THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 36, pp. 25036–25045, September 5, 2008

The Survival-promoting Peptide Y-P30 Enhances Binding of Pleiotrophin to Syndecan-2 and -3 and Supports Its Neuritogenic Activity*

Received for publication, February 5, 2008, and in revised form, June 6, 2008 Published, JBC Papers in Press, July 3, 2008, DOI 10.1074/jbc.M800963200

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Y-P30 is a polypeptide produced by peripheral blood mononuclear cells of the maternal immune system during pregnancy. The peptide passes the blood-placenta barrier and accumulates in neurons of the developing infant brain, where it enhances survival of thalamic neurons and displays neuritogenic activities. In this study, we identify pleiotrophin (PTN) and syndecan-2 and -3 as direct binding partners of Y-P30. PTN is known to promote neurite outgrowth of thalamic neurons due to its association with the proteoglycan syndecan-3. Via spontaneous oligomerization Y-P30 can capture large macromolecular complexes containing PTN and potentially syndecans. Accordingly, the neuritogenic activity of Y-P30 in thalamic primary cultures requires the presence of PTN in the media and binding to syndecans. Thus, we propose that the neurite outgrowth promoting actions of Y-P30 during brain development are essentially based on its association with the PTN/syndecan signaling complex. This identifies a new mechanism of communication between the nervous and the immune system that might directly affect the wiring of the brain during development.

Organotypic cultures of the thalamus need a peptide factor secreted from the cortex to survive for longer periods of time. In previous work we could identify Y-P30 as the crucial factor that is released from cortical neurons and required for the survival of thalamic cultures (1). Interestingly, Y-P30 (fragments of the peptide are also termed human cachexia factor (2), survival promoting peptide (3), or proteolysis-inducing factor (PIF)[2] (4)) is not synthesized in neural cells of the embryo but is a maternal blood-borne factor expressed by peripheral blood mononuclear cells (1). It is transported via the umbilical cord to the developing brain where it accumulates by a yet unknown mechanism in neurons of the cortex and the hippocampus (1). During the wiring of the fetal brain and in early postnatal development it is subsequently released from there. The factor derives from a larger precursor protein that after proteolytic cleavage gives rise to at least two bioactive peptides, dermcidin and Y-P30 (1, 5). Although dermcidin is an antimicrobial peptide produced as part of innate immunity in sweat glands (5), Y-P30 is virtually absent from the adult organism. However, during pregnancy Y-P30 expression is induced in peripheral blood mononuclear cell of the mother. In addition, the peptide can be induced in pathological states like nerve injury (1) and tumor growth (2, 6).

Based on these initial findings we hypothesized that the immune system of the mother might directly influence brain development of the infant via secretion of Y-P30 from maternal peripheral blood mononuclear cells. To further prove this hypothesis we set out to identify molecular mechanisms that might underlie the broad neurotrophic and neuritogenic effects of the peptide in the fetal brain. Part of this effort was the identification of pleiotrophin (PTN) as well as syndecans 2 and 3 as Y-P30-binding partners. PTN (also designated heparin-binding growth associated molecule, HB-GAM) is a secreted protein of 136 amino acids with lysine-rich domains at the N and C termini and two separate heparin-binding thrombospondin type-1 repeat domains linked internally by a short amino acid sequence (7, 8). PTN is a member of the midkine family and, like Y-P30, exhibits a broad spectrum of neuritogenic activities during brain development (7, 9–14). These actions appear to be related to signaling events elicited via binding to its neuronal receptor syndecan-3 (10, 14–15). In the present study we show that Y-P30 fosters the formation of large Y-P30/PTN oligomers that might increase the local concentration of Y-P30/PTN at their neuronal receptor syndecan. Moreover, the neuritogenic activity of the factor in thalamic primary cultures requires the PTN-syndecan interaction, suggesting that syndecan signaling might underlie many of the actions of Y-P30 in the infant brain.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Assay—Yeast two-hybrid screening was performed essentially as described in Helmuth *et al.* (16). For the screening a pre-transformed human fetal brain cDNA library in pACT2 (MATCHMAKER GAL-4 Two-Hybrid II, Clontech, Heidelberg, Germany, yeast strain AH 109) was used. As a bait construct the entire open reading frame of

* This work was supported by grants from the Bundes ministerium für Bildung und Forschung (Grant 01GA0505 to M. R. K. and E. D. G.), Deutsche Forschungsgemeinschaft (Grant SFB 779 TPB8), Leibniz-Society (Pakt für Forschung to M. R. K.), Land Sachsen-Anhalt (Grants N1/TP4/MRK and N2/TP5 to M. R. K. and E. D. G.), the Schram Foundation (to M. R. K.), and Fonds der Chemischen Industrie (to E. D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: PIF, proteolysis-inducing factor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HBSS, Hanks' balanced salt solution; MAP2, microtubuli-associated protein 2; MBP, maltose-binding protein; PBS, phosphate-buffered saline; PTN, pleiotrophin; TBS, Tris-buffered saline; YTH, yeast two hybrid.

