP $\alpha$ , CD45, associates with the membrane-cytoskeleton linker protein spectrin (Lokeshwar and Bourguignon, 1992; Iida et al., 1994), a binding partner of NCAM (Leshchyn'ska et al., 2003). To investigate if NCAM interacts with RPTP $\alpha$ , we analyzed the distribution of both proteins in cultured hippocampal neurons. NCAM and RPTP $\alpha$  partially colocalized along neurites, and both proteins accumulated in growth cones where clusters of NCAM partially overlapped with accumulations of RPTP $\alpha$  (Fig. 2 A). To verify whether or not NCAM interacts with RPTP $\alpha$ , 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 RPTP $\alpha$  localization (mean correlation between NCAM and RPTP $\alpha$  localization being  $0.3 \pm 0.05$  and  $0.6 \pm 0.03$  in neurons treated with nonspecific IgG or NCAM antibodies, respectively; Fig. 2 B), indicating that RPTP $\alpha$  partially redistributed to NCAM clusters and suggesting that NCAM and RPTP $\alpha$  form a complex. Because antibodies against RPTP $\alpha$  were directed against its intracellular domain, RPTP $\alpha$  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 RPTP $\alpha$  to NCAM clusters may represent redistribution of intracellular carriers containing RPTP $\alpha$ . To analyze whether or not NCAM associates with RPTP $\alpha$  in the plasma membrane, we transfected neurons with RPTP $\alpha$  containing the HA tag in the extracellular domain and induced clustering of NCAM and HA-RPTP $\alpha$  with antibodies against NCAM and the HA tag. HA-RPTP $\alpha$  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 RPTP $\alpha$  in the brain by coimmunoprecipitation. RPTP $\alpha$  coimmunoprecipitated with NCAM from brain homogenates (Fig. 2 D), confirming that NCAM associates with RPTP $\alpha$ . Interestingly, we found that the level of RPTP $\alpha$  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 RPTP $\alpha$ .

### NCAM140 is the most potent RPTP $\alpha$ -binding NCAM isoform

To identify the NCAM isoform interacting with RPTP $\alpha$ , we expressed NCAM120, NCAM140, and NCAM180 in CHO cells and analyzed their association with RPTP $\alpha$  by coimmunoprecipitation.



**Figure 3. Intracellular domain of NCAM140 directly interacts with the intracellular domain of RPTP $\alpha$ .** (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 RPTP $\alpha$ . Note that NCAM isoforms were expressed in approximately equal amounts whereas RPTP $\alpha$  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 of the intracellular domains of NCAM140, NCAM180, or CHL1 were bound to beads. Beads were incubated with equal concentrations of intracellular domains of RPTP $\alpha$  and the extent of RPTP $\alpha$  intracellular domain binding was determined by Western blotting using polyclonal antibodies against RPTP $\alpha$ . Intracellular domains of RPTP $\alpha$  interacted with intracellular domains of NCAM140, and to a lesser extent NCAM180, but not with intracellular domains of CHL1. (C) Intracellular domains of NCAM140, NCAM180, or CHL1 were bound to plastic and assayed by

nonprecipitation. CHO cells endogenously express RPTP $\alpha$  that was detected with RPTP $\alpha$  antibodies as a band with a molecular mass identical to RPTP $\alpha$  detected in brain homogenates (unpublished data). Although transfected CHO cells expressed NCAM120, NCAM140, and NCAM180 in similar amounts, RPTP $\alpha$  coimmunoprecipitated only with NCAM140 (Fig. 3 A). However, after prolonged exposure of the film we could also detect RPTP $\alpha$  in NCAM180 immunoprecipitates (unpublished data). RPTP $\alpha$  did not coimmunoprecipitate with NCAM120. We conclude that RPTP $\alpha$  associates predominantly with NCAM140 and to a lesser extent with NCAM180.

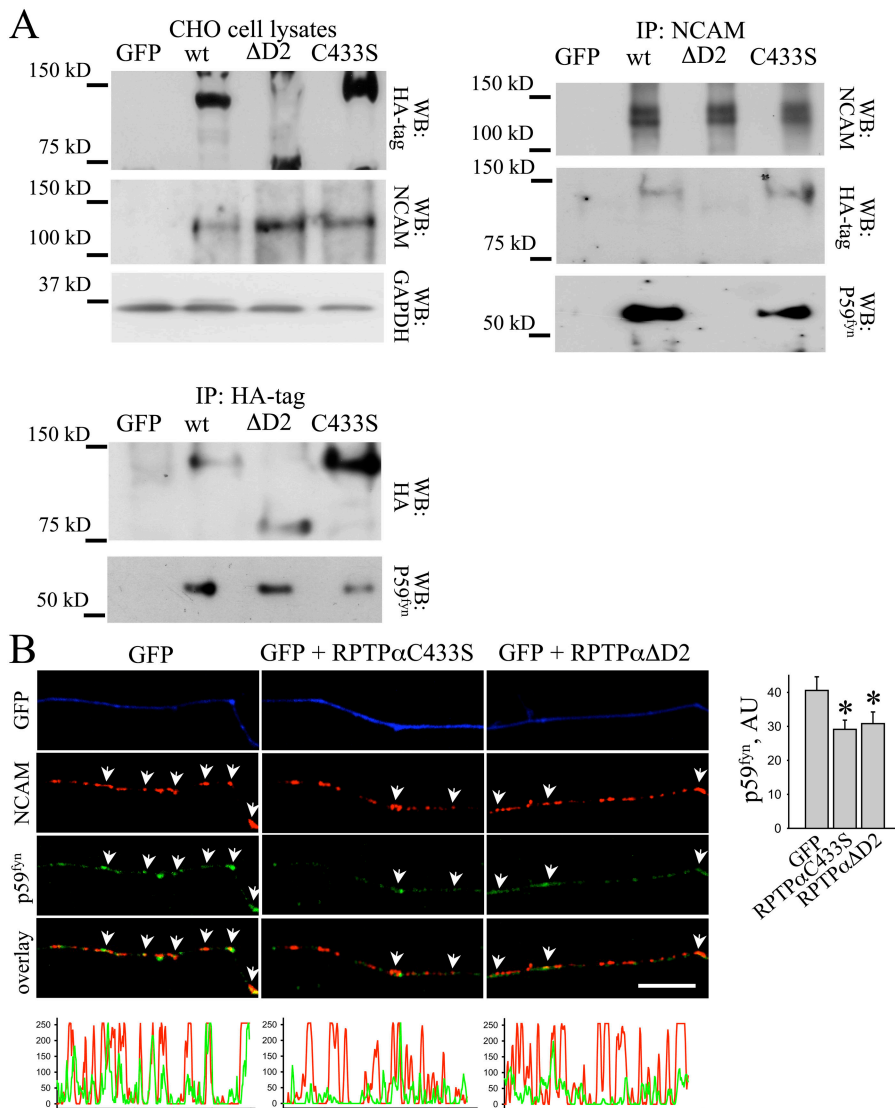
Inability of NCAM120, the GPI-linked NCAM isoform without the intracellular domain, to bind RPTP $\alpha$  suggested that the intracellular domain of NCAM is involved in the formation of a complex between NCAM and RPTP $\alpha$ . Furthermore, the extracellular domain of NCAM (NCAM-Fc) did not bind to RPTP $\alpha$  in brain lysates, confirming that the extracellular domain of NCAM does not bind to RPTP $\alpha$  (unpublished data). To verify that the NCAM intracellular domain interacts directly with the intracellular domain of RPTP $\alpha$ , we analyzed binding of the recombinant intracellular domain of RPTP $\alpha$  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 RPTP $\alpha$  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 RPTP $\alpha$  and NCAM140 was severalfold stronger than between the intracellular domains of RPTP $\alpha$  and NCAM180 (Fig. 3 B). To confirm this finding, we examined the direct interaction between the intracellular domains of RPTP $\alpha$  and NCAM180 or NCAM140 by ELISA. Intracellular domain of RPTP $\alpha$  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). No binding with the intracellular domain of CHL1 was observed (Fig. 3 C). We conclude that NCAM binds directly to RPTP $\alpha$  via the intracellular domain, with NCAM140 being the most potent RPTP $\alpha$ -binding NCAM isoform.

