Neural cell adhesion molecule (NCAM) association with PKCβ2 via β1 spectrin is implicated in NCAM-mediated neurite outgrowth

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Introduction

In hippocampal neurons and transfected CHO cells, neural cell adhesion molecule (NCAM) 120, NCAM140, and NCAM180 form Triton X-100–insoluble complexes with β1 spectrin. Heteromeric spectrin (αβ) binds to the intracellular domain of NCAM180, and isolated spectrin subunits bind to both NCAM180 and NCAM140, as does the β1 spectrin fragment encompassing second and third spectrin repeats (β12–3). In NCAM120-transfected cells, β1 spectrin is detectable predominantly in lipid rafts. Treatment of cells with methyl-β-cyclodextrin disrupts the NCAM120–spectrin complex, implicating lipid rafts as a platform linking NCAM120 and spectrin. NCAM140/NCAM180–β1 spectrin complexes do not depend on raft integrity and are located both in rafts and raft-free membrane domains. PKCβ2 forms detergent-insoluble complexes with NCAM140/NCAM180 and spectrin. Activation of NCAM enhances the formation of NCAM140/NCAM180–spectrin–PKCβ2 complexes and results in their redistribution to lipid rafts. The complex is disrupted by the expression of dominant-negative β12–3, which impairs binding of spectrin to NCAM, implicating spectrin as the bridge between PKCβ2 and NCAM140 or NCAM180. Redistribution of PKCβ2 to NCAM–spectrin complexes is also blocked by a specific fibroblast growth factor receptor inhibitor. Furthermore, transfection with β12–3 inhibits NCAM-induced neurite outgrowth, showing that formation of the NCAM–spectrin–PKCβ2 complex is necessary for NCAM-mediated neurite outgrowth.
Spectrin is now recognized as a ubiquitous scaffolding protein that acts in conjunction with a variety of adaptor proteins to organize membrane microdomains on both the plasma membrane as well as on intracellular organelles. Spectrin can also link membranes and membrane protein complexes to filamentous actin or to microtubule transport motors (dynein–dynactin and some kinesins) (for reviews see Hirokawa, 1998; De Matteis and Morrow, 2000). The functional unit of spectrin is an α,β heterodimer, although homopolymeric forms exist (Bloch and Morrow, 1989). Two spectrin genes encode α-type subunits, and five genes encode the β spectrins. Spectrin–membrane interactions are mediated by both protein–protein and protein–lipid interactions. The most studied interactions operate through ankyrin. These join spectrin to a variety of membrane receptors. The most studied interactions operate through ankyrin. They join spectrin to a variety of membrane receptors, including Na,K-ATPase, voltage-gated Na+ channel, and tyrosine phosphate phosphatase CD45. Ankyrin-independent association with proteins, such as complexes of cadherin–catenin and NCAM180–spectrin, as well as with cortical actin, offers additional pathways of membrane interaction. Finally, many β spectrins contain a pleckstrin homology domain. This domain mediates a direct interaction of spectrin with phosphatidylinositol 4,5-bisphosphate (PtdInsP2) and other acidic phospholipids.

Different β spectrin isoforms play specific roles in the formation of unique membrane microdomains. For example, whereas βII spectrin, the most widely distributed isoform, tends to be fairly uniformly distributed over the plasma membrane of neurons and other cells, βI spectrin sorts to specific organelles (De Matteis and Morrow, 2000) and to organized plasma membrane domains, such as the motor end plate of skeletal muscle (Bloch and Morrow, 1989), the postsynaptic density of cerebellar neurons (Malchiodi-Albedi et al., 1993), or to CD45-rich patches in T cells (Pradhan and Morrow, 2002). βI spectrin transcripts are now recognized in many nonerythroid cells, including neurons, lymphocytes, and epithelial cells. βIII spectrin is another widely expressed spectrin associated with intracellular organelles and the plasma membrane. Given the close association of spectrin with membrane-associated receptor clusters in muscle, neurons, and lymphocytes, it is likely that spectrin may trap or stabilize proteins at specific loci in neural membranes. However, little is known of the mechanisms that guide spectrin to membrane domains, or the consequences of its participation in cell interactions.

In the present study, and given our earlier observations that NCAM180 associates with spectrin in the brain (Pollerberg et al., 1986, 1987), we have hypothesized that NCAM might initiate the segregation of spectrin to localized membrane microdomains. We have chosen here to study βI spectrin, as it is the isoform most often associated with discrete receptor and organelle compartments, and is the form most prominent in postsynaptic densities. We find evidence for a direct interaction of the cytoplasmic domain of NCAM180 and NCAM140 with this spectrin and their colocalization in discrete membrane clusters. Building on observations that spectrin binds activated PKC (Rodriguez et al., 1999) and that PKC mediates NCAM-dependent neurite outgrowth (Kolvaa et al., 2000a), we show that PKCB2, an isoform enriched in neurons, interacts with NCAM140 and NCAM180 via spectrin in a fibroblast growth factor receptor (FGFR)–dependent manner. Activation of NCAM results in a redistribution of the NCAM–βI spectrin–PKCB2 complex to lipid-enriched microdomains to mediate neurite outgrowth.

