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Cortactin-Src Kinase Signaling Pathway Is Involved in N-syndecan-dependent Neurite Outgrowth*

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Tarja Kinnunen‡§¶‖, Marko Kaksonen‡§¶, Juhani Saarinen, Nisse Kalkkinen**, H. Benjamin Peng‡‡, and Heikki Rauvala‡§**

*From the ‡Laboratory of Molecular Neurobiology, Institute of Biotechnology, §Department of Biosciences, Division of Biochemistry, **Protein Chemistry Laboratory, Institute of Biotechnology, University of Helsinki, P. O. Box 56, 00014 Helsinki, Finland and the ‡‡Department of Cell Biology and Anatomy, University of North Carolina, Chapel Hill, North Carolina 27599*

N-syndecan (syndecan-3) was previously isolated as a cell surface receptor for heparin-binding growth-associated molecule (HB-GAM) and suggested to mediate the neurite growth-promoting signal from cell matrix-bound HB-GAM to the cytoskeleton of neurites. However, it is unclear whether N-syndecan would possess independent signaling capacity in neurite growth or in related cell differentiation phenomena. In the present study, we have transfected N18 neuroblastoma cells with a rat N-syndecan cDNA and show that N-syndecan transfection clearly enhances HB-GAM-dependent neurite growth and that the transfected N-syndecan distributes to the growth cones and the filopodia of the neurites. The N-syndecan-dependent neurite outgrowth is inhibited by the tyrosine kinase inhibitors herbimycin A and PP1. Biochemical studies show that a kinase activity, together with its substrate(s), binds specifically to the cytosolic moiety of N-syndecan immobilized to an affinity column. Western blotting reveals both c-Src and Fyn in the active fractions. In addition, cortactin, tubulin, and a 30-kDa protein are identified in the kinase-active fractions that bind to the cytosolic moiety of N-syndecan. Ligation of N-syndecan in the transfected cells by HB-GAM increases phosphorylation of c-Src and cortactin. We suggest that N-syndecan binds a protein complex containing Src family tyrosine kinases and their substrates and that N-syndecan acts as a neurite outgrowth receptor via the Src kinase-cortactin pathway.

HB-GAM[1] was initially isolated from neonatal rat brain as a neurite outgrowth-promoting protein, the expression of which in brain corresponds to the stage of rapid axonal growth (1). Molecular cloning of full-length cDNA identified a novel secretory sequence (2). The same cDNA sequence was reported for pleiotrophin, a protein suggested to be mitogen for fibroblastic cells (3, 4). The HB-GAM/pleiotrophin sequence shares approximately 50% homology with the midkine protein involved in retinoic acid-induced cell differentiation (5–7).

The expression of HB-GAM in the axon pathways of the brain and in the basement membranes outside of brain (8–9) and the neurite outgrowth-promoting property of HB-GAM *in vitro* (see Refs. 1 and 4) have suggested interaction of matrix-associated HB-GAM with a cell surface receptor. Furthermore, HB-GAM is expressed at the surface of developing muscle cells and is suggested to play a role in the development of nerve/muscle contacts (10–12).

N-syndecan (syndecan-3) has recently been isolated from detergent extracts of perinatal rat brain as a receptor or co-receptor for HB-GAM using recombinant HB-GAM as an affinity matrix (13). N-syndecan is localized at the surface of neurites and their growth cones in rat primary neurons growing on HB-GAM-coated matrix *in vitro* (13). Furthermore, HB-GAM and N-syndecan are spatiotemporally co-expressed in developing rat brain (14).

The cell surface N-syndecan interacts with HB-GAM through its heparan sulfate chains (15). This interaction is enhanced by assembly of the heparan sulfates in the protein moiety and is specific, as soluble N-syndecan and N-syndecan-derived glycosaminoglycans, in contrast to several other glycosaminoglycans, inhibit HB-GAM-induced neurite outgrowth *in vitro* (15). Anti-N-syndecan antibodies also inhibit specifically the interaction of HB-GAM with brain neurons (13).

N-syndecan is a member of the syndecan gene family of transmembrane heparan sulfate proteoglycans that are expressed in cell- and development-specific patterns (16). The four syndecans (syndecan-1, -2, -3, and -4) have structurally variable extracellular domains, but a high degree of conservation is found on their cytoplasmic domains (for reviews, see Refs. 17–20). The highly conserved cytoplasmic domain suggests an important function for it. This could occur either by association of syndecans directly with the cytoskeleton or with second messenger pathways. The core protein of syndecan-1 has been shown to colocalize with actin in syndecan-1-transfected Schwann cells (21), and syndecan-4 has been shown to localize to focal contacts (22).

The functions of cell surface proteoglycans and their interactions with the extracellular matrix have been widely studied (for reviews, see Refs. 23–25). In this paper, we study the HB-GAM-induced signals mediated by N-syndecan to the cytoplasm. We have transfected N18 neuroblastoma cells with an N-syndecan cDNA and show that cells expressing N-syndecan respond to HB-GAM by growing neurites, whereas the parental cells or mock-transfected cells respond weakly if at all. We have purified proteins from detergent extracts of rat brain and show that kinase activity together with substrate(s) associates with the N-syndecan cytosolic domain. Biochemical studies show that a protein complex containing Src family kinases (c-Src and Fyn) and their substrate(s) binds to N-syndecan. Cell-biological studies show that phosphorylation of both c-Src and cortactin is

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¶ Contributed equally to this work.
‖ To whom correspondence should be addressed. Tel.: 358-9-70859062; Fax: 358-9-70859068.

[1] The abbreviations used are: HB-GAM, heparin-binding growth-associated molecule; PAGE, polyacrylamide gel electrophoresis; RAGE, receptor for advanced glycation end products.

increased when N-syndecan is ligated by HB-GAM. We suggest that N-syndecan associates with the Src-cortactin pathway and thus mediates neurite outgrowth induced by extracellular matrix-bound HB-GAM.