#### RPTP $\alpha$ binds NCAM140 via the D2 domain and links NCAM140 to p59<sup>fyn</sup>

To identify the part of the intracellular domain of RPTP $\alpha$  responsible for the interaction with NCAM140, we coexpressed, in CHO cells, NCAM140 together with the wild-type form of RPTP $\alpha$  (wtRPTP $\alpha$ ), RPTP $\alpha$  lacking the D2 domain (RPTP $\alpha$  $\Delta$ D2), or catalytically inactive form of RPTP $\alpha$  containing a mutation within the D1 catalytic domain (RPTP $\alpha$ C433S) and analyzed binding of NCAM140 to these RPTP $\alpha$  mutants by coimmunoprecipitation. All transfected

ELISA for the ability to bind increasing concentrations of RPTP $\alpha$  intracellular domains. Binding to BSA served as a control. Mean values (OD 405)  $\pm$  SEM ( $n = 6$ ) are shown. Intracellular domains of RPTP $\alpha$  bound to intracellular domains of NCAM140 and with a lower affinity to intracellular domains of NCAM180, but not to intracellular domains of CHL1.





**Figure 4. RPTP $\alpha$  interacts with NCAM140 via D2 domain and links NCAM140 to p59<sup>fyn</sup>.** (A) Lysates from CHO cells cotransfected with NCAM140 and wild-type (wt) RPTP $\alpha$ , RPTP $\alpha$ ΔD2, RPTP $\alpha$ C433S, or GFP were probed with antibodies against HA tag and NCAM by Western blot. NCAM140 and RPTP $\alpha$  constructs are expressed at approximately equal amounts in transfected CHO cells. Labeling for GAPDH was included as loading control. NCAM (IP: NCAM) or RPTP $\alpha$  (IP: HA) immunoprecipitates were probed with antibodies against HA tag and p59<sup>fyn</sup>. wtRPTP $\alpha$  and RPTP $\alpha$ C433S but not RPTP $\alpha$ ΔD2 coimmunoprecipitated with NCAM140. RPTP $\alpha$ C433S and RPTP $\alpha$ ΔD2 inhibited coimmunoprecipitation of p59<sup>fyn</sup> with NCAM140. p59<sup>fyn</sup> coimmunoprecipitated with wtRPTP $\alpha$  and RPTP $\alpha$ ΔD2 but to a lower extent with RPTP $\alpha$ C433S. (B) Hippocampal neurons transfected with RPTP $\alpha$ C433S, RPTP $\alpha$ ΔD2, or GFP were incubated live with NCAM antibodies to cluster NCAM and fixed and labeled with antibodies against p59<sup>fyn</sup>. Note that redistribution of p59<sup>fyn</sup> to NCAM clusters (arrows) was reduced in neurons transfected with RPTP $\alpha$ C433S or RPTP $\alpha$ ΔD2. Bar, 10  $\mu$ m. The corresponding profiles show NCAM and p59<sup>fyn</sup> labeling intensities along transfected neurites. The histogram shows mean labeling intensity of p59<sup>fyn</sup> in NCAM clusters. Transfection with RPTP $\alpha$ C433S or RPTP $\alpha$ ΔD2 reduced association between p59<sup>fyn</sup> and NCAM. Mean values  $\pm$  SEM ( $n > 20$  neurons) are shown in arbitrary units (AU). \*,  $P < 0.05$ ,  $t$  test.

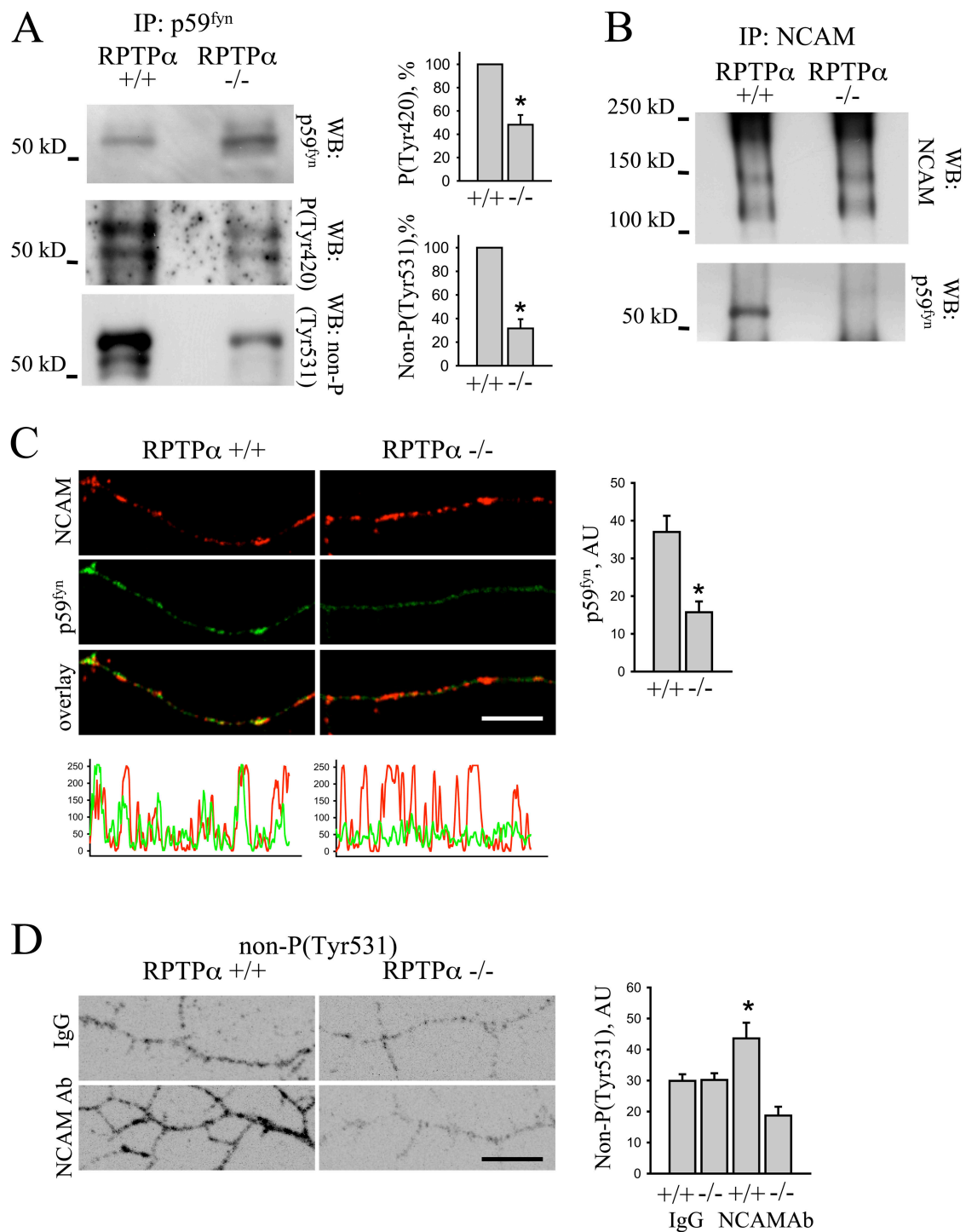
RPTP $\alpha$  constructs contained the HA tag to distinguish them from endogenous RPTP $\alpha$ . As seen for endogenous RPTP $\alpha$ , transfected wtRPTP $\alpha$  coimmunoprecipitated with NCAM140 (Fig. 4 A). Similar amounts of RPTP $\alpha$ C433S coimmunoprecipitated with NCAM140, whereas RPTP $\alpha$ ΔD2 did not coimmunoprecipitate (Fig. 4 A), indicating that the D2 domain is required for the interaction between RPTP $\alpha$  and NCAM140.

Remarkably, among the major NCAM isoforms, only NCAM140 forms a complex with p59<sup>fyn</sup> (Beggs et al., 1997) that we found to correlate with its ability to bind RPTP $\alpha$  (see the previous section). RPTP $\alpha$  directly interacts with p59<sup>fyn</sup> (Bhandari et al., 1998). Accordingly, p59<sup>fyn</sup> coimmunoprecipitated with wtRPTP $\alpha$  from transfected CHO cells (Fig. 4 A). Approximately the same amount of p59<sup>fyn</sup> coimmunoprecipitated with RPTP $\alpha$ ΔD2 (Fig. 4 A), indicating that this truncated construct also binds p59<sup>fyn</sup> probably via the D1 domain. In accordance with previous reports, p59<sup>fyn</sup> showed reduced ability to bind RPTP $\alpha$ C433S, a catalytically inactive mutant of RPTP $\alpha$  (Fig. 4 A; Zheng et al., 2000).