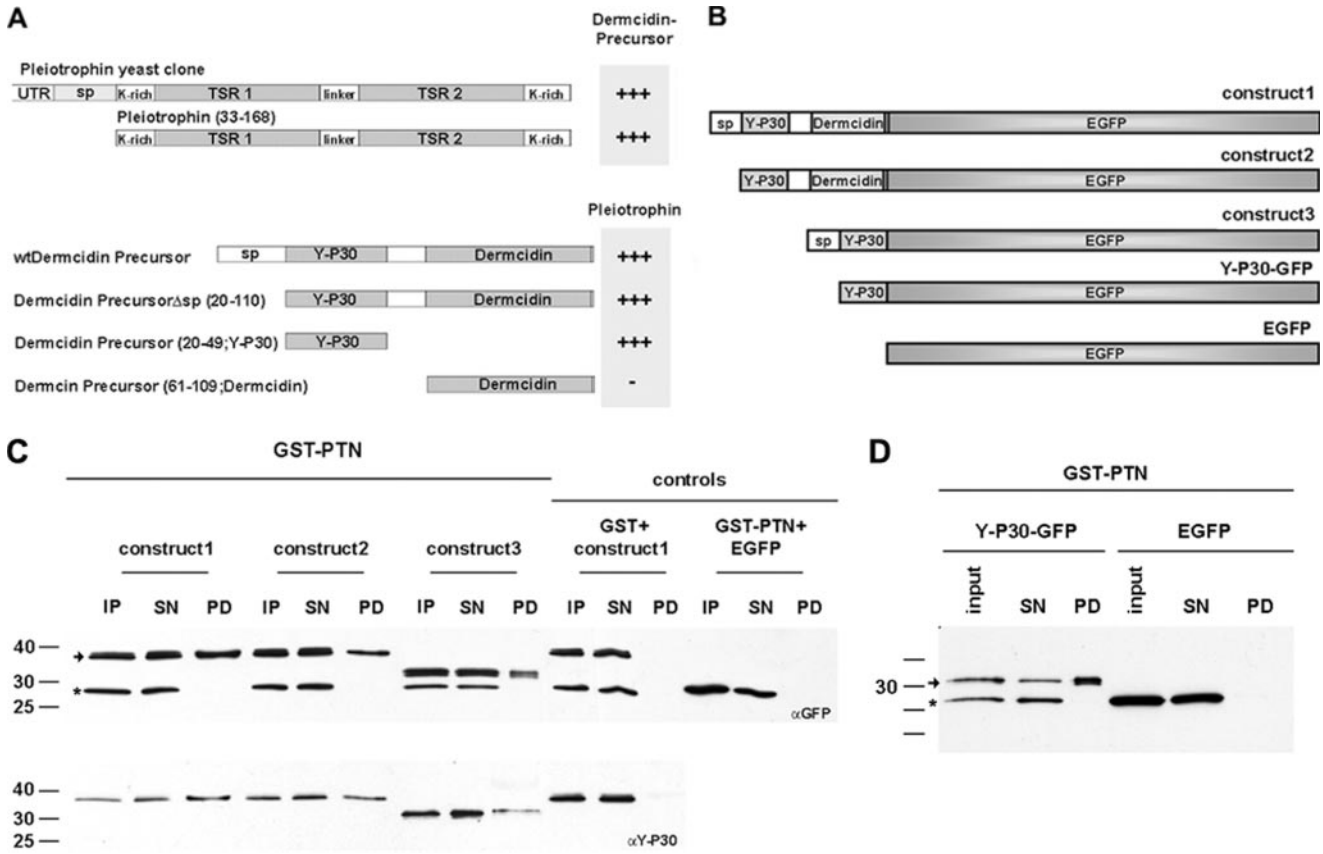

FIGURE 1. **Y-P30 associates with PTN (*A*) mapping of the PTN-Y-P30 interaction using the yeast-two-hybrid system.** *A*, deletion constructs of the Y-P30 precursor protein were tested for the interaction with PTN. Note that the signal peptide together with the 5′-untranslated region were removed from the PTN clones originally identified in the YTH screen prior to this analysis. The YTH interactions were qualitatively verified using Minimal SD Agar Base with the appropriate Dropout Supplement and quantitatively via the induction of the β-galactosidase reporter gene activity. +++, blue colonies within 1 h; −, no signal after 6 h. *B–D*, pull-down assay with GST-PTN and various YP30-GFP fusion proteins. Recombinant PTN was expressed in *E. coli* (BL21+) as GST fusion protein, purified, and immobilized on glutathione-Sepharose. The different Y-P30-GFP fusion proteins used for this assay are depicted in the scheme of figure (*B*). *C*, constructs 1–3 were overexpressed in COS-7 cells. The binding of YP30-GFP fusion proteins to PTN was confirmed by the detection with monoclonal GFP-antibodies (BabCO). Please note that only the upper band represents YP30-GFP fusion proteins. This is illustrated in the *lower panel* where the same samples were processed on another blot with a rabbit Y-P30 antibody. The lower band is most likely a degradation product. *D*, Y-P30-GFP or GFP without the Y-P30 fusion part was purified using GFP-immunoaffinity columns before the MBP pulldown assay was performed. MBP-PTN binds directly to purified Y-P30-GFP. Note that the Y-P30-GFP fusion construct contains only the 30 amino acids of Y-P30 (*B*). No binding of MBP-PTN to GFP was found. All experiments were repeated three times independently. Note that the full-length recombinant protein is marked with an *arrow*; an *asterisk* is used to label a putative degradation product (most likely the GFP part) in C+D. *IP*, input; *SN*, supernatant; *PD*, pulldown assay. Percentage of loading IP: 10%; SN: 10%; PD: 33% of total volume.

the dermcidin precursor cDNA (AF144011) was cloned in-frame into pAS2–1 and pGBKT7 (MATCHMAKER GAL-4 Two-Hybrid III, Clontech), respectively. A total of 2.8×10^6 co-transformants were screened.