EXPERIMENTAL PROCEDURES

Cloning of N-syndecan cDNA and Construction of an N-syndecan Expression Vector—N-syndecan cDNA was isolated from a rat hippocampal cDNA library (Stratagene), using a fragment of N-syndecan cDNA produced by polymerase chain reaction as a probe (14). A cDNA of 5.5 kilobases was isolated and confirmed by sequencing to correspond to an N-syndecan sequence (26). A 1.8-kilobase *Eco*RI-*Xho*I restriction fragment containing a 47-base pair leader, the complete 1329-base pair coding sequence, and a 419-base pair 3′-untranslated region was ligated into the pHβA-pr1-neo vector (27), which was cut with *Eco*RI and *Xho*I restriction enzymes. pHβA-pr1-neo contains human β-actin promoter, which drives a strong ubiquitous expression and a neomycin resistance gene.

Transfection of N18 Neuroblastoma Cells—N18 cells were grown in Dulbecco's modified Eagle's medium + 10% fetal calf serum. The cells were transfected by lipofection using Transfectam reagent (IBF Biotechnics). G418 (600 μg/ml; Life Technologies, Inc.) was added to the medium after 48 h to select for stably transfected clones. Individual G418-resistant clones were picked and screened for N-syndecan expression by Northern blotting as described (14) using the 1.8-kilobase N-syndecan cDNA as a probe. Two clones having the highest level of expression were chosen for further study. Control cells were transfected with vector only, and two G418-resistant clones were chosen randomly. N-syndecan-transfected and mock-transfected clones have been cultured under identical conditions.

Neurite Outgrowth Assays—Subconfluent N18 cultures were used for neurite outgrowth assay. Assays were done essentially as described before (28). Cells were detached by incubation in phosphate-buffered saline for 15 min followed by vigorous pipeting with a Pasteur pipette. Microtiter wells were coated by incubating with HB-GAM solution (5 μg/ml) for 20 h at +4 °C. Cells were plated on HB-GAM-coated wells (5×10^3 cells/well) and grown in serum-free Dulbecco's modified Eagle's medium containing 10 mg/ml bovine serum albumin for 24 h and fixed with 2% glutaraldehyde for 30 min. Proportion of neurite-bearing cells was counted with inverted microscope using phase contrast. Cell processes longer than two cell diameters were counted as neurites. For microphotography, the cells were seeded on microscope coverslips coated with HB-GAM.

Immunocytochemistry—Cells were grown on coverslips as in neurite outgrowth assays for 24 h. They were fixed with 4% paraformaldehyde and blocked with 10% fetal calf serum in phosphate-buffered saline for 1 h. Cells were incubated with polyclonal anti-N-syndecan antibodies (5 μg/ml; Ref. 14) for 1 h at room temperature in blocking solution. Binding of the antibodies was detected with TRITC-labeled goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc.). Olympus Provis 70 microscope with a 60 × apochromat objective was used for microscopy. Micrographs were taken using a Photometrics SenSys CCD camera.

Isolation of N-syndecan Binding Proteins—The peptide corresponding to the cytosolic domain of rat N-syndecan with an N-terminal cysteine (CRMKKKDEGSYTLEEPKQASVTYQKPDKQEEFYA) was synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (ABI 433) and purified with high performance liquid chromatography. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of the purified peptide confirmed the sequence expected from the chemical synthesis. The peptide was coupled to Thiopropyl Sepharose 6B (Pharmacia Biotech Inc.) according to the manufacturer's instructions. Residual active groups of thiopropyl-Sepharose were blocked by reaction with 2-mercaptoethanol at low pH (4.5) at which the aliphatic disulfides that link the protein with the gel remain intact. Forebrains from 4- to 6-day-old rats were homogenized in ice-cold extraction buffer containing 25 mM Tris-HCl, pH 8.5, 130 mM NaCl, 1% Nonidet P-40, with 1 mM NaF, 1 mM Na_3VO_4 as phosphatase inhibitors, and 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.6 μg/ml leupeptin, and 0.7 μg/ml pepstatin as protease inhibitors. The homogenate was extracted for 1 h and centrifuged at 100,000 × *g* for 1 h. The supernatant was precleared with HiTrap heparin affinity column (Pharmacia) and applied to the peptide affinity column. The column was washed with 20 column volumes of the extraction buffer with 0.1% Nonidet P-40, 5 column volumes of the extraction buffer with 0.1% Nonidet P-40 containing 2% bovine serum albumin, followed by washing with 5 column volumes of the extraction buffer with 0.1% Nonidet P-40. The column was further washed with 10 column volumes of each 0.2 M and 0.3 M NaCl. Bound proteins were eluted with 2.5 column volumes of 1 mg/ml of the peptide corresponding to full-length cytosolic domain of rat N-syndecan (NS-1). In some experiments, the column was eluted in parallel with a synthetic peptide corresponding to the membrane proximal region of the syndecan cytosolic domain (NS-2, 1 mg/ml, RMKKKDEGSYC; Multiple Peptide Systems) in the extraction buffer with 0.1% Nonidet P-40. As a control, the column was eluted with a peptide corresponding to the cytosolic domain of rat receptor for advanced glycation end products (RAGE, Ref. 29 and GenBank accession number L33413; CRKRQPRLEERKAPESQEDEEERAELNQSEEAEMPENGAGGP). After elution with the peptide, the column was further washed with 1 and 2 M NaCl. In some experiments, a blank Sepharose matrix column was run in parallel and analyzed for binding components.

N-syndecan and coeluting proteins were purified by HB-GAM affinity chromatography as described (13).