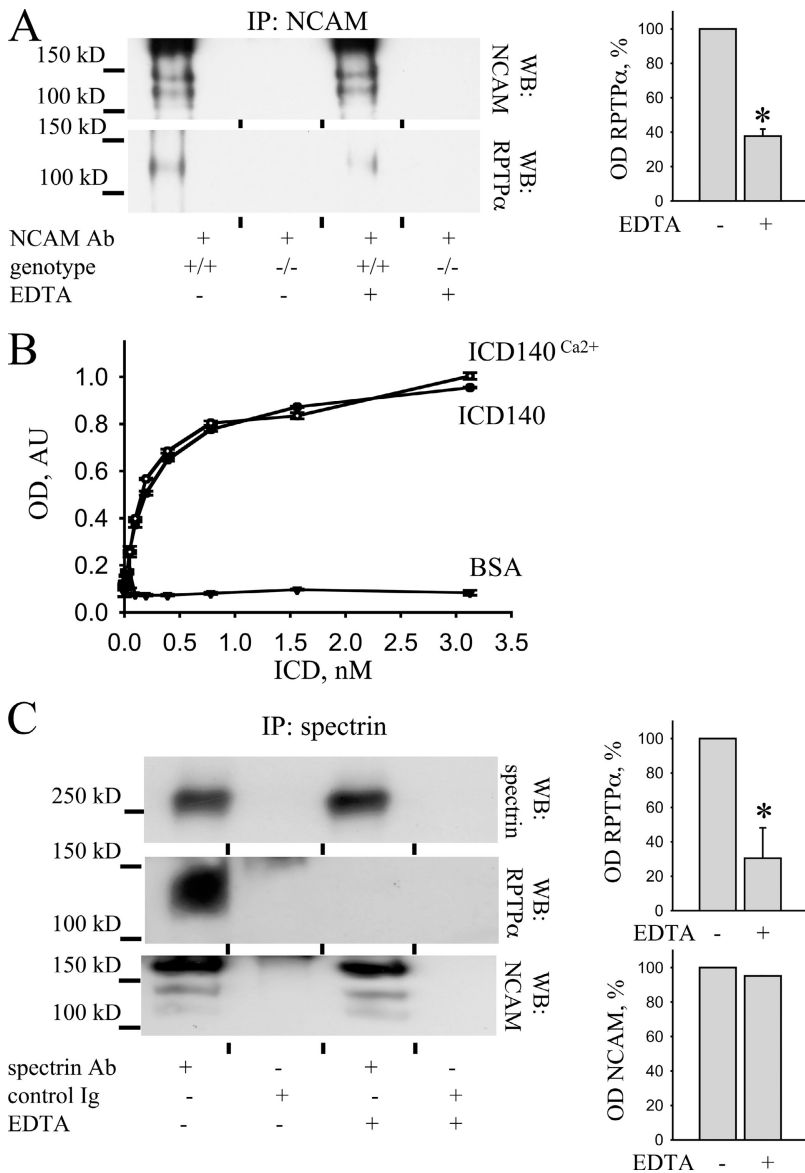
To analyze the role of RPTP $\alpha$  in NCAM140–p59<sup>fyn</sup> complex formation, we coimmunoprecipitated p59<sup>fyn</sup> with

NCAM140 in the presence of RPTP $\alpha$  mutants. In CHO cells cotransfected with NCAM140 and wtRPTP $\alpha$ , p59<sup>fyn</sup> coimmunoprecipitated with NCAM140 (Fig. 4 A). The amount of p59<sup>fyn</sup> coimmunoprecipitated with NCAM140 was reduced in cells cotransfected with RPTP $\alpha$ C433S (Fig. 4 A), correlating with the reduced ability of this catalytically inactive RPTP $\alpha$  mutant to bind p59<sup>fyn</sup> (see previous paragraph; Zheng et al., 2000). When NCAM140 was cotransfected with RPTP $\alpha$ ΔD2, p59<sup>fyn</sup> no longer coimmunoprecipitated with NCAM140 (Fig. 4 A). Because RPTP $\alpha$ ΔD2 binds p59<sup>fyn</sup> (Fig. 4 A), it is conceivable that this mutant, which does not bind NCAM140, competes with endogenous RPTP $\alpha$  for binding to p59<sup>fyn</sup> and thus inhibits NCAM140–p59<sup>fyn</sup> complex formation.

To extend this analysis to neurons, we transfected hippocampal neurons with GFP alone or cotransfected with GFP and RPTP $\alpha$ ΔD2 or RPTP $\alpha$ C433S and analyzed the redistribution of p59<sup>fyn</sup> to NCAM clusters after cross-linking NCAM with NCAM antibodies (Fig. 4 B). In neurons transfected with RPTP $\alpha$ ΔD2 or RPTP $\alpha$ C433S, the level of p59<sup>fyn</sup> in NCAM clusters was reduced by  $\sim 30\%$  when compared with GFP only transfected cells, suggesting that RPTP $\alpha$ ΔD2 or



**Figure 5. NCAM-p59<sup>fyn</sup> complex formation and NCAM-mediated p59<sup>fyn</sup> activation are abolished in RPTP $\alpha$ -deficient neurons.** (A) p59<sup>fyn</sup> immunoprecipitates from 4-d-old wild-type (RPTP $\alpha$ +/+) and RPTP $\alpha$ -deficient (RPTP $\alpha$ -/-) brain homogenates were probed by Western blot with antibodies against total p59<sup>fyn</sup> protein, p59<sup>fyn</sup> dephosphorylated at Tyr-531, or p59<sup>fyn</sup> phosphorylated at Tyr-420. Levels of p59<sup>fyn</sup> dephosphorylated at Tyr-531 and p59<sup>fyn</sup> phosphorylated at Tyr-420 are reduced in RPTP $\alpha$ -deficient brains. Histograms 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. (B) NCAM immunoprecipitates (IP: NCAM) from wild-type (RPTP $\alpha$ +/+) and RPTP $\alpha$ -deficient (RPTP $\alpha$ -/-) brain homogenates were probed with antibodies against NCAM and p59<sup>fyn</sup> by Western blot. Note that p59<sup>fyn</sup> coimmunoprecipitates with NCAM in wild-type but not in RPTP $\alpha$ -deficient brains. (C) Wild-type and RPTP $\alpha$ -deficient hippocampal neurons were incubated live with NCAM antibodies to cluster NCAM. Cells were fixed and labeled with antibodies against p59<sup>fyn</sup>. Note that redistribution of p59<sup>fyn</sup> to NCAM clusters was reduced in RPTP $\alpha$ -deficient neurons. Bar, 10  $\mu$ m. The corresponding profiles show NCAM and p59<sup>fyn</sup> labeling intensities along neurites. The histogram shows mean labeling intensity of p59<sup>fyn</sup> in NCAM clusters. Mean values  $\pm$  SEM ( $n > 20$  neurons) are shown in arbitrary units (AU). \*,  $P < 0.05$ ,  $t$  test. (D) Wild-type and RPTP $\alpha$ -deficient hippocampal neurons were incubated live with nonspecific IgG or NCAM antibodies, and fixed and labeled with antibodies against p59<sup>fyn</sup> dephosphorylated at Tyr-531. Immunofluorescence signals were inverted to accentuate the difference in immunolabeling intensities between groups. Bar, 10  $\mu$ m. Note that application of NCAM antibodies increased levels of p59<sup>fyn</sup> dephosphorylated at Tyr-531 in wild type, but not in RPTP $\alpha$ -deficient neurons. The histogram shows mean labeling intensity of p59<sup>fyn</sup> dephosphorylated at Tyr-531 along neurites. Mean values  $\pm$  SEM ( $n > 20$  neurons) are shown in arbitrary units (AU). \*,  $P < 0.05$ ,  $t$  test.