Generation and Purification of Recombinant GST and MBP Fusion Proteins in Escherichia coli—The respective cDNAs were cloned either into pGEX5T1 (GE Healthcare, Uppsala, Sweden) or pMALc2x (New England BioLabs, Hertfortshire, UK) and subsequently transformed into *E. coli*, strain BL21C+ (Stratagene, Heidelberg, Germany). Overexpression of the recombinant proteins was performed according to the instructions of the manufacturers' protocols and published procedures (17).

Pulldown Assays—Glutathione-Sepharose-B4 coupled GST-PTN and GST were equilibrated in 1× TBS containing 0.1% Triton X-100. Transfected COS-7 cells expressing GFP-tagged Y-P30/dermcidin were extracted after freezing and five sonification pulses with 0.1% Triton X-100 in 1× TBS for 1 h and subsequently centrifuged at 20,000 × *g* and 4 °C for 20 min. The remaining supernatants were incubated with either 20 μl of glutathione-Sepharose-B4-bound GST-PTN or GST and gently shacked in an end-over-end mixer overnight at 4 °C. After three times washing with 1× TBS containing 0.1% Triton X-100 proteins were eluted by boiling in SDS-sample buffer.

Production of MBP-PTN, MBP-Y-P30, and GFP-tagged Syndecan-2 and -3—For the syndecan binding assays a 10 mM Hepes buffer (pH 7.4) containing 1 mM EGTA, 0.1 mM $MgCl_2$, and 250 mM NaCl was used. The amylose-bound MBP fusion proteins or MBP as a negative control were washed and equilibrated in buffer. GFP-tagged syndecan-3 and -2 were expressed in COS-7 cells. After two freezing-thawing cycles and five sonification pulses the proteins were extracted for 1 h with 1% Triton X-100 in Hepes buffer. Then the resulting homogenate was centrifuged for 20 min at 20,000 × *g* and 4 °C. The supernatants were diluted 1:5 with Hepes buffer and incubated with the respective amylose-bound MBP fusion proteins at 4 °C overnight. After three times extensive washing with 10 mM Hepes buffer (pH 7.4) containing 1 mM EGTA, 0.1 mM $MgCl_2$, 250 mM