Kinase Activity Assays and Immunoprecipitation—Peptide affinity fractions were concentrated with Microcon microconcentrators (Amicon) and dissolved in 100 mM Tris-HCl, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 0.25 mM Na_3VO_4, 2 mM dithiothreitol. 2 μCi of [γ-^{32}P]ATP (5000 Ci/mmol) in 75 mM $MnCl_2$, 0.5 mM cold ATP was added to the fractions, and the reaction mixtures were incubated at 30 °C for 10 min. An aliquot of the kinase reaction was diluted with kinase buffer and immunoprecipitated with anti-cortactin antibodies (Upstate Biotechnology, Inc.) overnight at +4 °C. The samples were then incubated with protein G-agarose beads (Sigma) for 3 h at +4 °C. Agarose beads were collected by centrifugation and washed twice with the kinase buffer; the bound immunocomplexes were eluted with SDS-PAGE sample buffer. Samples from the kinase activity assay and immunoprecipitations were subjected to SDS-PAGE and autoradiography.

Characterization of Proteins That Bind to the Cytosolic Moiety of N-syndecan—Proteins of the peptide affinity fractions were separated on 12% SDS-PAGE, and the proteins of interest were excised from the gel, cut to 1 × 1-mm pieces, destained, and dehydrated by washing twice with acetonitrile and dried in a vacuum centrifuge. Proteins were reduced by rehydrating the gel pieces in 10 mM dithiothreitol in 0.1 M NH_4HCO_3 for 30 min at 56 °C. The dithiothreitol solution was removed, and the proteins were alkylated by addition of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. Excess iodoacetamide was removed by washing with 0.1 M NH_4HCO_3, followed by dehydration with acetonitrile, and the samples were dried in a vacuum centrifuge. The proteins were digested by rehydrating the gel pieces with 10 μl of lysyl endopeptidase (40 ng/μl) in digestion buffer (0.1 M Tris, pH 9.2, containing 10% acetonitrile). Digestion was carried out overnight at 37 °C and stopped by acidification with trifluoroacetic acid, a final concentration of 1%.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed in the delayed extraction mode with a BIFLEX mass spectrometer (Bruker-Franzen Analytik, Germany), using a 337-nm nitrogen laser. A thin layer matrix preparation with saturated α-cyano-4-hydroxycinnamic acid in acetone was used (30). 0.5 μl of matrix was deposited on a stainless steel target plate, and 0.5 μl of sample was added on top of the matrix. External calibration was performed with angiotensin 2 and ACTH clip (Sigma).

For N-terminal sequence analysis, the peptides were extracted from the gel pieces with two 30-min washes with 0.1% trifluoroacetic acid in 60% acetonitrile and concentrated in a vacuum centrifuge. Extracted peptides were separated by reversed-phase chromatography using LCPackings 1 × 150-mm C8 column (packed with Vydac C8 300 Å, 5-μm material) on Pharmacia SMART system with a trifluoroacetic acid-acetonitrile solvent system. N-terminal sequence analysis was performed with an Applied Biosystems PROCISE494 sequencer.

Immunoblotting—Fractions eluted from the N-syndecan peptide column were separated on 10–20% SDS-PAGE and transferred to nitrocellulose filters. Immunoblotting was done essentially as described (1) using horseradish peroxidase-conjugated secondary antibodies detected with enhanced chemiluminescence (Amersham). Monoclonal antibody against cortactin, 4F11 (anti-p80/85), and monoclonal antibody to c-Src, GD11, were from Upstate Biotechnology Inc. Monoclonal antibody to Fyn (FYN-15) and polyclonal antibody to c-Src (SRC-2) were from Santa Cruz Biotechnology, Inc. Monoclonal antibody to class III β-tubulin (SDL.3DID) was from Sigma. N-syndecan was immunoblotted with polyclonal N-syndecan antibodies (14) as described (13).

Analysis of Src and Cortactin Phosphorylation—N-syndecan-transfected and mock-transfected N18 cells were cultured on HB-GAM-coated Petri dishes as described above. At times indicated, the cells were lysed with extraction buffer (see above) and centrifuged briefly to remove debris. The supernatant was incubated with anti-phosphotyrosine agarose beads (Upstate Biotechnology Inc.) overnight at +4 °C.

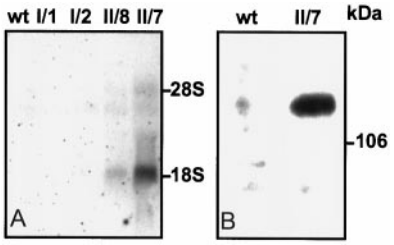

FIG. 1. **Expression of N-syndecan in transfected N18 cells.** *A*, Northern blot analysis of parental N18 cells (wt), mock-transfected cells (I/1 and I/2), and N-syndecan-transfected cells (II/8 and II/7). 20 μg of total RNA was loaded per lane. Ethidium bromide staining detected an equal amount of RNA on each lane. The transfected cell lines express mRNA of the expected size, whereas no expression of an endogenous N-syndecan mRNA can be detected (clones wt, I/1, and I/2). *B*, Western blotting of N-syndecan extracted from the parental cells and from the N-syndecan transfected cells. N-syndecan was bound to the HB-GAM affinity column, eluted with 0.5 M NaCl and deglycosylated by nitrous acid treatment prior to Western blotting. Affinity-purified anti-peptide antibodies detect the core protein of about 120 kDa in the transfected cells, whereas little, if any, expression is observed in the parental cells (see also Fig. 4).

The agarose beads were washed twice with the extraction buffer, and bound proteins were eluted with SDS-PAGE sample buffer and subjected to gradient SDS-PAGE and immunoblotting.