**Figure 6. NCAM-RPTP $\alpha$  complex formation is enhanced by spectrin in a Ca<sup>2+</sup>-dependent manner.** (A) NCAM immunoprecipitates obtained from brain homogenates of wild-type mice (+/+) in the presence or absence of 2 mM EDTA were probed by Western blot with antibodies against RPTP $\alpha$ . NCAM-deficient brains (-/-) were taken for control. Coimmunoprecipitation of RPTP $\alpha$  was inhibited by EDTA application. The histogram shows quantitation of the blots. (B) Intracellular domains of NCAM140 were bound to plastic and assayed by ELISA for the ability to bind increasing concentrations of RPTP $\alpha$  intracellular domains in the absence of Ca<sup>2+</sup> or in the presence of 2 mM Ca<sup>2+</sup>. Binding to BSA served as a control. Mean values (OD 405)  $\pm$  SEM ( $n = 6$ ) are shown. Binding of intracellular domains of RPTP $\alpha$  to intracellular domains of NCAM140 did not depend on Ca<sup>2+</sup>. (C) Spectrin immunoprecipitates obtained from brain homogenates of wild-type mice in the absence or presence of 2 mM EDTA were probed by Western blot with antibodies against RPTP $\alpha$  or NCAM. Non-immune rabbit Ig (control Ig) were used for control. Coimmunoprecipitation of RPTP $\alpha$  with spectrin was inhibited by EDTA, whereas coimmunoprecipitation of NCAM did not depend on Ca<sup>2+</sup>. Histograms show quantitation of the blots. For histograms in A and C, OD in the presence of Ca<sup>2+</sup> was set to 100% and mean values  $\pm$  SEM ( $n = 6$ ) are shown. \*,  $P < 0.05$ , paired  $t$  test.

RPTP $\alpha$ C433S inhibit NCAM-p59<sup>fyn</sup> complex formation by competing with endogenous RPTP $\alpha$ . The combined observations indicate that NCAM140-p59<sup>fyn</sup> complex formation correlates with the ability of NCAM140-associated RPTP $\alpha$  to bind to p59<sup>fyn</sup>, implicating RPTP $\alpha$  as a linker between NCAM140 and p59<sup>fyn</sup>.

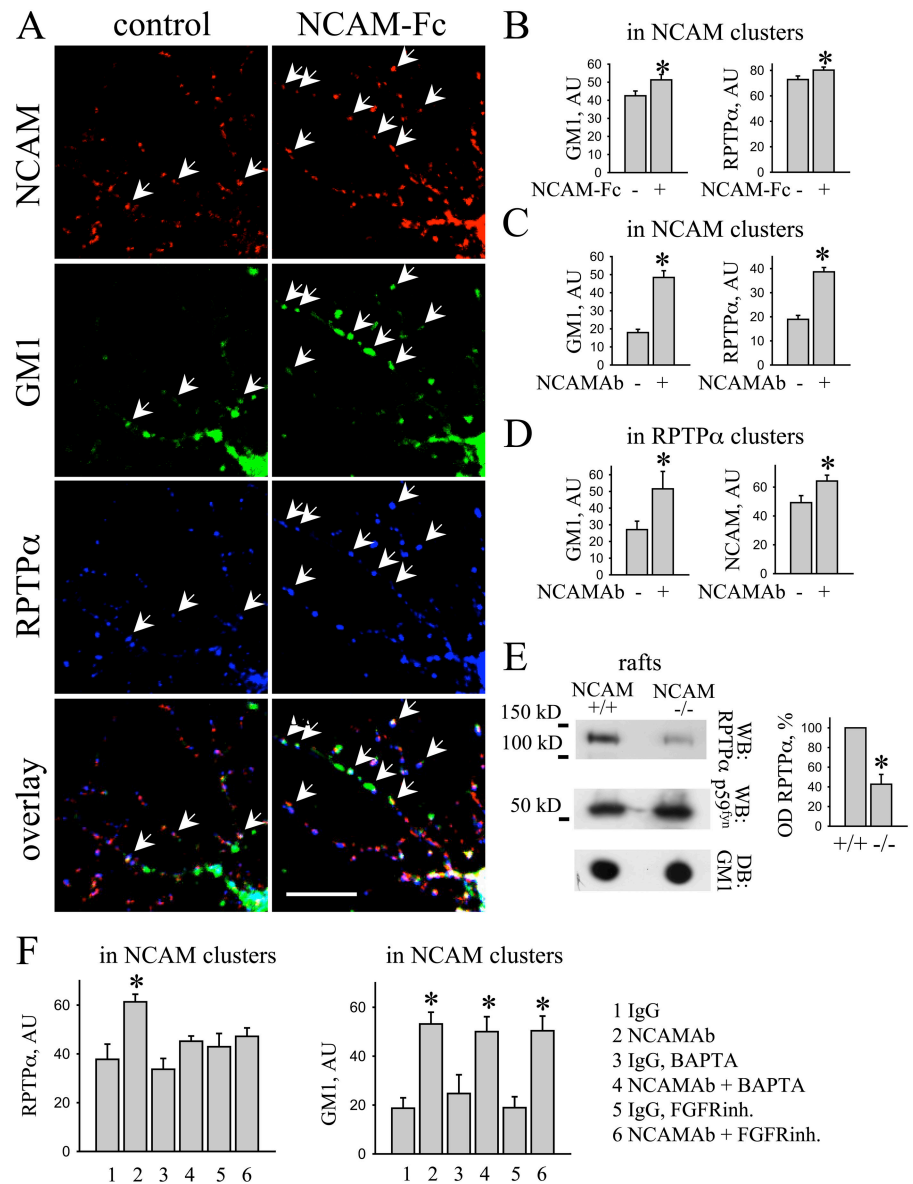
#### Association between NCAM and p59<sup>fyn</sup> and NCAM-mediated p59<sup>fyn</sup> activation are abolished in RPTP $\alpha$ -deficient neurons

To substantiate further our finding that RPTP $\alpha$  is a linker protein between NCAM and p59<sup>fyn</sup>, we analyzed p59<sup>fyn</sup> activation and association of p59<sup>fyn</sup> with NCAM in RPTP $\alpha$ -deficient brains. As for NCAM-deficient brains, levels of p59<sup>fyn</sup> dephosphorylated at Tyr-531 and levels of p59<sup>fyn</sup> phosphorylated at Tyr-420 were reduced in brain homogenates of RPTP $\alpha$ -deficient mice (Fig. 5 A), further suggesting that RPTP $\alpha$  plays a role in NCAM-mediated p59<sup>fyn</sup> activation in the brain. To analyze the role of RPTP $\alpha$  in the formation of the complex be-

tween NCAM and p59<sup>fyn</sup>, we immunoprecipitated NCAM from wild-type and RPTP $\alpha$ -deficient brains and probed immunoprecipitates with antibodies against p59<sup>fyn</sup>. Whereas p59<sup>fyn</sup> coimmunoprecipitated with NCAM from wild-type brains, p59<sup>fyn</sup> did not coimmunoprecipitate with NCAM from RPTP $\alpha$ -deficient brains (Fig. 5 B). Furthermore, when NCAM was clustered at the surface of wild-type and RPTP $\alpha$ -deficient cultured hippocampal neurons, levels of p59<sup>fyn</sup> were significantly reduced in NCAM clusters in RPTP $\alpha$ -deficient neurons when compared with wild-type cells (Fig. 5 C), indicating that RPTP $\alpha$  is required for complex formation between NCAM and p59<sup>fyn</sup>.

NCAM clustering at the cell surface induces rapid p59<sup>fyn</sup> activation (Beggs et al., 1997). To analyze whether or not RPTP $\alpha$  is required for NCAM-induced p59<sup>fyn</sup> activation, we treated live hippocampal neurons from wild-type and RPTP $\alpha$ -deficient mice with NCAM antibodies and analyzed levels of p59<sup>fyn</sup> dephosphorylated at Tyr-531 along neurites of the stimulated neurons. Clustering of NCAM increased levels of Tyr-531-dephosphorylated p59<sup>fyn</sup> along neurites of wild-type neu-

**Figure 7. NCAM activation induces redistribution of RPTP $\alpha$  to lipid rafts.** (A–D and F) Control hippocampal neurons and neurons incubated with corresponding reagents were extracted in cold 1% Triton X-100 and labeled with antibodies against NCAM and RPTP $\alpha$  together with FITC-labeled cholera toxin to visualize GM1-containing lipid rafts. (A) Note increased overlap of NCAM with RPTP $\alpha$  and GM1 after NCAM-Fc application when compared with control neurons (arrows). Bar, 10  $\mu$ m. (B and C) Histograms show mean intensities of RPTP $\alpha$  and GM1 in NCAM clusters in control and NCAM-Fc (B) or NCAM antibody (C)-treated neurons. (D) Histograms show mean intensities of NCAM and GM1 in RPTP $\alpha$  clusters in control and NCAM antibody-treated neurons. (E) Lipid rafts obtained from wild-type (+/+) or NCAM-deficient brains (-/-) were probed by Western blot (WB) with antibodies against RPTP $\alpha$  or p59<sup>lyn</sup> or by dot blot (DB) with cholera toxin. Note reduced amount of RPTP $\alpha$  in rafts from NCAM-deficient brains. The histogram shows quantitation of the RPTP $\alpha$  levels in lipid rafts. OD in wild type was set to 100% and mean values  $\pm$  SEM ( $n = 6$ ) are shown. \*,  $P < 0.05$ , paired  $t$  test. (F) Hippocampal neurons were incubated with nonspecific IgG or NCAM antibody alone, or in the presence of BAPTA-AM or FGFR inhibitor. Graphs show mean labeling intensity of RPTP $\alpha$  and GM1 in NCAM clusters. Note that treatment with BAPTA-AM and FGFR inhibitor abolished redistribution of RPTP $\alpha$  to NCAM clusters but not redistribution of NCAM to GM1-positive rafts. Histograms (B–D and F) show mean values  $\pm$  SEM ( $n > 50$ ) in arbitrary units (AU). \*,  $P < 0.05$ ,  $t$  test.



rons by  $\sim 60\%$  (Fig. 5 D). However, NCAM-mediated p59<sup>lyn</sup> activation was completely abolished in RPTP $\alpha$ -deficient neurons (Fig. 5 D), demonstrating that RPTP $\alpha$  is required for NCAM-mediated p59<sup>lyn</sup> activation.