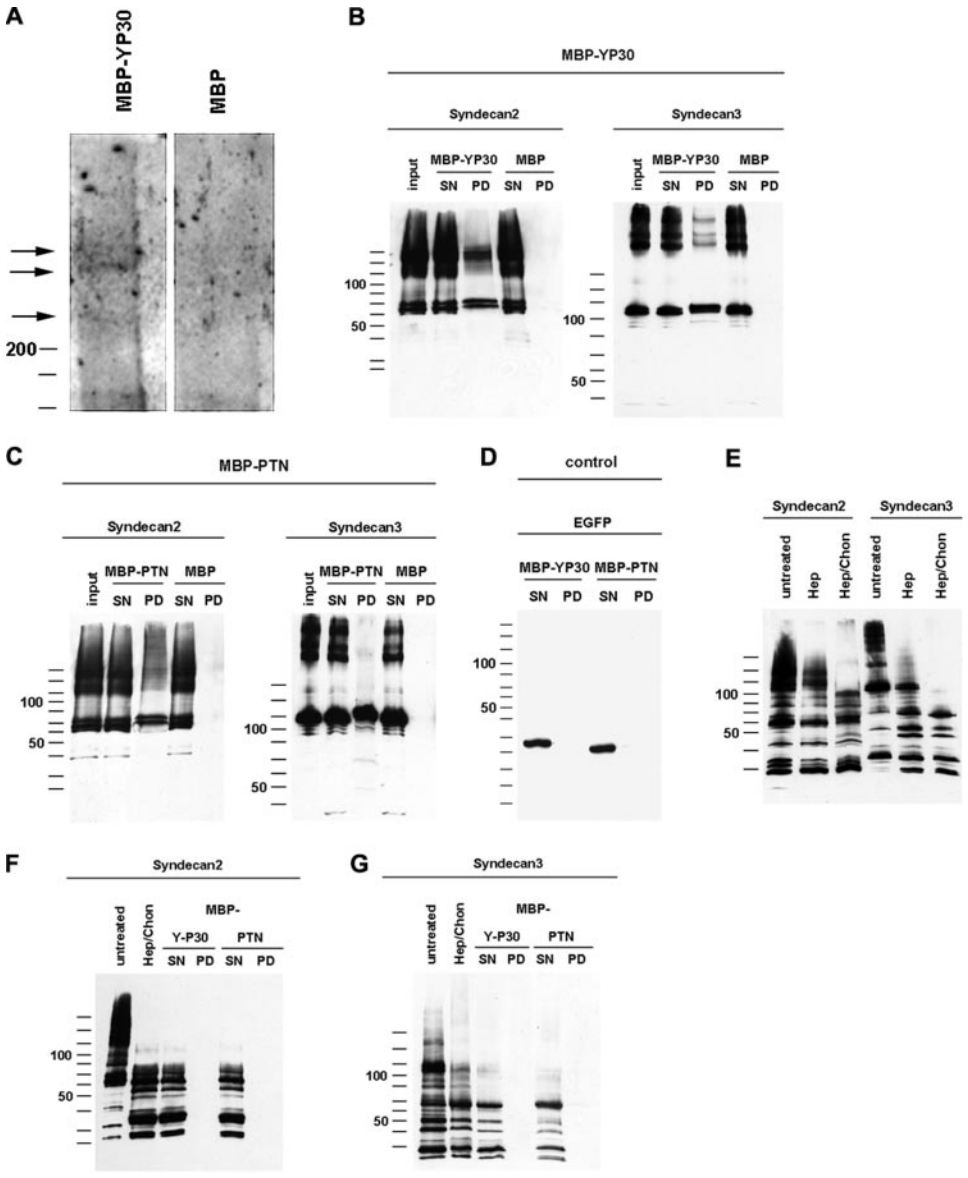

FIGURE 2. **Y-P30 and PTN interact both with syndecan-2 and -3.** *A*, both syndecan-2 as well as syndecan-3 were pulled down by MBP-Y-P30 from rat brain tissue. The *three arrows* indicate syndecan-immunoreactive bands. Neither syndecan-2 nor syndecan-3 binds to the MBP-control. *B*, pulldown assay with MBP-Y-P30 and syndecan-2-GFP and syndecan-3-GFP shows a clear association between Y-P30 and syndecans. No binding of GFP to MBP-Y-P30 was found in control experiments. *C*, pulldown assay with MBP-Y-P30 and syndecan-2-GFP and syndecan-3-GFP. Pulldown assay of syndecan-3 confirms previously published data. Interestingly also syndecan-2 binds to MBP-PTN, and no binding to the MBP-control matrix was observed. *D*, GFP-control protein extracted in a similar manner like syndecan-GFP constructs does not bind to MBP-Y-P30 or MBP-PTN. All experiments were three to five times repeated independently. *E*, syndecan-GFP fusion proteins were overexpressed in COS-7 cells and solubilized as described above. Treatment with heparitinase I or a mixture of heparitinase I and chondroitinases A–C leads to the cleavage of heparan and chondroitin sulfate side chains and a disappearance of high molecular mass syndecan bands above 100 kDa. *F*, the deglycosylated syndecans were used for further pulldown assays with Y-P30/PTN. The binding for both syndecans, 2 and 3, was completely abolished after deglycosylation. *G*, similarly the interaction between MBP-PTN and both syndecans requires the presence of heparan-sulfate side chains. The experiments were independently repeated two to three times. *IP*, input; *SN*, supernatant; *PD*, pulldown assay. Percentage of loading IP: 10%; SN: 10%; PD: 33% of total volume.

NaCl, and 0.2% Triton X-100 the proteins were eluted by boiling in SDS sample buffer. To test whether the heparan sulfate side chains of syndecans are responsible for these interactions syndecan-containing cell extracts were treated with 20 milliunits/ml heparitinase I (Sigma) and 0.2 unit/ml chondroitinase A, B, and C (Sigma) before pull-down experiments were carried out.

SDS-PAGE and Western Blot Experiments—For SDS-PAGE or immunoblot experiments protein fractions were solubilized with 4× SDS sample buffer (250 mM Tris-HCl, pH 6.8, 1% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.004% bromphenol blue), cooked for 5 min, and then separated on 5–20% SDS-polyacrylamide gradient gels (except for syndecan-3 where 4–12% gradient gels were used). After blotting the membranes were blocked with 5% dry milk and 0.1% Tween 20 in 1× TBS for 2 h. Subsequently, the membranes were incubated at 4 °C overnight with primary antibodies in 1× TBS containing 0.1% Tween 20.

Primary Thalamic Cell Cultures—Rat thalami were prepared from embryos (Long Evans rats) at embryonic day E16 and transferred into ice-cold Hanks' balanced salt solution, without Mg^{2+}/Ca^{2+} (HBSS, Invitrogen, Karlsruhe, Germany). After washing three times with 5 ml of HBSS, 2 ml of HBSS containing 0.5% trypsin (Sigma) was added to the tissue, and the the mixture was incubated for 20 min at 37 °C. Next, tissue was washed again five times with 5 ml of HBSS and finally transferred into 2-ml tubes with HBSS, containing 0.01% DNase-I (Invitrogen). For dissociation thalamic tissue was three times pressed slowly through a 0.9-mm gauged needle followed by three passages through a 0.45-mm gauged needle. The remaining cell suspension was poured through a 70-μm cell strainer (BD Biosciences, San Jose, CA) into a 50-ml tube and filled up with 18 ml of Dulbecco's modified Eagle's medium (Invitrogen). After estimating cell quantity, the suspension was diluted with Dulbecco's modified Eagle's medium to a density of 150,000 cells/ml. Finally 500 μl of this suspension were placed in each well of a 24-well plate, containing lipid-free, baked, and coated coverslips. Cell cultures were incubated at 37 °C and 5% CO_2 and 95% air humidity and kept in culture for 18 h.

Coverslips were coated first with poly-D-lysine (Sigma), washed three times of extensively (2× sterile distilled H_2O, 1× HBSS), and air dried. A second coating on top of this was per-

formed with 10 μg/ml laminin (Sigma) in PBS supplemented with 4 μg/ml recombinant PTN (R&D Systems, Minneapolis, MN), 6 μg/ml Y-P30 (synthetic peptide), or a mixture of both. The coverslips were then incubated for 2 h at 37 °C, 5% CO_2 and 95% air humidity, and subsequently extensively washed with sterile distilled H_2O and HBSS(−).