RESULTS

N-syndecan Transfected to N18 Cells Mediates Tyrosine Kinase-dependent Neurite Outgrowth—N18 mouse neuroblastoma cells were transfected with an N-syndecan expression vector, which contains N-syndecan cDNA under the control of human β-actin promoter (27). Single G418-resistant clones were picked, and the level of N-syndecan expression was studied by Northern hybridization. Two clones (II/7 and II/8) having the highest expression level were chosen for further analysis (Fig. 1*A*). Two separate G418-resistant clones of N18 cells transfected with vector without cDNA were chosen as controls. Extraction of the transfected cells with detergent and analysis using HB-GAM affinity chromatography showed that binding of the transfected N-syndecan to HB-GAM was similar to the binding of the endogenous protein (13) in that it was reversed at about 0.5 M NaCl. The core protein size of about 120 kDa was detected for the transfected N-syndecan (Fig. 1*B*), as was previously found for the brain N-syndecan (13). The intensity of the 120-kDa core protein band was 2.5-fold higher in the clone (II/7) found to express N-syndecan at a higher level in Northern blotting as compared with the clone having a lower expression level (II/8).

N18 neuroblastoma cells can be induced to differentiate into neuron-like cells by serum starvation (31). They respond strongly to extracellular matrix molecules such as laminin and fibronectin by spreading and growing processes. On HB-GAM, they spread poorly and grow only very few processes. N18 cells express very little, if any, N-syndecan (Fig. 1).

To study the effect of N-syndecan expression on neurite growth, the parental cell line and the mock-transfected and the N-syndecan-transfected cell lines were grown for 24 h on HB-GAM-coated surfaces. Phase contrast microscopy revealed an obvious difference in the N-syndecan-transfected cells as compared with the nontransfected or mock-transfected cells. In particular, the transfected cells with a higher N-syndecan expression level grew long neurites extending several diameters of the cell soma (Fig. 2). Most of the neurite-extending cells were mono- or bipolar. The N-syndecan-expressing clones had approximately three and nine times more neurite-bearing cells as compared with the parental and mock-transfected cells (Figs. 2 and 3), depending on the level of N-syndecan expression. The number of adherent cells after the 24-h culture did not vary between clones (not shown).

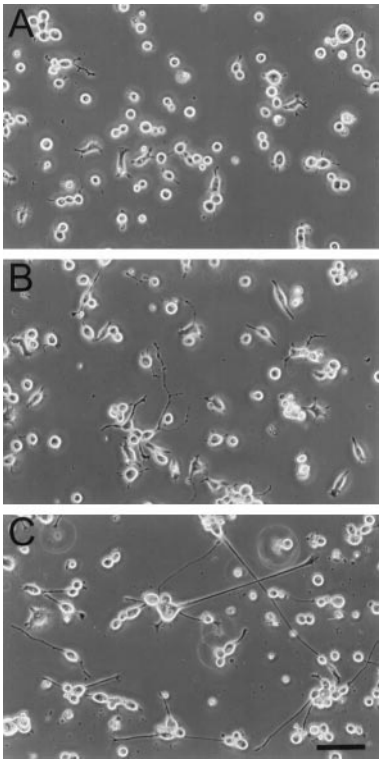

FIG. 2. **Phase contrast micrographs of N18-cells.** Parental N18 cells (*A*), clone II/8 (*B*), and clone II/7 (*C*) were grown on HB-GAM-coated coverslips for 24 h in serum-free medium. *Scale bar*, 100 μm.

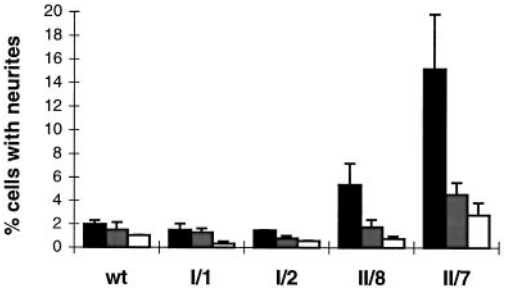

FIG. 3. **Neurite outgrowth in the different cell clones on HB-GAM-coated matrix in the absence and presence of tyrosine kinase inhibitors.** Proportions of neurite-bearing cells in parental (wt), mock-transfected (I/1 and I/2), and N-syndecan-transfected (II/8 and II/7) clones were measured. Cells were grown for 24 h on HB-GAM-coated culture wells (*black bars*). Neurite outgrowth in the N-syndecan-transfected clones is inhibited by 1 μM herbimycin A (*gray bars*) and by 1 μM PP1 (*white bars*). The data represent mean values (+S.E.) from four independent experiments (*black bars*) and mean values (+S.E.) from three independent experiments for inhibition assays (*gray* and *white bars*).

As neurite growth is known to be regulated by phosphorylation, effect of the tyrosine kinase inhibitor herbimycin A on N18 cells was tested. Herbimycin A at the concentration of 1 μM had no effect on the proportion of neurite-bearing cells in parental and vector-transfected cells but reduced HB-GAM-induced neurite outgrowth in N-syndecan expressing clones almost to the background level (Fig. 3). As biochemical experiments suggested that Src-kinase(s) interact with N-syndecan (see below), a selective inhibitor of the Src family kinases (PP1; Ref. 32) was also tested. PP1 also inhibited N-syndecan-dependent neurite outgrowth virtually completely (Fig. 3).