#### Formation of the complex between RPTP $\alpha$ and NCAM is enhanced by Ca<sup>2+</sup>

Coimmunoprecipitation experiments were performed either in the presence of Ca<sup>2+</sup> or with 2 mM EDTA, a Ca<sup>2+</sup>-sequestering agent. Whereas RPTP $\alpha$  coimmunoprecipitated with NCAM from brain homogenates under both conditions, coimmunoprecipitated complexes were reduced by  $\sim 60\%$  in the presence of EDTA (Fig. 6 A), suggesting that Ca<sup>2+</sup> promotes formation of the NCAM–RPTP $\alpha$  complex. These results are in accordance with findings of Zeng et al. (1999), who found that NCAM and RPTP $\alpha$  did not coimmunoprecipitate in the presence of EDTA. To analyze if the direct interaction between NCAM and RPTP $\alpha$  is Ca<sup>2+</sup> dependent, we assayed binding of

the intracellular domain of NCAM140 to the intracellular domain of RPTP $\alpha$  by ELISA in the presence or absence of Ca<sup>2+</sup> (Fig. 6 B), showing that the direct interaction is Ca<sup>2+</sup> independent and suggesting that additional binding partners of NCAM and/or RPTP $\alpha$  may enhance complex formation in a Ca<sup>2+</sup>-dependent manner. Spectrin, which directly interacts with the intracellular domain of NCAM (Leshchyn'ska et al., 2003) and contains a Ca<sup>2+</sup> binding domain (De Matteis and Morrow, 2000), is one of the possible candidates. Indeed, RPTP $\alpha$  coimmunoprecipitated with spectrin from brain homogenates (Fig. 6 C). In the presence of 2 mM EDTA, RPTP $\alpha$  coimmunoprecipitating with spectrin was reduced by  $\sim 80\%$  (Fig. 6 C), whereas coimmunoprecipitation of NCAM with spectrin did not depend on Ca<sup>2+</sup> (Fig. 6 C). We conclude that RPTP $\alpha$  directly interacts with NCAM in a Ca<sup>2+</sup>-independent manner. However, formation of the complex is enhanced by Ca<sup>2+</sup>-dependent cross-linking of NCAM140 and RPTP $\alpha$  via spectrin.

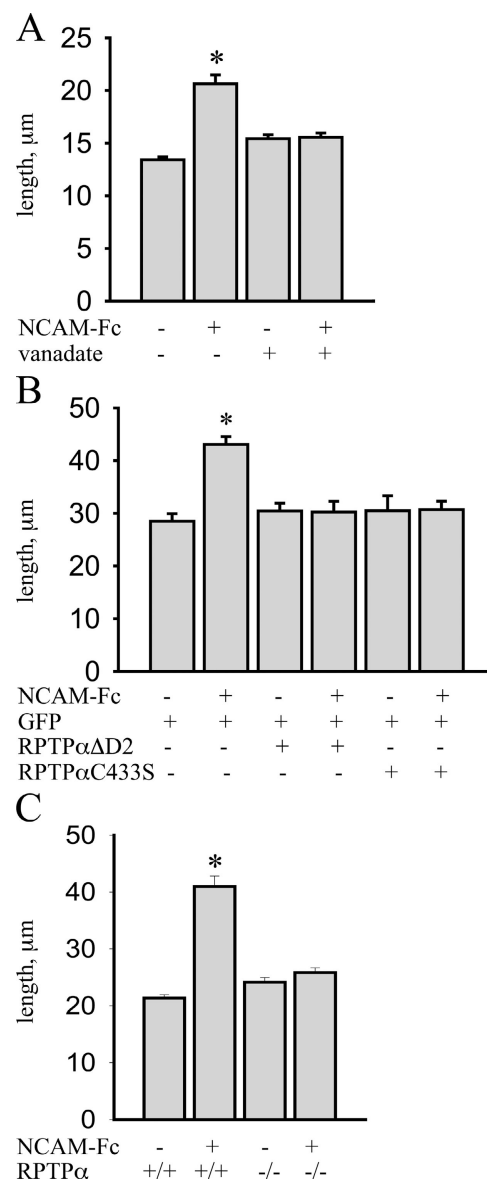


### The NCAM-RPTP $\alpha$ complex redistributes to lipid rafts after NCAM activation

Whereas p59<sup>fyn</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 RPTP $\alpha$  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 RPTP $\alpha$  only partially overlapped with the lipid raft marker ganglioside GM1 (Fig. 7 A), further confirming that RPTP $\alpha$  and p59<sup>fyn</sup> are segregated at the sub-cellular 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 RPTP $\alpha$  to lipid rafts and thus activate raft-associated p59<sup>fyn</sup>. To verify this hypothesis, we studied association of NCAM and RPTP $\alpha$  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 RPTP $\alpha$  in NCAM clusters, indicating that NCAM activation promoted NCAM–RPTP $\alpha$  complex formation (Fig. 7, A–C). Furthermore, NCAM activation also increased GM1 levels in detergent-insoluble clusters of RPTP $\alpha$  (Fig. 7 D), confirming that NCAM-associated RPTP $\alpha$  also redistributed to lipid rafts and suggesting that NCAM recruits RPTP $\alpha$  to lipid rafts. To further analyze this possibility, we compared levels of RPTP $\alpha$  in lipid rafts in brains of wild-type and NCAM-deficient mice. Indeed, RPTP $\alpha$  was reduced by  $\sim 60\%$  in lipid rafts isolated from NCAM-deficient brains (Fig. 7 E), confirming that NCAM plays a role in RPTP $\alpha$  targeting to lipid rafts. The levels of p59<sup>fyn</sup> were increased in NCAM-deficient lipid rafts (100% and  $124 \pm 7.6\%$  in wild-type and NCAM deficient rafts, respectively) probably reflecting increased levels of p59<sup>fyn</sup> in NCAM-deficient brains. Levels of GM1 were not different in lipid rafts from wild-type and NCAM-deficient brains (100% and  $103.3 \pm 6.8\%$  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 RPTP $\alpha$ to lipid rafts is enhanced by NCAM-induced FGF receptor (FGFR)-dependent increase in intracellular Ca<sup>2+</sup>