Results

NCAM120, NCAM140, and NCAM180 form a 1% Triton X-100–insoluble complex with βI spectrin
To identify cytoskeletal components interacting with NCAM, we performed double immunofluorescence labeling of cultured hippocampal neurons using antibodies against all NCAM isoforms along with markers of microtubules, microfilaments, or spectrin. The NCAM and βI spectrin (hereafter called spectrin) distributions along neurites were similar (see Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200303020/DC1), with clusters of NCAM and spectrin localized along neurites and growth cones. After Triton X-100 extraction, 93.9 ± 0.615% of the total spectrin immunofluorescence was detected in detergent-insoluble clusters of NCAM (n = 9 neurons, 66 neurites). Conversely, the overall NCAM pattern was largely distinct from the microtubule and microfilament distributions (Fig. S1). When microtubules or microfilaments were disrupted by vinblastine or latrunculin B, respectively (and this was confirmed by labeling with tubulin antibodies and Texas red-X phalloidin), NCAM clusters remained detergent insoluble, implying that neither actin nor tubulin was responsible for NCAM’s detergent insolubility (unpublished data). Application of the drugs (latrunculin for 24 h, vinblastine for 5 h) did not have any visible effect on the morphology of neurons, in accordance with previously published data (Allison et al., 1998, 2000). Depolymerization of microtubules and actin microfilaments did not have any effect on the colocalization of NCAM and spectrin and their association in detergent-insoluble complexes (unpublished data).

Previous studies have demonstrated an interaction of NCAM180 with spectrin (Pollerberg et al., 1986, 1987). To extend this analysis, CHO cells and hippocampal neurons from an NCAM-deficient mouse were transfected with NCAM120, NCAM140, or NCAM180. Immunofluorescence analysis revealed that all three NCAM isoforms colocalized with spectrin, both in CHO cells and in neurons (Fig. 1, A and B). Cells transfected with NCAM (versus GFP alone) also accumulated more spectrin (Fig. 1, C and D). This was also observed in the brains of wild-type versus NCAM-deficient mice (Fig. 1 E). As spectrin is stabilized when incorporated into a detergent-resistant membrane cytoskeleton (Molitoris et al., 1996), we examined the impact of NCAM expression on spectrin’s detergent solubility. In CHO cells expressing any of the three major NCAM isoforms, the 0.1% Triton X-100–insoluble fraction was enriched in spectrin, whereas there was no effect on the detergent-soluble fraction (see Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200303020/DC1). We conclude that all major NCAM isoforms promote spectrin’s incorporation into a detergent-insoluble membrane skeleton.

To establish that NCAM could direct the distribution of spectrin in neurons, we used antibodies against all NCAM isoforms to cluster cell surface NCAM in live hippocampal...
neurons and analyzed the impact on spectrin (Fig. 2 A). Clustering of endogenous NCAM in wild-type neurons was accompanied by coredistribution of spectrin. In neurons from NCAM-deficient mice, the same coredistribution of spectrin was achieved when the neurons were transfected with NCAM120, NCAM140, or NCAM180. Moreover, spectrin coimmunoprecipitated with NCAM from brain homogenates, and NCAM180, NCAM140, and NCAM120 coimmunoprecipitated with spectrin (Fig. 2, B and C). NCAM180, NCAM140, or NCAM120 also coimmunoprecipitated with spectrin from transfected CHO cells (Fig. 2, D and E), confirming that the major NCAM isoforms are associated with spectrin in both normal brain and transfected cells. In these studies, NCAM180 was the most potent isoform precipitating spectrin. NCAM140 and NCAM120 precipitated 69.77% and 74.68%, respectively, of the amount of spectrin that coprecipitated with NCAM180 (set to 100%) under comparable conditions in three independent experiments.

Figure 1. NCAM120, NCAM140, and NCAM180 colocalize with spectrin and increase its steady-state level. Double immunostaining of (A) CHO cells and (B) hippocampal neurons from NCAM−/− mice transfected with NCAM120, NCAM140, or NCAM180 with antibodies against NCAM and spectrin. Note the colocalization of all NCAM isoforms with spectrin. Density profiles of NCAM and spectrin immunofluorescence intensity calculated across CHO cells (dashed lines) or along neurites overlap. Spectrin immunofluorescence intensity relative to nontransfected cells in the same field or to GFP only–transfected cells (n > 30) is significantly higher in NCAM-transfected (C) CHO cells and (D) hippocampal neurons. (E) Levels of spectrin were increased in brain homogenates from wild-type versus NCAM-deficient mice, as assayed by immunoblotting with antibodies against spectrin. Mean values ± SEM from five independent experiments are shown. AU, arbitrary units. *, P < 0.05 (paired t test). Bars: (low power) 20 μm; (high power) 5 μm.
The NH₂-terminal region of βI spectrin