N-syndecan Distributes to Growth Cones and Their Filopodia—To evaluate N-syndecan expression at the plasma membrane level, N-syndecan was immunostained in different cell clones using affinity-purified anti-peptide antibodies that bind

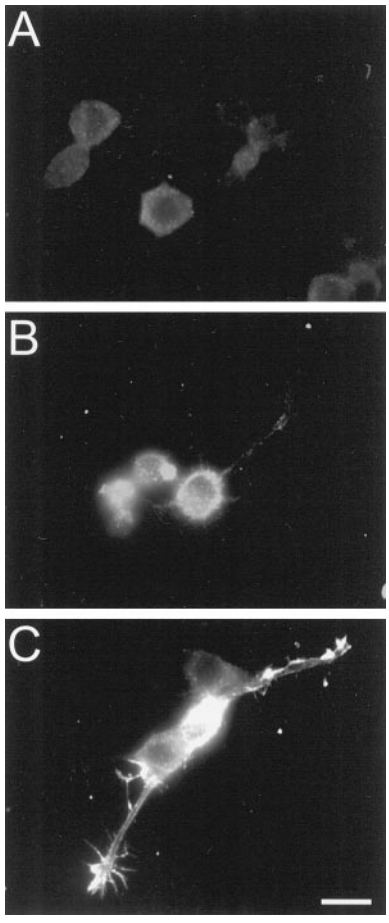

FIG. 4. **Localization of N-syndecan in N18 cells grown on HB-GAM matrix.** Parental N18 cells (*A*), clone II/8 cells (*B*), and clone II/7 (*C*) cells were stained with affinity-purified anti-peptide antibodies against N-syndecan (5 μg/ml). Staining is seen in the transfected cells (*B* and *C*) in the cell soma and in neurites. Staining in the neurites is localized to growth cone and filopodia. Parental cells (*A*) show faint staining, which is probably nonspecific. *Scale bar*, 20 μm.

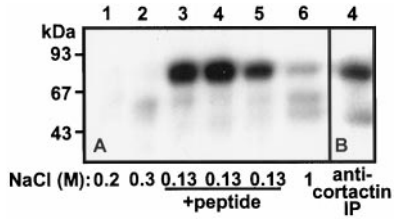

FIG. 5. **Kinase activity and its substrate bind to the cytosolic moiety of N-syndecan.** *A*, N-syndecan cytosolic peptide coupled to Sepharose (2 mg of peptide/ml of affinity matrix) was used as a peptide affinity column. Crude rat brain (postnatal days 4–6) extract was fractionated using the affinity column and eluted with increasing molarity of NaCl (*lanes 1* and *2*), followed by elution with the full-length cytosolic peptide (1 mg/ml) in physiological NaCl (*lanes 3–5*). 2 μCi of [γ-^{32}P]ATP was added to the affinity fractions in kinase buffer and incubated at 30 °C for 10 min, and the fractions were subjected to gradient SDS-PAGE and autoradiography. *B*, an aliquot of the kinase activity reaction shown on *lane 4* was immunoprecipitated with anti-cortactin antibodies (4F11).

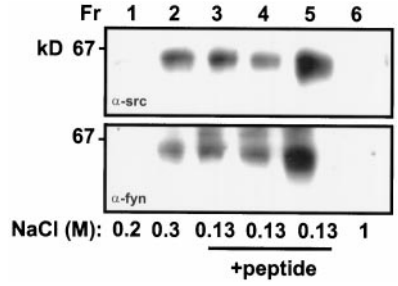

FIG. 6. **Src family kinases associate with N-syndecan.** The peptide affinity fractions were immunoblotted with anti-Src (GD11) and anti-Fyn (Fyn-15) antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and detected with ECL.

to the N-terminal sequence of the core protein. The expression level of N-syndecan at the cell surface apparently correlated to the expression level detected by Northern blotting (compare Figs. 1 and 4). N-syndecan was detected in the cell body and in neurites, especially in growth cones and in filopodia (Fig. 4).

Kinase Activity and Its Substrate(s) Bind Specifically to the Cytosolic Moiety of N-syndecan—To detect possible kinase activity that binds to N-syndecan, crude extracts of postnatal rat brain were analyzed using an affinity column to which the cytosolic moiety of N-syndecan had been coupled (Fig. 5*A*). The affinity column was extensively washed, after which the components binding specifically to the column were displaced using the cytosolic peptide in the elution buffer. An intrinsic kinase activity was consistently detected in the fractions displaced from the column by the free peptide (Fig. 5*A*). The activity remained bound to the column that was washed from a physiological to a somewhat higher salt (0.3 M) concentration but was displaced by the free peptide in a buffer containing physiological salt. Some remaining activity was eluted from the column by a high salt concentration (Fig. 5*A*).

The major phosphorylated component in the N-syndecan-binding, kinase-active fractions migrated at 80 kDa on SDS-PAGE (Fig. 5*A*). As Src family tyrosine kinases occur in the same fractions (see below), this phosphorylated band might be due to cortactin (p80/85) that has been identified as a major substrate of pp60^{src} (33). Immunoprecipitation experiments revealed that cortactin indeed is the major phosphorylated component in the fractions containing intrinsic kinase activity (Fig. 5*B*). These results suggest that a kinase activity, together with its substrate(s), associates with the cytosolic domain of N-syndecan.

Src and Fyn Tyrosine Kinases Associate with N-syndecan Cytosolic Tail—The HB-GAM-induced neurite outgrowth from N-syndecan-transfected N18 cells was inhibited by herbimycin and PP1 (see above). This suggests involvement of tyrosine kinase(s) in the N-syndecan-mediated neurite growth. The major non-receptor tyrosine kinases of the growth cones (where N-syndecan is expected to mediate neurite growth (see above)) belong to the Src family kinases (34, 35). Western blotting of the fractions that bind to the cytosolic moiety of N-syndecan detected both c-Src and Fyn in the kinase-active fractions (Fig. 6). Both c-Src and Fyn peak in the fractions eluting with the peptide, but their interaction with the affinity column can be partially reversed already at 0.3 M NaCl (Fig. 6). The substrate sites of the kinases thus seem to bind more tightly to the affinity column because in the intrinsic kinase assay the activity associates more rigorously to the fractions that are displaced by the N-syndecan peptide (compare with Fig. 5*A*).