Immunocytochemistry and Morphological Analysis—Rat thalamic cell cultures were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany), washed three times with 10 mM PBS, and preincubated for 90 min at 4 °C in blocking solution (10 mM PBS, 10% normal horse serum, PAA Laboratories, Pasching, Austria), 2% bovine serum albumin (Sigma), 5% sucrose (Merck), and 0.3% Triton X-100 (Sigma). Afterward cells were incubated at 4 °C overnight with primary antibodies in blocking solution (mouse anti-MAP-2, 1:1000, Sigma). Next day, the cultures were washed three times with 10 mM PBS containing 0.3% Triton X-100 and incubated with secondary antibodies (Cy3-conjugated rabbit anti mouse IgG (H+L), 1:1500 (Dianova, Hamburg, Germany). Finally they were washed twice with 10 mM PBS containing 0.3% Triton X-100, once with 10 mM PBS, and subsequently covered with Mowiol (Merck, Darmstadt, Germany). Images were taken using a Zeiss-AxioplanII imaging fluorescence microscope (Zeiss, Jena, Germany), a Spot RT camera, and Meta View software (Visitron Systems, Puchheim, Germany). Morphological analysis was carried out with ImageJ (National Institutes of Health). Numbers and lengths of neurites were evaluated using Sholl analysis. Data are presented as mean ± S.E. Levels of $p < 0.05$ were considered as statistically significant.

Surface Plasmon Resonance Binding Studies—Syndecan binding to MBP-PTN was analyzed by surface plasmon resonance using the Biacore 2000 system and CM5 sensor chips (Biacore AB, GE Healthcare, Uppsala, Sweden). MBP-PTN was coupled to the carboxymethylated Dextran matrix of one sensor chip cell according to the manufacturer's instructions. A second cell of the sensor chip was prepared as a reference matrix by coupling equimolar amounts of MBP alone. After equilibrating the sensor chip with flow buffer HBS-EP (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) at a flow rate of 5 μl/min the Dextran matrix was activated for 7 min with 50 mM *N*-hydroxysuccinimide/200 mM *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide at a flow rate of 5 μl/min. Subsequently MBP-PTN and MBP were immobilized separately on the surface of the respective sensor chip by applying ligand solution (10–15 μg/ml of the respective ligand in 10 mM sodium acetate, pH 5.0) for 7 min. Finally, the excess of reactive groups was deactivated with 1 M ethanolamine hydrochloride, pH 8.5 (7 min at a flow rate of 5 μl/min). The final immobilization levels of the respective ligands were 7–8 ng/mm^2 cell surface. For binding studies of syndecan-2 and -3 the purified proteins were diluted to the indicated concentrations with HBS-EP.

Expression of GFP-tagged Syndecan-2 and -3 in COS-7 Cells—COS-7 cells were grown in Dulbecco's modified Eagle's medium plus (Invitrogen) at 37 °C, 5% CO_2 and transfected with Lipofectamine 2000 (Invitrogen) at ~80% confluence according to the manufacturer's instructions. Transfection efficiencies and expression rates were monitored by fluorescent