To investigate whether the interaction of NCAM with spectrin was direct, the intracellular domains of NCAM180 (IC180) and NCAM140 (IC140) were immobilized on plastic, and their ability to capture purified erythrocyte spectrin (βI) was measured by semiquantitative assay. Interestingly, the intact spectrin dimer bound selectively to IC180, but not IC140 (Fig. 3 A). However, when the subunits of the erythrocyte spectrin heterodimer were dissociated, both the βI and βII chains bound not only IC180, but also IC140 (Fig. 3, B and C). There was no binding of either subunit to BSA or to the intracellular domains of a different cell adhesion molecule, a close homologue of L1 (CHL1) (unpublished data). To verify whether binding of IC180 and IC140 to βI spectrin monomers was physiologically relevant, we analyzed the oligomeric state of βI spectrin bound to brain membranes, using Western blotting of membrane fractions after PAGE under nondenaturing conditions (Fig. 3 E). Two immunoreactive spectrin bands were evident: a complex of ~520 kD, presumably representing spectrin heterodimers and possibly βI spectrin homodimers (Bloch and Morrow, 1989), and more importantly, a single band at ~240 kD, representing βI spectrin monomers. This membrane-bound βI spectrin monomeric pool amounted to ~13% of the total membrane-associated spectrin.

The NH₂ terminus of βI spectrin contains sequences highly homologous to spectrin repeats located at the COOH terminus of the αI subunit. This region also exhibits homology with α-actinin (Byers et al., 1989; Winkelmann et al., 1990). Thus, although the binding to the isolated αI spectrin was not likely to be biologically meaningful, given the paucity of αI transcripts in either epithelial cells or neurons, the fact that this subunit could bind suggested that the biologi-
cally important ligand site might reside within homologous sequences in the NH$_2$-terminal repeats of βI spectrin. To verify that NCAM bound in a biologically significant way to this region of βI spectrin, a series of spectrin truncation mutants were expressed in hippocampal neurons and in CHO cells. Neurons transfected with a GFP fusion construct encoding the NH$_2$-terminal region of βI spectrin and its first five repeat units (GFP–βI$_{N,5}$) demonstrated tight co-localization of NCAM with the GFP-labeled fragment (see Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200303020/DC1), indicating that the βI$_{N,5}$ fragment bound the NCAM complex in vivo. This was confirmed when βI$_{N,5}$ was communoprecipitated with NCAM in CHO cells after they were cotransfected with FLAG epitope-labeled βI$_{N,5}$ (FLAG–βI$_{N,5}$) and either NCAM140 or NCAM180 (Fig. 3 G). A larger spectrin fragment containing βI$_{N,5}$ fused to the ankyrin-binding region (repeats 14–15, βI$_{N,5,14,15}$) also communoprecipitated with NCAM140 and NCAM180 (unpublished data). Spectrin βI fragments encompassing repeats 1–2, 3–5, or 14–15 alone, or the actin- and dynactin-binding domain alone within the NH$_2$ terminus, did not precipitate with NCAM (Fig. 3 G), whereas the βI spectrin fragment containing 2–3 spectrin repeats communoprecipitated with NCAM140 and NCAM180. We conclude that the 2–3 homologous repeat units of βI spectrin are necessary and sufficient to bind to the intracellular domains of NCAM140 and NCAM180 in living cells.

**Lipid rafts are necessary for the interaction of NCAM120 with βI spectrin**

GPI-anchored NCAM120 is confined mainly to lipid rafts that are insoluble in cold 1% Triton (Kramer et al., 1999; He and Meiri, 2002). The coincidence of spectrin with NCAM120 (Fig. 1) suggested that spectrin also associates with lipid rafts, as it does in erythrocytes (Salzer and Prohaska, 2001). CHO cells and NCAM–/– neurons were thus transfected with NCAM120, NCAM140, or NCAM180 and treated with 5 mM methyl-β-cyclodextrin (MCD), which disintegrates lipid rafts. The relationship of spectrin to NCAM was then examined by immunofluorescence and

![Figure 3](http://www.jcb.org/cgi/content/full/jcb.200303020/DC1)

**Figure 3. The intracellular (IC) domains of NCAM180 and NCAM140 interact with the NH$_2$-terminal region of spectrin.**

(A–C) Increasing concentrations of the IC domain of NCAM180 or NCAM40 were bound to plastic and assayed by ELISA for their ability to bind heterodimeric human erythrocyte spectrin (αβI) or isolated αI and βI spectrin subunits. Binding to BSA served as a control. Mean values ± SEM from six independent experiments are shown. Note that heterodimeric spectrin bound well to IC180, whereas the isolated subunits bound to both IC180 and IC140. (D) Coomassie blue staining of the purified αβI dimers and the isolated αI and βI spectrin subunits. (E) Isolated brain membranes assayed for βI spectrin by immunoblotting after PAGE under nondenaturing conditions. Note the low molecular weight band representing βI spectrin monomers. (F) Schematic alignment of βI spectrin fragments. For comparison, the βI21 and βI22 isoforms of βI spectrin are shown. These differ by alternative mRNA splicing at their COOH terminus, which contains the pleckstrin homology (PH) domain. ABD, actin-binding domain; MAD1, membrane association domain 1; ANK, ankyrin-binding domain. (G) Lysates of CHO cells transfected with NCAM180 or NCAM140 together with FLAG-labeled spectrin fragments were immunoblotted with FLAG antibodies to confirm approximately equal expression of each of the constructs (top). Lysates from NCAM180 (middle)– and NCAM140 (bottom)–transfected cells were immunoprecipitated (IP) with NCAM antibodies and immunoblotted with FLAG antibodies to detect which of the spectrin constructs would precipitate. Note that only βI$_{N,5}$ and βI$_{K,2}$ are present in the NCAM precipitates.
coimmunoprecipitation (Fig. 4). MCD did not disturb the plasma membrane association of spectrin with NCAM140 or NCAM180. After MCD treatment, NCAM140 precipitated 74 ± 7.32% (n = 3) of the amount of spectrin that coprecipitated with NCAM180 (set to 100%). This level was similar to the amount precipitated in cells not treated with MCD. In contrast, when rafts were dispersed by MCD, the association of NCAM120 with spectrin was lost. In NCAM120-transfected CHO cells treated with MCD, the spectrin distribution internalized and shifted to a more perinuclear appearance (Fig. 4 A), presumably reflecting its association with intracellular organelles (De Matteis and Morrow, 2001). We conclude that NCAM120 interacts indirectly with spectrin through lipid rafts.