Tubulin, Cortactin, and a 30-kDa Protein Are Major Components Binding to the Cytosolic Moiety of N-syndecan—Coomassie Blue staining of the fractions binding specifically to the affinity column detected consistently three major proteins. A major protein migrating at about 50 kDa on SDS-PAGE, a doublet migrating at about 30 kDa, and an 80-kDa protein eluted specifically with the peptide (Fig. 7). Sequences of endoproteinase Lys-C peptides from the 50-kDa protein were read as EDAANNYARGHYTIGK, EIIDLVLDRIRK, and GHYTEGAELVDSVLDVVK, and they match accurately with the amino acid sequences of rat α- and β-tubulin (36, 37). The two different tubulin sequences indicate that both α- and β-tubulin

FIG. 7. **Proteins that bind specifically to the N-syndecan cytoplasmic moiety.** The peptide affinity column was eluted as in Fig. 5. After elution with the cytosolic peptide, the column was further washed with 1 and 2 M NaCl. *Lane 1*, total brain extract applied to the column. *Lanes 2* and *3*, elution with increasing molarity of NaCl. *Lanes 4–6*, elution of the column with the cytosolic peptide. *Lanes 7* and *8*, elution with 1 and 2 M NaCl, respectively. Proteins were separated on 10–20% SDS-PAGE and visualized by Coomassie Blue. The 43-kDa component that elutes nonspecifically to all fractions is due to actin.

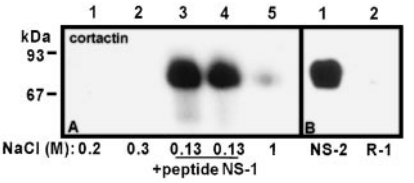

FIG. 8. **Cortactin elutes from the affinity column specifically with the N-syndecan peptide.** *A*, crude rat brain extract was fractionated using the peptide affinity column as in Figs. 5–7. The column was eluted with the full-length cytoplasmic peptide (NS-1), and fractions were immunoblotted with monoclonal anti-cortactin antibody, 4F11. *B*, cortactin eluted from the peptide affinity column also with a peptide corresponding to the membrane proximal domain of syndecan (NS-2), whereas rat RAGE cytoplasmic peptide (R-1) did not elute cortactin from the syndecan column. *Lanes 1* and *2* in *B* correspond to *lane 4* in *A*.

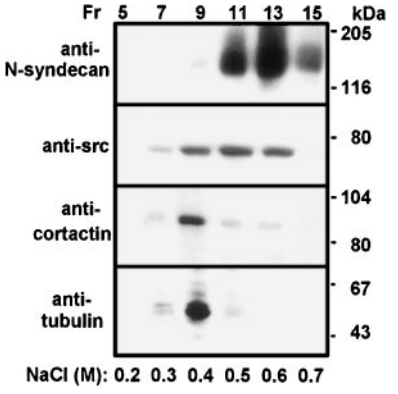

FIG. 9. **Association of c-Src and cortactin with N-syndecan in tissue.** Crude extract of rat brain was passed through the HB-GAM affinity column that was eluted with a linear NaCl gradient. The affinity fractions were immunoblotted with polyclonal anti-N-syndecan and anti-Src (SRC-2) antibodies and monoclonal anti-cortactin (4F11) and class III β-tubulin (SDL.3DID) antibodies.

are present in the complex associating with N-syndecan. A monoclonal antibody against the neuron-specific, class III β-tubulin also identified this band in Western blotting (not shown). It is noteworthy that the 50-kDa band corresponding to tubulin in protein staining (Fig. 7) and in immunoblotting elutes specifically with the peptide, whereas the 43-kDa actin band detected by protein staining (Fig. 7) and by immunostaining (not shown) elutes nonspecifically in all fractions.

The 30-kDa protein could not be identified by peptide mass fingerprinting (38), suggesting that the sequence of this protein is not available in the databases searched. The 80-kDa protein was identified as cortactin by immunoprecipitation from the kinase assay and by Western blotting with monoclonal antibody 4F11 (see above and Fig. 8).

Displacement of the binding components from the affinity column was also tested using a membrane proximal cytosolic peptide (RMKKKDEGSYC; NS-2), a polypeptide region that has the same sequence in all known syndecans. This peptide eluted the same set of binding components as effectively as the full-length cytosolic peptide (shown for cortactin in Fig. 8*B*).

RAGE, an immunoglobulin superfamily member, mediates neurite outgrowth (39) and binds an overlapping, though a distinct set of proteins when used as an affinity matrix in the same way as the N-syndecan cytosolic peptide.[2] Experiments were therefore carried out to elute the N-syndecan-binding components using the full-length cytosolic peptide of RAGE. This peptide did not elute any of the components binding to the N-syndecan peptide (shown for cortactin in Fig. 8*B*).

C-Src, Cortactin, and Tubulin Copurify with N-syndecan from Brain Tissue—To study the association of c-Src, cortactin, and tubulin with N-syndecan in tissue, detergent extracts of rat brain were passed through an HB-GAM affinity column. After extensive washing, the affinity column was eluted with a linear NaCl gradient. The elution patterns of c-Src, cortactin, and tubulin partially overlapped with the N-syndecan elution pattern (Fig. 9). The binding of cortactin, tubulin, and c-Src to N-syndecan seems to be slightly weaker than that of N-syndecan to HB-GAM, as association of cortactin, c-Src, and tubulin to the column was reversed with a slightly lower NaCl concentration (0.3–0.6 M for cortactin and Src, and 0.3–0.5 M for tubulin) than the binding of N-syndecan (0.45–0.7 M). Co-elution of cortactin and tubulin with c-Src thus agrees with the prior findings that both of these proteins are co-immunoprecipitated with c-Src from cells (40, 41).