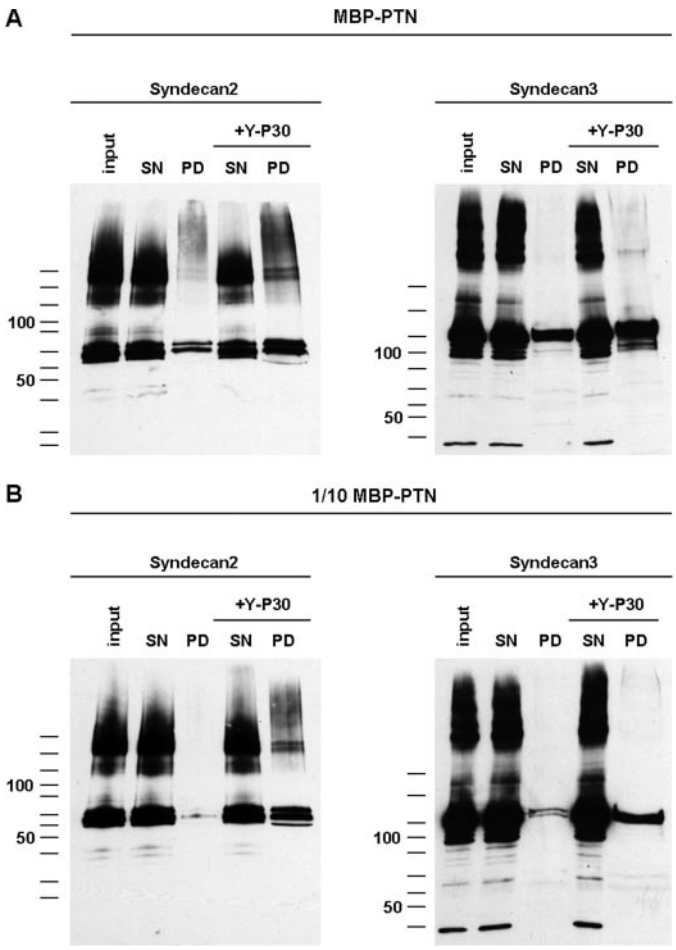

FIGURE 3. **The presence of Y-P30 enhances the binding of syndecan-2 and -3 to PTN in a pulldown assay.** *A*, supplementation of Y-P30 at an equimolar concentration like PTN leads to no competition for binding to syndecan-2 and syndecan-3 in a pulldown assay with MBP-PTN. The intensity of syndecan bands appears to be even more intense. *B*, to clarify this observation, only 10% of MBP-PTN was used for the pulldown assay. Without supplementation of YP30 neither for syndecan-2 nor for syndecan-3 binding to MBP-PTN was detectable under these conditions. In contrast, both syndecans could be pulled down, when the same amount of Y-P30 was added to the assay buffer as indicated in *A*. The experiments were independently repeated two to three times. *SN*, supernatant; *PD*, pulldown assay. Percentage of loading SN: 10%; PD: 33% of total volume.

microscopy. To purify GFP-tagged syndecans, cell pellets were shock frozen in liquid nitrogen and resuspended in 200 μl of 10 mM Hepes buffer (pH 7.5) containing 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, and an EDTA-free protease inhibitor mixture (Complete Mini, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany). The suspension was again shock frozen, thawed, and treated five times with two-second strokes of sonification. Afterward the suspension was shaken for 1 h in an end-over-end mixer at 4 °C and centrifuged at 20,000 × *g* and 4 °C for 20 min. From supernatants GFP-tagged syndecans were purified using the μMACS™ epitope *Taq* protein isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following the manufacturer's instructions. Samples were eluted with 0.1 M triethylamine and neutralized immediately. The buffer of the protein samples was exchanged against HBS-EP using Zeba™ Desalt Spin Columns (Pierce) according to the manufacturer's protocol.