**NCAM120 promotes association of spectrin exclusively with rafts, whereas NCAM140 and NCAM180 recruit spectrin both to rafts and nonraft areas**

Whereas GPI-anchored NCAM120 is present mainly in lipid rafts, NCAM140 and NCAM180 segregate to both rafts and raft-free areas of the plasma membrane (Niethammer et al., 2002). To investigate whether NCAM can direct the association of spectrin with lipid rafts, CHO cells and NCAM120, NCAM140, or NCAM180 and extracted with cold 1% Triton X-100. After this treatment of CHO cells, NCAM-120 was detectable in coarse dispersed clusters, whereas NCAM140 and NCAM180 remained in distinct clusters (Fig. 5 A). Spectrin colocalized with all of these detergent-insoluble clusters (Fig. 5 A). The same was observed in neurons (Fig. 5, B and E). To confirm that NCAM120 promoted the association of spectrin exclusively with lipid rafts, whereas NCAM180 and NCAM140 collected spectrin in both rafts and raft-free areas, we labeled detergent-treated neurons with fluorescein-conjugated cholera toxin B subunit, which marks GM1-rich areas. All NCAM120–spectrin complexes colocalized with GM1-positive areas (Fig. 5, B–E). Conversely, only a subset of the clusters containing spectrin and NCAM140 or NCAM180 colocalized with GM1 (Fig. 5, B–E). In astrocytes present in our cultures, NCAM120, the predominant isoform expressed in glial cells
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(Bhat and Silberberg, 1986), colocalized with spectrin, and NCAM120 expressed in NCAM−/− astrocytes also colocalized with spectrin. Both NCAM120 and spectrin formed detergent-insoluble clusters that colocalized with GM1 (unpublished data), suggesting an association between NCAM120 and spectrin, albeit indirect, in glial cells.

NCAM140 and NCAM180 form a complex with PKCβ2

The pleckstrin homology domain of β1 spectrin interacts with activated PKCβ2 (Rodriguez et al., 1999). To investigate whether spectrin may link PKCβ2 to NCAM, the distribution of these proteins was examined in hippocampal neurons (Fig. 6 A). NCAM colocalized with PKCβ2 along neurites and in growth cones, where clusters of NCAM overlapped with intensely labeled accumulations of PKCβ2. Similar results were also obtained with CHO cells and NCAM−/− neurons transfected with NCAM120, NCAM140, or NCAM180 (unpublished data). Clustering of NCAM by the application of antibodies to all NCAM isoforms to live neurons redistributed PKCβ2 to NCAM clusters, confirming their coassociation in a detergent-insoluble complex (Fig. 6 A). When neurons were extracted with 1% Triton X-100, 74.6 ± 1.3% (n = 11 neurons, 84 neurites) of the total PKCβ2 immunofluorescence remained associated with NCAM clusters, confirming their coassociation in a detergent-insoluble complex (Fig. 6 A). Moreover, PKCβ2 was coimmunoprecipitated with NCAM from mouse brain homogenates (Fig. 6 B). When precipitation was performed with antibodies against PKCβ2, only NCAM140 and NCAM180 were coimmunoprecipitated (Fig. 6 C). Also in CHO cells, PKCβ2 coimmunoprecipitated with NCAM140 and NCAM180. NCAM140 precipitated 58.7 ± 8.92% (n = 3) of the amount of PKCβ2 that coprecipitated with NCAM180. PKCβ2 did not coimmunoprecipitate with NCAM120 (Fig. 6 D). Given that spectrin and NCAM120 ...
do coprecipitate (Fig. 2), the lack of PKC\(_\beta_2\) in the spectrin–NCAM120 raft complex suggested that there may be competing interactions that can favor the release of activated PKC\(_\beta_2\) from spectrin.