Phosphorylation of c-Src and Cortactin Is Enhanced in N-syndecan-transfected N18 Cells upon Growth on HB-GAM—The effect of HB-GAM on phosphorylation of Src kinases and their substrates was studied using N-syndecan-transfected N18 cells and mock-transfected cells. The N-syndecan-transfected (II/7) and the vector-transfected (I/2) cells were grown on an HB-GAM matrix for various time periods, after which the cells were lysed and immunoprecipitated with anti-phosphotyrosine agarose. The precipitated materials were subjected to Western blotting with c-Src and cortactin antibodies. C-Src and cortactin phosphorylation was repeatedly enhanced upon interaction of N-syndecan-transfected N18 cells with an HB-GAM-coated matrix (Fig. 10). The increase in c-Src phosphorylation reached its maximum by 20 min, decreasing gradually to 60 min in culture on HB-GAM. Cortactin phosphorylation was also enhanced in transfected N18 cells, following that of c-Src and remaining increased up to 40 min of cells in culture on HB-GAM (Fig. 10*A*). This suggests that c-Src phosphorylation in the N-syndecan-transfected N18 cells activates the Src kinase with the consequence of cortactin phosphorylation. In contrast to the N-syndecan-transfected cells, only a slight change in phosphorylation of c-Src and cortactin was observed in mock-transfected cells. Direct Western blotting of the cell lysates showed that the expression levels of c-Src and cortactin did not change significantly in the experiments (data not shown). Increase of cortactin and Src phosphorylation in N-syndecan transfected cells is hardly a result of overexpression, as the expression level of N-syndecan in the transfected cells is actually lower than in brain cells,[3] and the same effect is also seen in C6 rat glioma cells, which express endogenous N-syndecan.[3]

[2] H. Huttunen and H. Rauvala, unpublished results.

[3] T. Kinnunen and H. Rauvala, unpublished results.

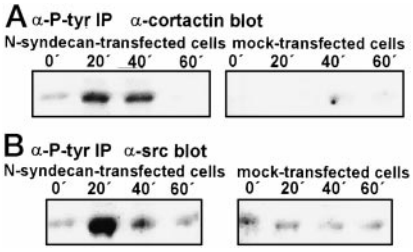

FIG. 10. **Ligation of N-syndecan by HB-GAM enhances phosphorylation of c-Src and cortactin.** N-syndecan-transfected (clone II/7) and mock-transfected (clone I/2) cells were grown on HB-GAM for the time periods indicated. The cell lysates were immunoprecipitated with anti-phosphotyrosine agarose and immunoblotted with anti-cortactin (*A*) and anti-Src (GD11) (*B*) antibodies.

DISCUSSION

Previous studies have demonstrated that N-syndecan acts as a receptor or a coreceptor in HB-GAM-induced neurite outgrowth in perinatal rat brain neurons (13–15). The present results show that N-syndecan, stably transfected to N18 neuroblastoma cells, clearly enhances neurite outgrowth upon cell contact with HB-GAM-containing substrate. This effect can hardly be explained by increased adhesiveness only, as the neurite outgrowth response in the cell type used does not display any direct relationship to adhesiveness of the substrate. This was previously demonstrated by studying the neurite outgrowth response in N18 cells on many different substrates with varying degrees of adhesiveness (28). The finding that the tyrosine kinase inhibitors herbimycin and PP1 inhibit neurite outgrowth in N-syndecan-transfected cells suggests that the transfection indeed influences a signal transduction pathway.

If N-syndecan is coupled to a tyrosine kinase pathway in cells, biochemical evidence of such a pathway should be provided. To this end, we have studied interactions of the cytosolic moiety of N-syndecan with proteins in crude extracts of perinatal brain and cultured cells. The cytosolic moiety of N-syndecan and of other syndecans is a reasonable candidate to interact with cytosolic signal transduction pathway(s) because it is highly conserved across different syndecans and across different species in a given syndecan (17–20).

Interestingly, the cytosolic domain of N-syndecan immobilized to an affinity column binds from crude extracts a phosphorylating activity containing both kinase(s) and its substrate(s). Displacement of this intrinsic kinase activity from the affinity column by the free cytosolic peptide in solution indicates that binding of this protein complex to N-syndecan is specific. Because only the cytosolic moiety of N-syndecan was used in the experiments, the activity is expected to be due to nonreceptor cytosolic kinase(s). We suspected that the activity might be due to Src family tyrosine kinase(s) that are the major nonreceptor tyrosine kinases in the growth cones of neurons (34), where N-syndecan is localized and where it is expected to interact with a kinase pathway to mediate neurite growth. Furthermore, the Src family kinases have been implicated in several studies in the regulation of the cytoskeleton during cell motility phenomena and during adhesion-related signaling in general (for a review, see Ref. 42). Western blotting using a set of monoclonal antibodies indeed demonstrated the presence of both c-Src and Fyn in the kinase-active fractions that bind to N-syndecan.

The major component that was phosphorylated by the intrinsic kinase activity in the fractions that bind to the cytosolic tail of N-syndecan migrated on SDS-PAGE as an 80-kDa component. If the intrinsic kinase activity is due to Src family kinase(s), this component is likely to be cortactin (p80/85), a filamentous (F) actin-binding protein that was initially identified as the major phosphorylated protein in Src-transformed cells (33). In agreement with this premise, monoclonal anti-cortactin antibodies immunoprecipitated the 80-kDa protein phosphorylated by the intrinsic kinase activity; in addition, cortactin was detected by Western blotting in the N-syndecan-binding fractions. Cortactin was also detected in fractions eluted with a peptide corresponding to the membrane proximal region of the syndecan cytoplasmic domain but not in the fractions eluted with a peptide corresponding to RAGE cytoplasmic domain, indicating that the binding is specific. The occurrence of cortactin in the signal transduction pathway that connects to N-syndecan is in agreement with the suggested role of N-syndecan as a neurite growth receptor, as recent genetic studies using mouse embryos suggest that Src plays a biological role in the regulation of cytoskeletal structures via phosphorylation of cortactin (43). Cortactin is known to co-immunoprecipitate with Src (40) and is localized at the leading edge of motile fibroblasts where actin undergoes dynamic reorganization (44, 45). Consistent with the suggested role in the leading edge function is the observation that cortactin is also concentrated in the developing neuromuscular synapses (46) and in the growth cones of developing rat brain neurons.[4]