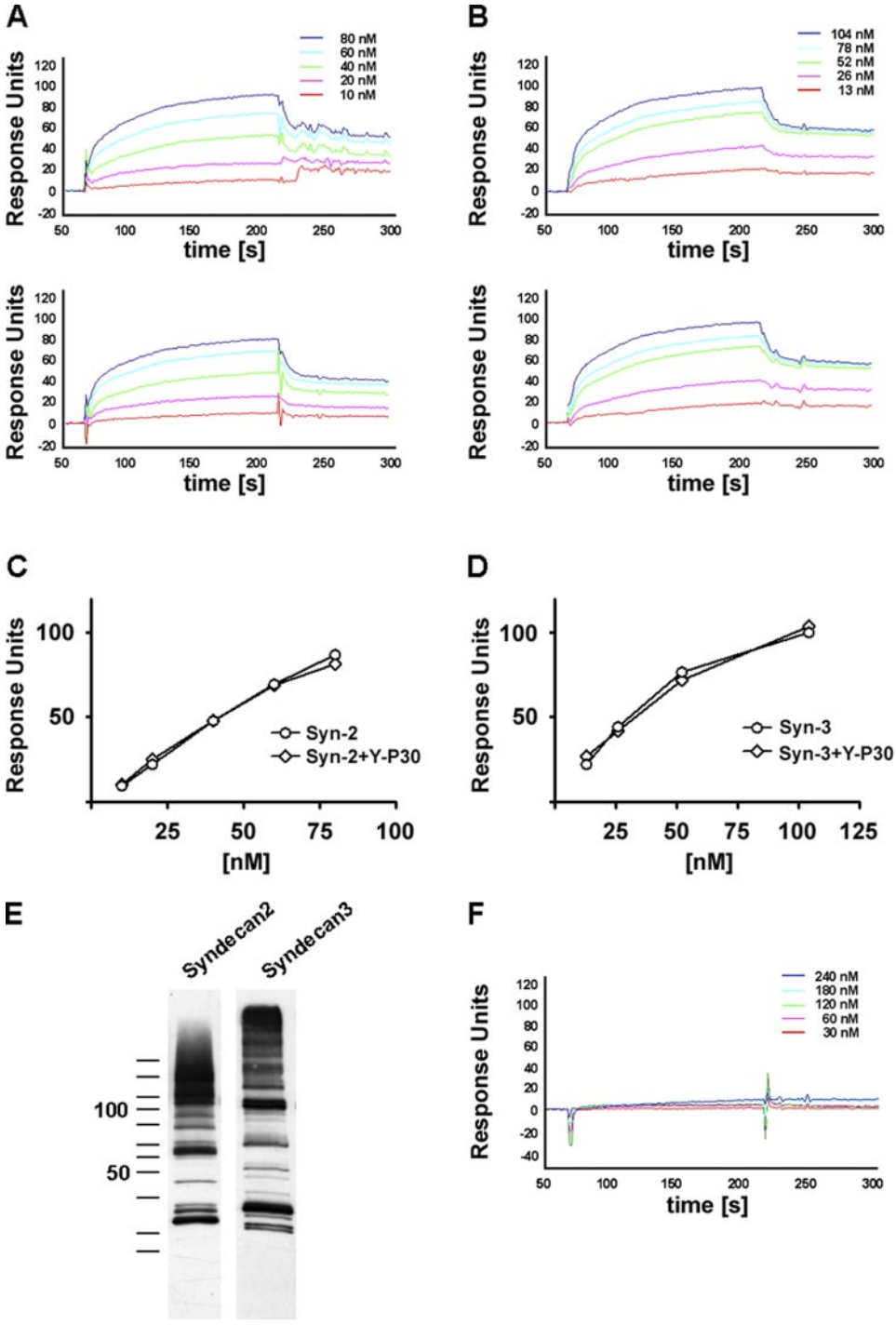

FIGURE 4. **Y-P30 does not increase the affinity of syndecans to PTN as evidenced by surface plasmon resonance.** *A* and *B*, MBP-PTN and MBP, respectively, were coupled via amide binding to the dextran matrix of the sensor chip CM5 and analyzed in a Biacore 2000 (Biacore, GE Healthcare). Binding of syndecans to PTN was monitored as response units, whereby the signal from the reference surface (MBP) was directly subtracted from the PTN-syndecan sensorgram. *C* and *D*, supplementing the syndecan-2 (*A*) and syndecan-3 (*B*) analyte solution with Y-P30 had no effect to the binding on PTN under these conditions. Results of the sensorgrams are blotted against the used concentration of syndecan-2 (*C*) and syndecan-3 (*D*). *E*, depicted is an immunoblot showing the purified syndecan-GFP proteins used for surface plasmon resonance. The blot was processed utilizing monoclonal GFP-antibodies (BabCO). *F*, no increase of response units was seen when GFP purified in a similar manner like Syndecan-GFP was used as analyte solution with the MBP-PTN coupled sensor chip.

Gel-filtration Assays—Equimolar amounts of purified GST-PTN and Y-P30 peptide were preincubated in 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, and 0.2% Triton X-100 for 2 h at 4 °C, and 0.8 ml of the solution was loaded onto a pre-equilibrated Hiload™16/60-Superdex™ 200 column (Amersham Biosciences). Equilibration and running buffer were used as above. The protein complexes were separated by size at a flow rate of 0.2 ml/min and collected in 1.5-ml fractions. Fractions were lyophilized and redissolved in 150 μl of distilled water. Finally, the samples were solubilized in SDS sample buffer and analyzed by immunoblots. Complex sizes were calculated after calibrating the column with different reference proteins.

RESULTS

Y-P30 Binds to PTN—Y-P30 is taken up by neurons and subsequently released into the extracellular space by yet unknown mechanisms (1). To identify potential binding partners involved in these processes we performed a yeast-two-hybrid screen using a pretransformed fetal human brain cDNA library and the entire Y-P30-dermcidin precursor as bait. Six independent clones contained the complete open reading frame of the midkine PTN. Removal of the untranslated region and the signal peptide did not affect binding of PTN to the Y-P30-dermcidin precursor (Fig. 1*A*). PTN binds exclusively to the 30 amino acids encoding the Y-P30 peptide fragment as revealed by shortening the Y-P30-dermcidin cDNA (Fig. 1*A*) accordingly.

Because the PTN-Y-P30 interaction physiologically should take place extracellularly we sought to confirm the yeast-two-hybrid data independently. To this end we performed GST-PTN pulldown assays with different Y-P30-dermcidin-GFP fusion constructs heterologously expressed in COS-7 cells (Fig. 1*B*). PTN specifically pulls down a Y-P30-containing GFP-tagged protein (Fig. 1*C*). In controls no binding of untagged GFP to GST-PTN or *vice versa* GST-Sepharose to Y-P30-dermcidin precursor tagged with GFP was found (Fig. 1*C*). Moreover, like in yeast cells neither the presence of dermcidin nor the N-terminal signal peptide of the Y-P30-dermcidin precursor was

essential for the interaction in this assay (Fig. 1*C*). To further prove that the interaction between PTN and Y-P30 is direct we produced and purified a bacterially expressed MBP-PTN fusion protein and a Y-P30-GFP expressed in COS-7 cells (Fig. 1, *B* and *D*). In pulldown experiments we could confirm a direct binding of Y-P30-GFP to a MBP-PTN-amylose matrix (Fig. 1*D*). No binding of GFP alone was observed in control experiments (Fig. 1*D*).

PTN and Y-P30 Associate with Syndecan-2 and -3—In previous work we identified Y-P30 immunoreactivity in neurons (1), whereas PTN is mainly expressed by stem cells and glia during development (18–19). Because Y-P30 and PTN are secreted polypeptides their interaction *in vivo* in the embryonic and neonatal brain should occur in the extracellular space. During brain development PTN has a broad spectrum of neurotrophic activities linked to neurite outgrowth and neuronal differentiation (7, 9–13). Several studies suggest that binding to the heparan sulfate side chains of the cell surface receptor syndecan-3 mediates these neurite outgrowth-promoting activities (14–15, 20–21). Because Y-P30 displays similar activities like PTN in neuronal cultures we next tested whether both peptides associate with the most abundant syndecan family members in brain, *i.e.* syndecan-2 and -3.