**NCAM activation promotes the formation of NCAM–spectrin–PKC\(_\beta_2\) complexes and enhances their association with lipid rafts**

NCAM-mediated neurite outgrowth depends on the activation of PKC (Kolkova et al., 2000a) and on the phosphorylation of one of its major substrates, GAP43 (Meiri et al., 1998). The phosphorylated forms of GAP43-like proteins accumulate in lipid rafts (Laux et al., 2000; He and Meiri, 2002), where they reorganize the cytoskeleton (Aarts et al., 1999; Laux et al., 2000). Palmitoylation of NCAM140 and its localization in lipid rafts is critical for NCAM-mediated neurite outgrowth (Niethammer et al., 2002). To study the dynamics of NCAM association with spectrin and PKC\(_\beta_2\), we performed a set of experiments aimed to estimate the amount of spectrin and PKC\(_\beta_2\) associated with NCAM at resting conditions and in response to NCAM activation. We also investigated the localization of NCAM–spectrin–PKC\(_\beta_2\) complexes with regard to lipid rafts before and after NCAM activation using GM1 as a raft marker. Activation of NCAM with NCAM-Fc or antibodies against NCAM resulted in a recruitment of PKC\(_\beta_2\) to NCAM140/NCAM180 and the formation of 1% Triton X-100–insoluble complexes (Fig. 7, A and B; Fig. 8 D). Furthermore, the recruitment of PKC\(_\beta_2\) to NCAM clusters correlated with an increase in the amount of PKC\(_\beta_2\) in detergent-insoluble clusters of NCAM (Fig. 7, A and B; Fig. 8 D). Activation of NCAM resulted also in an increase of GM1 fluorescence intensity associated with NCAM clusters, suggesting that there was a redistribution of NCAM–spectrin–PKC\(_\beta_2\) complexes into lipid rafts (Fig. 7, A and B).
NCAM associates with PKC\(_{\beta 2}\) via \(\beta I\) spectrin

NCAM140 and NCAM180 bind PKC\(_{\beta 2}\) via spectrin in an FGFR–dependent manner

To verify whether spectrin is indeed a linker protein between NCAM and PKC\(_{\beta 2}\), we transfected neurons with the \(\beta I_{2-3}\) construct that deletes spectrin’s pleckstrin homology domain, the binding site for PKC\(_{\beta 2}\), but retains the NCAM-binding site. The redistribution of PKC\(_{\beta 2}\) to NCAM clusters induced by the application of NCAM antibodies to live cells was significantly inhibited in cells transfected with the \(\beta I_{2-3}\) construct when compared with GFP-transfected cells (Fig. 8, A and B). In CHO cells cotransfected with \(\beta I_{N-5}\) or \(\beta I_{2-3}\) constructs, coprecipitation of endogenous spectrin and PKC\(_{\beta 2}\) with NCAM180 or NCAM140 was also blocked both at resting conditions and in response to NCAM activation (Fig. 8, D and E). Conversely, spectrin mutants, \(\beta I_{N-2}\) and \(\beta I_{N-5,5}\), that did not bind IC140 or IC180 did not disturb coprecipitation (Fig. 8 E). We conclude that PKC\(_{\beta 2}\) is associated indirectly with NCAM140 or NCAM180 via its interaction with spectrin.

NCAM-mediated activation of PKC occurs via the FGFR (Kolkova et al., 2000a). Inhibition of the FGFR with the specific inhibitor PD173074 blocked redistribution of PKC\(_{\beta 2}\) to detergent-insoluble NCAM clusters in response to NCAM activation (Fig. 8 C). It also significantly inhibited coprecipitation of PKC\(_{\beta 2}\) with NCAM140/NCAM180 from CHO cells (Fig. 8 D). By contrast, inhibition of the FGFR did not affect the ability of NCAM to recruit spectrin and redistribute to lipid rafts (Fig. 8, C and D).

Formation of a complex between PKC\(_{\beta 2}\), spectrin, and NCAM is implicated in NCAM-mediated neurite outgrowth

To investigate whether the association of PKC\(_{\beta 2}\) with NCAM140 and NCAM180 was functionally important for NCAM-mediated outgrowth, we compared neurite length in hippocampal neurons from wild-type mice cotransfected gradient centrifugation. Fraction 1 contained the low-density lipid rafts, whereas fraction 4 contained the high-density rafts. As described previously (He and Meiri, 2002), the amount of NCAM140/NCAM180 associated with lipid rafts gradually increased from fraction 2 to fraction 4 both in rafts isolated from brain (Fig. 7 C) and from growth cones (Fig. 7 D). All fractions (fractions 1–4) isolated from brain also contained NCAM120 that probably represents glia-associated NCAM (Fig. 7 C). PKC activity correlated in these fractions with the presence of NCAM140 and NCAM180.
NCAM140 and NCAM180 bind PKCβ2 via spectrin in an FGFR-dependent manner. (A) Wild-type neurons were transfected either with GFP or GFP together with the βI<sub>2–3</sub> construct. NCAM was clustered by application of NCAM antibodies to live cells. Note the reduced redistribution of PKCβ2 to NCAM clusters in neurons cotransfected with βI<sub>2–3</sub>. Bar, 10 μm. NCAM clusters were outlined, and the level of PKCβ2 immunoreactivity was measured within the outlines (B). Mean values ± SEM are shown (n > 20 neurites). * P < 0.05 (paired t test).