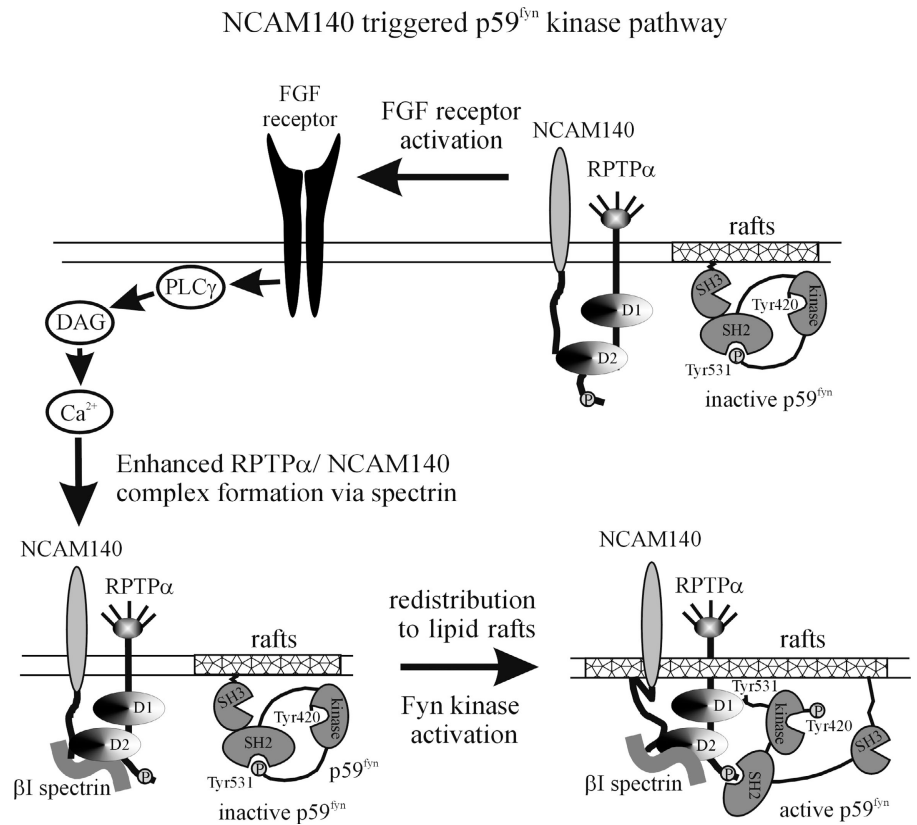
NCAM activation increases intracellular Ca<sup>2+</sup> concentrations via a FGFR-dependent mechanism (Walsh and Doherty, 1997; Kamiguchi and Lemmon, 2000; Juliano, 2002). This increase in intracellular Ca<sup>2+</sup> may account for the enhanced association between NCAM and RPTP $\alpha$  after NCAM activation (Fig. 7, A–D) because of spectrin-mediated cross-linking of NCAM140 and RPTP $\alpha$  (Fig. 6). Interestingly, NCAM activation also induces redistribution of NCAM-associated spectrin to lipid rafts (Leshchyns'ka et al., 2003). To analyze the



**Figure 8. NCAM-mediated neurite outgrowth depends on RPTP $\alpha$  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 RPTP $\alpha$ C433S or RPTP $\alpha$ ΔD2 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 RPTP $\alpha$ C433S or RPTP $\alpha$ ΔD2. (C) Lengths of the longest neurites were measured in wild-type (+/+) and RPTP $\alpha$ -deficient (-/-) neurons not treated or treated with NCAM-Fc. NCAM-Fc increased neurite length in wild-type but not in RPTP $\alpha$ -deficient neurons. For A–C, mean values  $\pm$  SEM are shown ( $n > 150$  neurons; \*,  $P < 0.05$ ,  $t$  test). Experiments were performed two times with the same effect.

role of FGFR and Ca<sup>2+</sup> in the recruitment of RPTP $\alpha$  to an NCAM complex, we estimated levels of RPTP $\alpha$  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.,

Figure 9. **A proposed model of NCAM140-mediated p59<sup>fyn</sup> activation cascade.** Before NCAM activation, NCAM140 and RPTP $\alpha$  located predominantly in raft-free areas are segregated from p59<sup>fyn</sup>, which predominantly associates with lipid rafts. NCAM activation induces FGFR-dependent increase in intracellular Ca<sup>2+</sup> that enhances RPTP $\alpha$ -NCAM140 complex formation via spectrin as a cross-linking platform. Additionally, NCAM activation results in the redistribution of the complex to lipid rafts due to NCAM palmitoylation. In lipid rafts, RPTP $\alpha$  binds and dephosphorylates p59<sup>fyn</sup> resulting in p59<sup>fyn</sup> activation, which, in turn, promotes neurite outgrowth.



2001), or a specific FGFR inhibitor (Niethammer et al., 2002; Leshchyn'ska et al., 2003). Whereas NCAM activation increased levels of RPTP $\alpha$  and GM1 in NCAM clusters (Fig. 7 F), treatment with BAPTA-AM or FGFR inhibitor abolished recruitment of RPTP $\alpha$  to NCAM clusters in response to NCAM activation (Fig. 7 F). In accordance with previous findings (Leshchyn'ska 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 RPTP $\alpha$  associated with NCAM under nonactivated conditions (Fig. 7 F). We conclude that, whereas at resting conditions Ca<sup>2+</sup> does not play a major role in the interaction between NCAM and RPTP $\alpha$ , NCAM-induced FGFR-dependent elevations of intracellular Ca<sup>2+</sup> levels strengthen the interactions between NCAM and RPTP $\alpha$  in response to NCAM activation, most likely via spectrin (see the section Formation of the complex between RPTP $\alpha$  and NCAM is enhanced by Ca<sup>2+</sup>).

#### NCAM-induced neurite outgrowth depends on NCAM association with RPTP $\alpha$

NCAM-induced neurite outgrowth depends on p59<sup>fyn</sup> activation (Kolkova et al., 2000), suggesting that NCAM association with RPTP $\alpha$  may be involved. To analyze the role of protein tyrosine phosphatases in NCAM-induced neurite outgrowth, we incubated cultured hippocampal neurons with 100  $\mu$ M 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 RPTP $\alpha$  in NCAM-induced neurite outgrowth, we transfected hippocampal neurons with the dominant-negative mutants of RPTP $\alpha$ . Both, RPTP $\alpha$  $\Delta$ D2, which does not bind NCAM but associates with p59<sup>fyn</sup>, and catalytically inactive RPTP $\alpha$ C433S, which associates with NCAM but binds p59<sup>fyn</sup> with a lower efficiency than endogenous RPTP $\alpha$ , inhibited association of NCAM with p59<sup>fyn</sup> by competing with endogenous RPTP $\alpha$  (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 RPTP $\alpha$  $\Delta$ D2 or RPTP $\alpha$ C433S remained unresponsive to NCAM-Fc stimulation (Fig. 8 B), indicating that RPTP $\alpha$  plays a major role in NCAM-induced neurite outgrowth. To confirm this finding, we analyzed NCAM-mediated neurite outgrowth in hippocampal neurons from RPTP $\alpha$ -deficient mice. Whereas NCAM-Fc enhanced neurite outgrowth in neurons from RPTP $\alpha$  wild-type littermates by  $\sim$ 100%, NCAM-Fc-induced neurite outgrowth was completely abolished in RPTP $\alpha$ -deficient neurons (Fig. 8 C), further confirming that RPTP $\alpha$  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 pp60<sup>c-src</sup> or p59<sup>fyn</sup>, 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 p59<sup>fyn</sup>, the receptor protein tyrosine phosphatase RPTP $\alpha$ , as a novel binding partner of NCAM. Activation of p59<sup>fyn</sup> is reduced in NCAM-deficient mice and interaction between NCAM and p59<sup>fyn</sup> is abolished in RPTP $\alpha$ -deficient brains. Interestingly, we found that the levels of p59<sup>fyn</sup> and RPTP $\alpha$  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, RPTP $\alpha$ , and p59<sup>fyn</sup>. NCAM-induced neurite outgrowth is completely abrogated in RPTP $\alpha$ -deficient neurons or in neurons transfected with dominant-negative RPTP $\alpha$  mutants, indicating that RPTP $\alpha$  links NCAM to p59<sup>fyn</sup> both physically and functionally.

### Role of Ca<sup>2+</sup> in NCAM-RPTP $\alpha$ -p59<sup>fyn</sup> complex formation

Interactions between NCAM and RPTP $\alpha$  and NCAM-RPTP $\alpha$ -p59<sup>fyn</sup> complex formation leading to neurite outgrowth are tightly regulated (Fig. 9). First, whereas direct interaction between NCAM and RPTP $\alpha$  is Ca<sup>2+</sup> independent, NCAM-RPTP $\alpha$  complex formation is enhanced by Ca<sup>2+</sup>-dependent cross-linking via spectrin. Remarkably, NCAM activation results in an increase in intracellular Ca<sup>2+</sup> concentration via influx through Ca<sup>2+</sup> channels or release from intracellular stores, and may thus provide a positive feedback loop between NCAM activation and NCAM-RPTP $\alpha$  complex formation involving spectrin. RPTP $\alpha$  binding to spectrin may also elevate RPTP $\alpha$  enzymatic dephosphorylation activity (Lokeshwar and Bourguignon, 1992). Interestingly, NCAM activation also induces activation of PKC (Kolkova et al., 2000; Leshchyn'ska et al., 2003), which is known to phosphorylate RPTP $\alpha$  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 p59<sup>fyn</sup> via RPTP $\alpha$  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 RPTP $\alpha$ -mediated p59<sup>fyn</sup> activation is achieved by segregation of RPTP $\alpha$  and p59<sup>fyn</sup> to different subdomains in the plasma membrane (Fig. 9). Approximately 90% of all RPTP $\alpha$  molecules in the brain are located in a lipid raft-

free environment and are thereby segregated from lipid raft-associated p59<sup>fyn</sup> 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 (Petroni and Sap, 2000), the mechanisms that target receptor protein tyrosine phosphatases to lipid rafts have remained unclear. We show that levels of raft-associated RPTP $\alpha$  in the NCAM-deficient brain are reduced, and NCAM redistribution to lipid rafts in response to NCAM activation also induces redistribution of RPTP $\alpha$  to lipid rafts via its NCAM association. The combined observations indicate that NCAM plays a role in recruiting NCAM-associated RPTP $\alpha$  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-RPTP $\alpha$ -p59<sup>fyn</sup> 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 RPTP $\alpha$ -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 RPTP $\alpha$  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 p59<sup>fyn</sup> protein were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies against Tyr-527-dephosphorylated or