Three major polypeptides were consistently identified by protein staining in the N-syndecan-binding fractions that were displaced from the affinity column by the free cytosolic peptide: a doublet migrating at 30 kDa, a 50-kDa band, and an 80-kDa band that is due to cortactin (see above). The 30-kDa component has not yet been isolated in amounts sufficient for sequencing studies. However, the amounts isolated were sufficient for peptide-mass fingerprinting, but screening of the peptide masses from the 30-kDa band against a data base containing fragments from over 50,000 proteins (38) did not identify this polypeptide. The 30-kDa polypeptide may thus be a novel component, and further studies are required to identify and characterize this protein.

Peptide sequencing showed that the 50-kDa band is due to α- and β-tubulins. Tubulin could not be eluted from the protein complex by extensive washing of the column, and it co-eluted with cortactin and Src when the full-length cytosolic peptide or its membrane proximal region were used to displace the protein complex from the affinity column. It therefore seems that tubulin is an intrinsic component in the protein complex binding to N-syndecan. The finding that tubulin, but not actin, occurs in the protein complex in the isolation method used is unexpected because microtubules are generally regarded as components of the central region of the growth cone, whereas the actin-containing cytoskeleton lines the plasma membrane. However, a fraction of tubulins is known to associate with the plasma membrane in the growth cones and to serve as a major Src substrate in developing neurons (47). In cells, c-Src has been shown to associate with tubulin by co-immunoprecipitation and by biochemical methods (41). Recent studies have indicated a regulatory role for tubulin in actin-based growth and guidance of neurites (48, 49). Microtubules have been shown to extend to the filopodia (50, 51), where N-syndecan is also localized. Furthermore, we have very recently found, using the antibodies that were also used in the present study, that N-syndecan and tubulin closely colocalize in early axon pathways of brain (52). On this basis, we have suggested that N-syndecan and class III β-tubulin interact during early pathway formation in tissue (52).

It seems probable that all components that were found to bind to the cytosolic moiety of N-syndecan, including the Src family kinases Src and Fyn, cortactin, tubulin, and the 30-kDa

[4] M. Kaksonen and H. Rauvala, unpublished results.

protein, do not bind directly but through linking protein(s) that makes a direct contact with N-syndecan. In agreement with this idea, binding only to the 30-kDa polypeptide was observed when the peptide affinity fractions were separated by SDS-PAGE and analyzed by overlaying with a labeled cytosolic peptide of N-syndecan.[3] Very recently, syntenin, a member of the PDZ protein superfamily, was shown to bind to the cytoplasmic C-terminal domain of syndecan by yeast two-hybrid screens and by ligand-overlay assay (53). Syntenin may thus function as an adaptor that couples syndecan to the cytoskeleton or cytoplasmic signaling proteins. However, further work is clearly warranted to understand the molecular organization of the components bound by the cytosolic moiety of N-syndecan.

For syndecan to be considered as a signaling receptor, it is necessary to show that the molecular complex associated with the cytosolic domain undergoes ligand-induced changes in cells. Interestingly, HB-GAM causes a transient increase in c-Src phosphorylation in the N-syndecan-transfected cells but not in the mock-transfected cells. It seems probable that the N-syndecan-dependent increase in Src phosphorylation also increases Src activity in cells, as a rise in the level of phosphorylation of the major Src substrate, cortactin, temporally parallels and follows the Src phosphorylation.

Asundi and Carey (54) have recently shown that N-syndecan resembles other transmembrane receptors in its ability to self-associate to dimeric and oligomeric forms in the plasma membrane. The spontaneously occurring self-association was shown to depend on a structural motif in the transmembrane and ectodomain flanking regions and to bear some resemblance to the self-association motif found in the platelet-derived growth factor receptor (PDGFR-B) and in β_3 integrin (54). Although the functional consequences of the N-syndecan self-association still remain unclear, it is tempting to speculate that this property is essential for the signaling property of N-syndecan observed in this study. In any case, a transmembrane configuration of N-syndecan is required for neurite outgrowth; soluble extracellular N-syndecan is actually inhibitory in neurite outgrowth (15). Further studies are in progress to elucidate the sequences in the ectodomain, transmembrane, and cytosolic domains that are required for signaling to the Src-cortactin pathway during process extension in neurons and in other cells.

Proteoglycans are generally regarded as storage sites for growth factors or as coreceptors in growth factor signaling but have not been considered as transmembrane receptors that would directly interact with cytosolic signaling pathways. However, syndecan-4, but not other members in the syndecan family, has been shown to localize to focal contacts of fibroblasts (22), suggesting a role in adhesion-dependent cell signaling. Recent studies (55, 56) have suggested that the cytosolic moiety of syndecan-4 binds and activates protein kinase C. This activity seems to be specific for syndecan-4 and was suggested to be due to the unique central portion in the cytosolic moiety of syndecan-4 (55). Because the cytosolic moiety in the syndecan family is highly conserved, one might expect that many, if not all, syndecan forms would also signal via a common cytosolic kinase pathway. The present finding that the conserved membrane proximal peptide is as active as the full-length peptide in displacing the signaling complex from N-syndecan suggests that also other syndecans may interact with the cortactin-Src kinase pathway.

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