Discussion

The results presented here establish a direct structural and functional linkage between βI spectrin (hereafter called spectrin) and NCAM180 and NCAM140. This linkage mediates the localized assembly of spectrin microdomains at points of NCAM concentration, the recruitment of activated PKCβ2 to these patches, and the subsequent trans-
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The location of activated PKCβ2 to detergent-insoluble lipid rafts. Spectrin-mediated coordination between NCAM and PKCβ2 is required to trigger NCAM-mediated neurite outgrowth in cultured neurons. Several lines of data support these conclusions. Specifically, (a) NCAM180 and NCAM140 are colocalized with spectrin and PKCβ2 in detergent-insoluble clusters in cultured hippocampal neurons and in CHO cells; (b) patching and capping of NCAM by antibodies induces a coincident redistribution of spectrin and PKCβ2; (c) NCAM antibodies coprecipitate NCAM180 and NCAM140 with spectrin and PKCβ2 and vice versa; (d) the recombinant intracellular domains of NCAM180 and NCAM140 bind to spectrin; (e) the steady-state levels of spectrin are up-regulated in cells expressing NCAM and down-regulated in NCAM-deficient mouse brains; and (f) the spectrin fragments βI<sub>1,5</sub> and βI<sub>2,3</sub> coprecipitate with NCAM180 and NCAM140, but block the recruitment of PKCβ2 to NCAM clusters at the cell surface, and suppress NCAM-mediated neurite outgrowth. Collectively, these findings suggest that spectrin participates in a complex interplay between NCAM organization, the activation of PKCβ2, and the control of neurite outgrowth.

We have previously shown that FGFR activation in non-raft membrane domains, followed by the recruitment of NCAM140 to lipid rafts via palmitoylation, is necessary for neurite outgrowth (Niethammer et al., 2002). The present results establish that the spectrin-mediated recruitment of activated PKCβ2 to lipid rafts is also a step along this pathway. Our concept of how this process works is presented in Fig. 9. Earlier work has established that PKC can be activated by diacylglycerol (Inoue et al., 1977; Kishimoto et al., 1980) via the FGFR (Walsh and Doherty, 1997; Kolkova et al., 2000a). We envision that the NCAM-mediated activation of the FGFR occurs independently of lipid rafts and induces the formation of a spectrin microdomain enriched in activated PKCβ2 that binds to spectrin’s pleckstrin homology domain. Subsequent palmitoylation of NCAM140 transfers the NCAM–spectrin–PKCβ2 complex to a lipid raft. It is also possible that direct palmitoylation of spectrin may contribute to this process (Das et al., 1997). Once in the lipid raft, the NCAM–spectrin–PKCβ2 complex encounters growth-associated and cytoskeletal control molecules that include GAP43, CAP23, and MARCKS (Lau et al., 2000; He and Meiri, 2002). These proteins, recruited to lipid rafts via myristoylation, are major substrates of PKC, and at least GAP43 is required for NCAM-stimulated neurite outgrowth (Meiri et al., 1998). Thus, the recruitment of the NCAM–spectrin–PKCβ2 complex to lipid rafts may initiate the activation of downstream cytoskeletal organizers that contribute to neurite outgrowth.

We have also shown that both NCAM140 and NCAM180 can each form a signaling complex with PKCβ2 via spectrin. These data are in agreement with our previous finding that both NCAM140 and NCAM180 can signal via the FGFR–PKC pathway (Niethammer et al., 2002). NCAM-mediated neurite outgrowth depends on the activa-

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**Figure 9.** Proposed model of NCAM–spectrin–PKCβ interactions in the NCAM-triggered PKC pathway. See text in Discussion.
ition of two signaling pathways. One pathway includes activation of PKC, whereas the other requires activation of fyn and FAK kinases (Beggs et al., 1997; Kolkova et al., 2000a). NCAM140, but not NCAM180, can signal independently of the FGFR by activating fyn/FAK kinases. Due to conformational constraints in NCAM180, its interaction with fyn is prevented (Beggs et al., 1997; Kolkova et al., 2000b; Niethammer et al., 2002). Thus, because NCAM140 can activate both signaling pathways, it alone is sufficient to stimulate NCAM-mediated neurite outgrowth when expressed in NCAM−/− neurons (Niethammer et al., 2002). NCAM180, on the other hand, would then be more instrumental in the accumulation of spectrin–PKCB2 complexes at sites of cell contacts, as proposed previously (Pollerberg et al., 1986, 1987) and indicated by the localization of NCAM-180 and spectrin, for instance, in postsynaptic densities (Persohn et al., 1989; Malchiodi-Albedi et al., 1993; Schuster et al., 1998). However, preliminary evidence suggests that in hippocampal neurons expressing both NCAM140 and NCAM-180, NCAM180 may amplify NCAM140-mediated activation of the FGFR–PKC pathway, and thus participate in NCAM-mediated neurite outgrowth (unpublished data).