Tyr-416-phosphorylated pp60<sup>src</sup> kinase that cross-react with Tyr-531-dephosphorylated or Tyr-420-phosphorylated p59<sup>fyn</sup> were obtained from Cell Signaling Technology. Secondary antibodies against rabbit, rat, and mouse Ig coupled to HRP, Cy2, Cy3, or Cy5 were obtained from Dianova.

#### 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 RPTP $\alpha$ -deficient mice, RPTP $\alpha$ -positive 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 LSM510; Carl Zeiss MicroImaging, Inc.), LSM510 software (version 3; Carl Zeiss MicroImaging, Inc.), and oil Plan-Neofluar 40 $\times$  objective (NA 1.3; Carl Zeiss MicroImaging, Inc.) at 3 $\times$  digital zoom. Contrast and brightness of the images were further adjusted in Photo-Point 9 (Corel Corporation).

#### Detergent extraction of cultured neurons

Cells washed in PBS were incubated for 1 min in cold microtubule-stabilizing buffer (2 mM MgCl<sub>2</sub>, 10 mM EGTA, and 60 mM Pipes, pH 7.0) and extracted 8 min on ice with 1% Triton X-100 in microtubule-stabilizing buffer as described previously (Ledezma et al., 1998). After washing with PBS, cells were fixed with cold 4% formaldehyde in PBS.

#### Colocalization analysis

Colocalization quantification was performed as described previously (Leshchyn'ska et al., 2003). In brief, an NCAM cluster was defined as an accumulation of NCAM labeling with a mean intensity at least 30% higher than background. NCAM clusters were automatically outlined using the threshold function of the Scion Image software (Scion Corporation). Within the outlined areas the mean intensities of NCAM, RPTP $\alpha$ , p59<sup>fyn</sup>, or GM1 labeling associated with NCAM cluster were measured. The same threshold was used for all groups. All experiments were performed two to three times with the same effect. Colocalization profiles were plotted using ImageJ software (National Institutes of Health).

#### DNA constructs

Rat NCAM140 and NCAM180/pcDNA3 were a gift of P. Maness (University of North Carolina, Chapel Hill, NC). Rat NCAM120 (a gift of E. Bock, University of Copenhagen, Copenhagen, Denmark) was subcloned into the pcDNA3 vector (Invitrogen) by two EcoRI sites. The EGFP plasmid was purchased from CLONTECH Laboratories, Inc. cDNAs encoding intracellular domains of NCAM140 and NCAM180 were as described previously (Sytnyk et al., 2002; Leshchyn'ska et al., 2003). The plasmid encoding the intracellular domain of RPTP $\alpha$  was a gift of C.J. Pallen. Wild-type RPTP $\alpha$ , RPTP $\alpha$ C433S, and RPTP $\alpha$ ΔD2 (containing RPTP $\alpha$  residues 1–516 [last 6 residues: KIYNKI]) were as described previously (den Hertog and Hunter, 1996; Blanchetot and den Hertog, 2000; Buist et al., 2000).

#### Online supplemental material

Details on cultures and transfection of hippocampal neurons and CHO cells, immunofluorescence labeling, ELISA and pull-down assay, coimmunoprecipitation, isolation of lipid enriched microdomains, gel electrophoresis, immunoblotting, and generation of RPTP $\alpha$ -deficient mice are given in online supplemental material. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200405073/DC1>.

We thank Achim Dahlmann and Eva Kronberg for genotyping and animal care, Drs. Patricia Maness and Elisabeth Bock for NCAM cDNAs, Dr. Catherine J. Pallen for antibodies against RPTP $\alpha$  and cDNA encoding the RPTP $\alpha$  intracellular domain, Dr. Harold Cremer for NCAM-deficient mice, and Dr. Christo Goridis for hybridoma clone H28.

This work was supported by Zonta Club, Hamburg-Alster (I. Leshchyn'ska), and Deutsche Forschungsgemeinschaft (I. Leshchyn'ska, V. Sytnyk, and M. Schachner).

Submitted: 12 May 2004

Accepted: 11 November 2004

## References

- Beggs, H.E., P. Soriano, and P.F. Maness. 1994. NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. *J. Cell Biol.* 127:825–833.
- Beggs, H.E., S.C. Baragona, J.J. Hemperly, and P.F. Maness. 1997. NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). *J. Biol. Chem.* 272:8310–8319.
- Bhandari, V., K.L. Lim, and C.J. Pallen. 1998. Physical and functional interactions between receptor-like protein-tyrosine phosphatase alpha and p59fyn. *J. Biol. Chem.* 273:8691–8698.
- Blanchetot, C., and J. den Hertog. 2000. Multiple interactions between receptor protein-tyrosine phosphatase (RPTP) alpha and membrane-distal protein-tyrosine phosphatase domains of various RPTPs. *J. Biol. Chem.* 275:12446–12452.
- Brown, M.T., and J.A. Cooper. 1996. Regulation, substrates and functions of src. *Biochim. Biophys. Acta.* 1287:121–149.
- Buhusi, M., B.R. Midkiff, A.M. Gates, M. Richter, M. Schachner, and P.F. Maness. 2003. Close homolog of L1 is an enhancer of integrin-mediated cell migration. *J. Biol. Chem.* 278:25024–25031.
- Buist, A., C. Blanchetot, L.G. Tertoolen, and J. den Hertog. 2000. Identification of p130cas as an in vivo substrate of receptor protein-tyrosine phosphatase alpha. *J. Biol. Chem.* 275:20754–20761.
- Cavallaro, U., J. Niedermeyer, M. Fuxa, and G. Christofori. 2001. N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. *Nat. Cell Biol.* 3:650–657.
- Cremer, H., R. Lange, A. Christoph, M. Plomann, G. Vopper, J. Roes, R. Brown, S. Baldwin, P. Kraemer, S. Scheff, et al. 1994. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature.* 367:455–459.
- Crossin, K.L., and L.A. Krushel. 2000. Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Dev. Dyn.* 218:260–279.
- De Matteis, M.A., and J.S. Morrow. 2000. Spectrin tethers and mesh in the biosynthetic pathway. *J. Cell Sci.* 113:2331–2343.
- den Hertog, J., and T. Hunter. 1996. Tight association of GRB2 with receptor protein-tyrosine phosphatase alpha is mediated by the SH2 and C-terminal SH3 domains. *EMBO J.* 15:3016–3027.
- den Hertog, J., C.E. Pals, M.P. Peppelenbosch, L.G. Tertoolen, S.W. de Laat, and W. Kruijer. 1993. Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. *EMBO J.* 12:3789–3798.
- den Hertog, J., S. Tracy, and T. Hunter. 1994. Phosphorylation of receptor protein-tyrosine phosphatase alpha on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 in vivo. *EMBO J.* 13:3020–3032.
- den Hertog, J., J. Sap, C.E. Pals, J. Schlessinger, and W. Kruijer. 1995. Stimulation of receptor protein-tyrosine phosphatase alpha activity and phosphorylation by phorbol ester. *Cell Growth Differ.* 6:303–307.
- Filipp, D., J. Zhang, B.L. Leung, A. Shaw, S.D. Levin, A. Veillette, and M. Julius. 2003. Regulation of Fyn through translocation of activated Lck into lipid rafts. *J. Exp. Med.* 197:1221–1227.
- Gennarini, G., M. Hirn, H. Deagostini-Bazin, and C. Goridis. 1984. Studies on the transmembrane disposition of the neural cell adhesion molecule N-CAM. The use of liposome-inserted radioiodinated N-CAM to study its transbilayer orientation. *Eur. J. Biochem.* 142:65–73.
- Harder, K.W., N.P. Moller, J.W. Peacock, and F.R. Jirik. 1998. Protein-tyrosine phosphatase alpha regulates Src family kinases and alters cell-substratum adhesion. *J. Biol. Chem.* 273:31890–31900.
- He, Q., and K.F. Meiri. 2002. Isolation and characterization of detergent-resistant microdomains responsive to NCAM-mediated signaling from growth cones. *Mol. Cell. Neurosci.* 19:18–31.
- Helmke, S., K. Lohse, K. Mikule, M.R. Wood, and K.H. Pfenninger. 1998. SRC binding to the cytoskeleton, triggered by growth cone attachment to laminin, is protein tyrosine phosphatase-dependent. *J. Cell Sci.* 111:2465–2475.
- Hubbard, S.R. 1999. Src autoinhibition: let us count the ways. *Nat. Struct. Biol.* 6:711–714.
- Iida, N., V.B. Lokeshwar, and L.Y. Bourguignon. 1994. Mapping the fodrin binding domain in CD45, a leukocyte membrane-associated tyrosine phosphatase. *J. Biol. Chem.* 269:28576–28583.
- Juliano, R.L. 2002. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Annu. Rev. Pharmacol. Toxicol.* 42:283–323.
- Kamiguchi, H., and V. Lemmon. 2000. IgCAMs: bidirectional signals underlying neurite growth. *Curr. Opin. Cell Biol.* 12:598–605.
- Kaplan, R., B. Morse, K. Huebner, C. Croce, R. Howk, M. Ravera, G. Ricca, M. Jaye, and J. Schlessinger. 1990. Cloning of three human tyrosine