It is interesting, in this respect, that NCAM180 co-immunoprecipitates spectrin with higher efficiency than NCAM140 or NCAM120. This phenomenon could account for our previous observation that NCAM180, but not NCAM140, coisolated with the spectrin by immunopurification chromatography (Pollerberg et al., 1986, 1987, note the purification procedure for NCAM140 and NCAM120 in these studies, which consisted of consecutive isolation of NCAM180 from the immunopurification-purified L1 fraction and of NCAM140 and NCAM120 from the residual fraction, thus possibly accounting for a depletion of spectrin in the latter fraction). The ability of spectrin to bind as the αβ heterodimer to the recombinant intracellular domains of NCAM180, but not of NCAM140, has been noted previously (Pollerberg et al., 1986, 1987). We attribute little significance to the in vitro binding of the isolated α1 spectrin subunit, as this isoform of spectrin is either nonexistent or present at extremely low levels in either neurons or epithelial cells. However, both heterodimeric, and to a lesser extent homopolymeric, β1 spectrins exist in neurons and in other cells, often as specialized membrane complexes, such as with the secretory pathway, at the postsynaptic density, or with lymphocyte receptors (Bloch and Morrow, 1989; De Matteis and Morrow, 2000). As demonstrated here, ~13% of the total membrane-bound β1 spectrin exists in a monomeric state. We thus believe that β1 spectrin could play a role in the control of NCAM-mediated signaling.

The question arises how NCAM120, the GPI-linked isoform of NCAM, associates with spectrin. It cannot do so directly, as it lacks an intracellular domain, yet it colocalizes and coimmunoprecipitates with spectrin in both neurons and in transfected CHO cells. Both NCAM120 and spectrin associate with lipid rafts, and this association is disturbed by the dispersal of rafts after treatment with MCD. We infer that a direct association with acidic lipids, possibly in rafts, may be mediated by spectrin’s pleckstrin homology domain, as detected in other studies (for reviews see De Matteis and Morrow, 2000; Muresan et al., 2001). As PKCB2 binds to the pleckstrin homology domain and as this domain is occupied by acidic lipids in rafts, the lack of this kinase in the NCAM120 immunoprecipitates can be accounted for. The functional role of spectrin’s association with NCAM120 is interesting in view of its predominant expression by astrocytes. NCAM120 is involved in signal pathways regulating astrocyte proliferation via glucocorticoid receptors (Krushel et al., 1998). These pathways are different from the signals regulating neurite outgrowth in neurons (Krushel et al., 1998). The intracellular glucocorticoid receptor binds to the heat shock protein and chaperone hsp70 (Moriyama et al., 2000), which in turn binds to spectrin (Di et al., 1995). Whether spectrin also provides a platform for glucocorticoid receptors and whether NCAM120 modulates this interaction are intriguing issues for further studies.

Materials and methods

Antibodies and toxins

Rabbit polyclonal antibodies against NCAM (Martini and Schachner, 1986) were used in immunoprecipitation, immunoblotting, and immunocytochemical experiments, and rat monoclonal antibodies H28 against mouse NCAM (Gennarini et al., 1984) were used in immunocytochemical experiments. Both antibodies react with the three major isoforms of NCAM. Mouse monoclonal antibodies 3B8 against the NCAM140 and NCAM180 intracellular domains (Developmental Studies Hybridoma Bank) were also used for immunocytochemical experiments. The hybridoma H28 clone was obtained from Christo Goridis (Developmental Biology Institute of Marseille, Marseille, France). We also used polyclonal rabbit antibodies against human erythrocyte spectrin and mouse monoclonal antibodies against PKCB2, tubulin, and FLAG epitope (Sigma-Aldrich). Secondary antibodies against rabbit, rat, and mouse IgG coupled to Cy2, Cy3, or Cy5 were from Dianova. Cholera toxin B subunit labeled with fluorescein and vincristine were purchased from Sigma-Aldrich, latrunculin B was from Calbiochem, and Texas red-X phalloidin was from Molecular Probes.

Cultures and transfection of hippocampal neurons and CHO cells

Cultures of hippocampal neurons were prepared from 1–3-d-old C57BL/6J mice or from NCAM-deficient (NCAM−/−) mice (Cremer et al., 1994) in N2 media at least nine generations onto the C57BL/6J background. Neurons were grown in 10% horse serum on glass coverslips coated with poly-l-lysine (100 μg/ml) in conjunction with laminin (20 μg/ml) (Dityatev et al., 2000). Transfection of hippocampal neurons and neurite outgrowth quantification were performed as previously described (Niethammer et al., 2002). CHO cells were maintained in Glasgow modified Eagle’s medium containing 10% fetal calf serum. Cells were transfected using Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s instruction.

Fluorescence labeling

Indirect immunofluorescence staining of fixed cells was performed as previously described (Dityatev et al., 2000). Clustering of NCAM was induced by incubating live cells for 15 min (5% CO2 at 37°C) with NCAM antibodies, visualized with secondary antibodies applied for 5 min. To visualize cholesterol-enriched microdomains, fluorescent cholera toxin B subunit (8 μg/ml) was applied to formaldehyde-fixed cells for 30 min at room temperature. Images were acquired using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.).

Colocalization analysis

For colocalization analysis, we defined an NCAM cluster 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. Within the outlined areas, the mean intensities of NCAM, spectrin, PKCB2, and GM1 labeling associated with an NCAM cluster were measured. The same threshold was used for all groups. In nontransfected neurons, GM1 clusters were outlined using the same procedure. To determine the total amount of spectrin or PKCB2, neurites were manually outlined, and the total fluorescence of spectrin or PKCB2, along the neurites was measured. Colocalization profiles were plotted using LSM510 software.
Detergent extraction, cholesterol depletion, and cytoskeleton disruption

Detergent extraction followed Lededema et al. (1998). Cells washed in PBS, pH 7.3, were incubated for 1 min in cold microtubule-stabilizing buffer (MSB; 2 mM MgCl₂, 10 mM EGTA, 60 mM Pipes, pH 7.0) and extracted 8 min on ice with 1% Triton X-100 in MSB. After washing, cells were fixed with cold 4% formaldehyde. To deplete cholesterol from lipid rafts, cultures were incubated for 15 min at 37°C with 5 mM MCD (Sigma-Aldrich) in culture medium. To disrupt microtubules, cell cultures were incubated with vincristine (5 μM) for 5 h before fixation (Allison et al., 2000). For disruption of actin filaments, cultures were incubated in 5 μM latrunculin B for 24 h before fixation (Allison et al., 1998).

Protein purification

The αβγ spectrin dimers were purified from erythrocyte ghosts (Shotton, 1998). Cells were washed three times in 15 volumes of 155 mM NaCl and then three times in 15 volumes of 155 mM sodium phosphate buffer, pH 7.6. Erythrocytes were lysed for 20 min in 15 volumes of 10 mM sodium phosphate buffer, pH 7.6. Erythrocyte ghosts were collected by centrifugation at 30,000 g for 10 min at 4°C, washed in ice-cold extraction buffer (1 mM EDTA, 10 μM DTT, pH 9.5), sonicated for 30 s, incubated for 60 min at 37°C with occasional rocking, and centrifuged at 230,000 g for 60 min at 4°C. The supernatant containing αβγ spectrin dimers and actin was further purified by gel filtration chromatography on Sepharose 4B in 1 mM Tris-HCl buffer, pH 8.0, containing 100 μM EDTA, 10 μM DTT, and 0.02% NaN₃.

The isolated spectrin α and β subunits of spectrin were purified according to Davis and Bennett (1983). The αβγ spectrin dimers were dialyzed against 10 mM sodium phosphate buffer, pH 6.3, containing 7 M urea, 0.02% NaN₃, and 0.05% Tween 20. Subunits were purified by 80 mM sodium phosphate buffer, pH 7.3, containing 7 M urea, 10 mM glycine, 0.05% Tween 20, and 1 mM DTT. β Spectrin was eluted by 250 mM sodium phosphate buffer, pH 7.1, containing 7 M urea, 10 mM glycine, 0.05% Tween 20, and 1 mM DTT. Spectrin was eluted with the appropriate primary antibodies followed by incubation with peroxidase-labeled secondary antibodies and visualized using Super Signal West Pico reagents (Pierce Chemical Co.) on BIOMAX film (Schleicher & Schuell) for 3 h at 250 mA. Immunoblots were incubated with the appropriate primary antibodies followed by incubation with peroxidase-labeled secondary antibodies and visualized using Super Signal West Pico reagents (Pierce Chemical Co.) on BIOMAX film (Sigma-Aldrich). Molecular weight markers were prestained protein standards from Bio-Rad Laboratories.

Online supplemental material

The supplemental material (available at http://www.jcb.org/cgi/content/full/jcb.200303102/DC1) includes images showing colocalization of NCAM and spectrin in detergent-insoluble clusters in neurons (Fig. S1). Western blots showing NCAM-mediated formation of a detergent-insoluble spectrin cytoskeleton (Fig. S2), and neurite outgrowth data showing the involvement of NCAM association with PKCβ via spectrin in NCAM-mediated neurite outgrowth (Fig. S3).

We thank Dr. Harold Cremer (Developmental Biology Institute of Marseille, Marseille, France) for his gift of NCAM-deficient mice. We are grateful to Achim Dahlmann, Galina Dityateva, and Eva Kronberg for genotyping, cell culture, and animal care, Drs. Ulrich Bornmann and Melanie Richter for purification of NCAM-Fc and intracellular domains of CHL1, NCAM140, and NCAM180, and Drs. Patricia Maness and Elisabeth Bock for the gift of NCAM cDNAs. Drs. Carol Cianci and Deepti Pradhan and Mr. Paul Stabach are thanked for their help with this paper and for assistance with the spectrin constructs. We thank Dr. Alexander Dityatev for helpful discussions and suggestions.

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PKC assay and immunoblotting

PKC activity was measured using the PepTag assay for nonradioactive detection according to the manufacturer’s instructions (Promega). Proteins separated by SDS-PAGE (8%) or PAGE (3–6%) (nondenaturing conditions) were electroblotted onto nitrocellulose transfer membrane (PROTRAN; Schleicher & Schuell) for 3 h at 250 mA. Immunoblots were incubated with the appropriate primary antibodies followed by incubation with peroxidase-labeled secondary antibodies and visualized using Super Signal West Pico reagents (Pierce Chemical Co.) on BIOMAX film (Sigma-Aldrich). Molecular weight markers were prestained protein standards from Bio-Rad Laboratories.

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