- phosphatases reveals a multigene family of receptor-linked protein-tyrosine-phosphatases expressed in brain. *Proc. Natl. Acad. Sci. USA*. 87:7000–7004.
- Kolkova, K., V. Novitskaya, N. Pedersen, V. Berezin, and E. Bock. 2000. Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. *J. Neurosci.* 20:2238–2246.
- Kramer, E.M., C. Klein, T. Koch, M. Boytinck, and J. Trotter. 1999. Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. *J. Biol. Chem.* 274:29042–29049.
- Krueger, N.X., M. Streuli, and H. Saito. 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. *EMBO J.* 9:3241–3252.
- Ledesma, M.D., K. Simons, and C.G. Dotti. 1998. Neuronal polarity: essential role of protein-lipid complexes in axonal sorting. *Proc. Natl. Acad. Sci. USA*. 95:3966–3971.
- Leshchyn'ska, I., V. Sytnyk, J.S. Morrow, and M. Schachner. 2003. Neural cell adhesion molecule (NCAM) association with PKC $\beta_2$  via  $\beta$ 1 spectrin is implicated in NCAM-mediated neurite outgrowth. *J. Cell Biol.* 161:625–639.
- Lokeshwar, V.B., and L.Y. Bourguignon. 1992. Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* 267:21551–21557.
- Niethammer, P., M. Delling, V. Sytnyk, A. Dityatev, K. Fukami, and M. Schachner. 2002. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neurogenesis. *J. Cell Biol.* 157:521–532.
- Paratcha, G., F. Ledda, and C.F. Ibanez. 2003. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell*. 113:867–879.
- Petrone, A., and J. Sap. 2000. Emerging issues in receptor protein tyrosine phosphatase function: lifting fog or simply shifting? *J. Cell Sci.* 113:2345–2354.
- Petrone, A., F. Battaglia, C. Wang, A. Dusa, J. Su, D. Zagzag, R. Bianchi, P. Casaccia-Bonnel, O. Arancio, and J. Sap. 2003. Receptor protein tyrosine phosphatase alpha is essential for hippocampal neuronal migration and long-term potentiation. *EMBO J.* 22:4121–4131.
- Ponniah, S., D.Z. Wang, K.L. Lim, and C.J. Pallen. 1999. Targeted disruption of the tyrosine phosphatase PTPalpha leads to constitutive downregulation of the kinases Src and Fyn. *Curr. Biol.* 9:535–538.
- Schmid, R.S., R.D. Graff, M.D. Schaller, S. Chen, M. Schachner, J.J. Hemperly, and P.F. Maness. 1999. NCAM stimulates the Ras-MAPK pathway and CREB phosphorylation in neuronal cells. *J. Neurobiol.* 38:542–558.
- Schmid, R.S., W.M. Pruitt, and P.F. Maness. 2000. A MAP kinase-signaling pathway mediates neurite outgrowth on L1 and requires Src-dependent endocytosis. *J. Neurosci.* 20:4177–4188.
- Su, J., L.T. Yang, and J. Sap. 1996. Association between receptor protein-tyrosine phosphatase RPTPalpha and the Grb2 adaptor. Dual Src homology (SH) 2/SH3 domain requirement and functional consequences. *J. Biol. Chem.* 271:28086–28096.
- Sytnyk, V., I. Leshchyn'ska, M. Delling, G. Dityateva, A. Dityatev, and M. Schachner. 2002. Neural cell adhesion molecule promotes accumulation of TGN organelles at sites of neuron-to-neuron contacts. *J. Cell Biol.* 159:649–661.
- Thomas, S.M., and J.S. Brugge. 1997. Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* 13:513–609.
- Tracy, S., P. van der Geer, and T. Hunter. 1995. The receptor-like protein-tyrosine phosphatase, RPTP alpha, is phosphorylated by protein kinase C on two serines close to the inner face of the plasma membrane. *J. Biol. Chem.* 270:10587–10594.
- van't Hof, W., and M.D. Resh. 1997. Rapid plasma membrane anchoring of newly synthesized p59fyn: selective requirement for NH<sub>2</sub>-terminal myristoylation and palmitoylation at cysteine-3. *J. Cell Biol.* 136:1023–1035.
- von Wichert, G., G. Jiang, A. Kostic, K. De Vos, J. Sap, and M.P. Sheetz. 2003. RPTP- $\alpha$  acts as a transducer of mechanical force on  $\alpha_5\beta_3$ -integrin-cytoskeleton linkages. *J. Cell Biol.* 161:143–153.
- Walsh, F.S., and P. Doherty. 1997. Neural cell adhesion molecules of the immunoglobulin superfamily: role in axon growth and guidance. *Annu. Rev. Cell Dev. Biol.* 13:425–456.
- Wang, Y., and C.J. Pallen. 1991. The receptor-like protein tyrosine phosphatase HPTP alpha has two active catalytic domains with distinct substrate specificities. *EMBO J.* 10:3231–3237.
- Williams, E.J., P. Doherty, G. Turner, R.A. Reid, J.J. Hemperly, and F.S. Walsh. 1992. Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. *J. Cell Biol.* 119:883–892.
- Wu, L., A. Buist, J. den Hertog, and Z.Y. Zhang. 1997. Comparative kinetic analysis and substrate specificity of the tandem catalytic domains of the receptor-like protein-tyrosine phosphatase alpha. *J. Biol. Chem.* 272:6994–7002.
- Yang, L.T., K. Alexandropoulos, and J. Sap. 2002. c-SRC mediates neurite outgrowth through recruitment of Crk to the scaffolding protein Sin/Efs without altering the kinetics of ERK activation. *J. Biol. Chem.* 277:17406–17414.
- Zeng, L., L. D'Alessandri, M.B. Kalousek, L. Vaughan, and C.J. Pallen. 1999. Protein tyrosine phosphatase  $\alpha$  (PTP $\alpha$ ) and contactin form a novel neuronal receptor complex linked to the intracellular tyrosine kinase fyn. *J. Cell Biol.* 147:707–714.
- Zeng, L., X. Si, W.P. Yu, H.T. Le, K.P. Ng, R.M. Teng, K. Ryan, D.Z. Wang, S. Ponniah, and C.J. Pallen. 2003. PTP $\alpha$  regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. *J. Cell Biol.* 160:137–146.
- Zheng, X.M., Y. Wang, and C.J. Pallen. 1992. Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. *Nature*. 359:336–339.
- Zheng, X.M., R.J. Resnick, and D. Shalloway. 2000. A phosphotyrosine displacement mechanism for activation of Src by PTPalpha. *EMBO J.* 19:964–978.
- Zheng, X.M., R.J. Resnick, and D. Shalloway. 2002. Mitotic activation of protein-tyrosine phosphatase alpha and regulation of its Src-mediated transforming activity by its sites of protein kinase C phosphorylation. *J. Biol. Chem.* 277:21922–21929.