To assess whether Y-P30 can bind to endogenous syndecan-3 we performed pulldown assays with bacterially expressed MBP-Y-P30 fusion protein and solubilized membrane fractions of early postnatal rat brain. Indeed syndecan-3 is precipitated with MBP-Y-P30 as detected by immunoblot analysis (Fig. 2*A*). No binding to the MBP matrix was observed in control experiments (Fig. 2*A*). The small size of Y-P30 and the poor quality of commercially available syndecan-3 antibodies precluded further co-immunoprecipitation experiments. To learn more about the interaction we therefore expressed Myc/GFP-tagged syndecan-2 and -3 heterologously in COS-7 cells. Like other proteoglycans both overexpressed Myc/GFP-tagged syndecans run as a smear in SDS-PAGE because of their variable decoration with heparan sulfate side chains (Fig. 2, *B* and *C*). Subsequent pulldown experiments revealed binding of MBP-PTN to syndecan-3 and syndecan-2. The latter interaction was not described previously. No binding to untagged MBP or to GFP alone was observed (Fig. 2*C*). Moreover, we observed a specific pulldown of both proteoglycans with the MBP-Y-P30 matrix (Fig. 2*B*). Of note, also smaller syndecan fragments were found in the pulldown (Fig. 2, *B* and *C*), which most likely represent degradation products from syndecan dimers (22) that are pulled down together. A GFP-control protein associated neither with the MBP-Y-P30 nor the MBP-PTN matrix (Fig. 2*D*). These data confirm the binding of both peptides to syndecan-2 and -3.

Previous studies suggested that the interaction of PTN with syndecan-3 requires the presence of the heparan sulfate side chains (10). We therefore sought to determine whether removal of the carbohydrate side chains with heparatinase and chondroitinase affects binding of PTN and Y-P30 to syndecan-2 and -3 (Fig. 2*D*). Indeed the interaction with MBP-PTN and MBP-Y-P30 was completely abolished after the incubation of GFP-tagged syndecan-2 and -3 with both enzymes (Fig. 2, *E*–*G*). Thus, both peptides seem to interact with the heparan/chondroitin sulfate moieties of both syndecans. Interestingly, the small fragments that most likely represent deglycosylated syndecan fragments (22) originating from syndecan dimers are also not present in the pulldown under these conditions (Fig. 2, *F* and *G*).

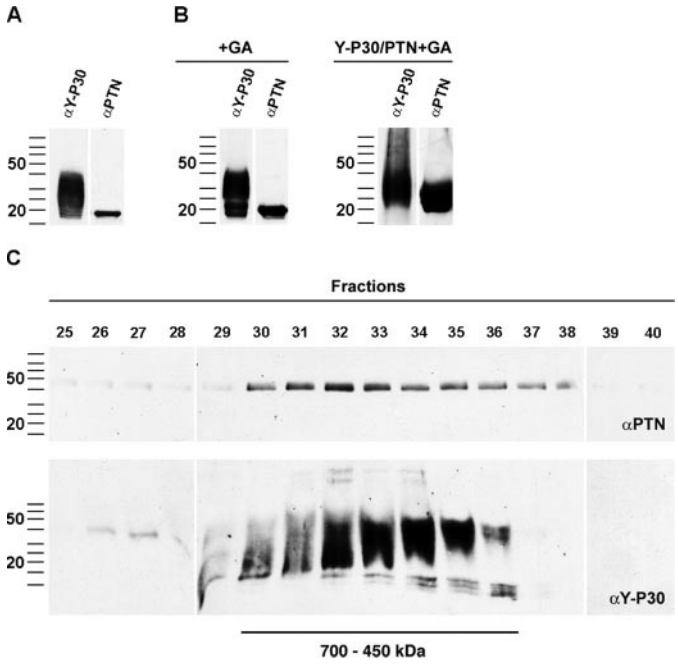

FIGURE 5. **Y-P30 and PTN built-up macromolecular complexes.** *A*, Y-P30 spontaneously oligomerizes *in vitro* to SDS-resistant complexes, whereas the recombinant PTN remains as a monomer at the expected size under such conditions. *B*, combined incubation of both peptides for 2 h at 4 °C and subsequent fixation with 0.5% glutaraldehyde demonstrate that both peptides form hetero-oligomer aggregates as shown in the *right panel*. Glutaraldehyde treatment of the single peptides shown in the *left panel* does not lead to extended oligomerization. *C*, hetero-oligomerization of Y-P30 and PTN could also be confirmed by gel-filtration assay. Fractions were collected at a volume of 1.5 ml, lyophilized till complete dryness, and redissolved in 150 μl of H_2O. Subsequently the fractions were solubilized in SDS-sample buffer and analyzed by Western blots with Y-P30 and PTN-specific antibodies. The molecular mass range of the Y-P30/PTN-positive fractions is indicated in the *panel*.

The Association of PTN and YP30 with Syndecans Is Not Competitive—To elucidate whether Y-P30 and PTN bind syndecans simultaneously or whether binding is competitive we next performed MBP-PTN pulldown assays in the presence of equimolar amounts of Y-P30. Notably, we found no evidence for a competition of both peptides for syndecan binding (Fig. 3*A*). On the contrary, addition of Y-P30 seemed to increase the association of PTN to GFP-syndecan-2 and -3 (Fig. 3*B*). A 1:10 dilution of the binding matrix resulted in almost undetectable syndecan levels in the pulldown assay (Fig. 3*B*). However, when we added Y-P30 a significant amount of GFP-syndecan-2 and -3 remained attached to the MBP-PTN matrix (Fig. 3*B*).

This latter finding led us to ask whether Y-P30 might increase the affinity of PTN binding to syndecans. To address this question we employed surface plasmon resonance measurements with MBP-PTN captured via amide binding to the dextran matrix of the sensor chip. GFP-tagged syndecan-2 and -3 were purified from COS-7 cells (Fig. 4*E*). To screen for the relative affinity of GFP-syndecan-2 and -3 in the presence or absence of Y-P30 a single sensor chip was used with defined analyte concentration. We initially proved the specificity of the

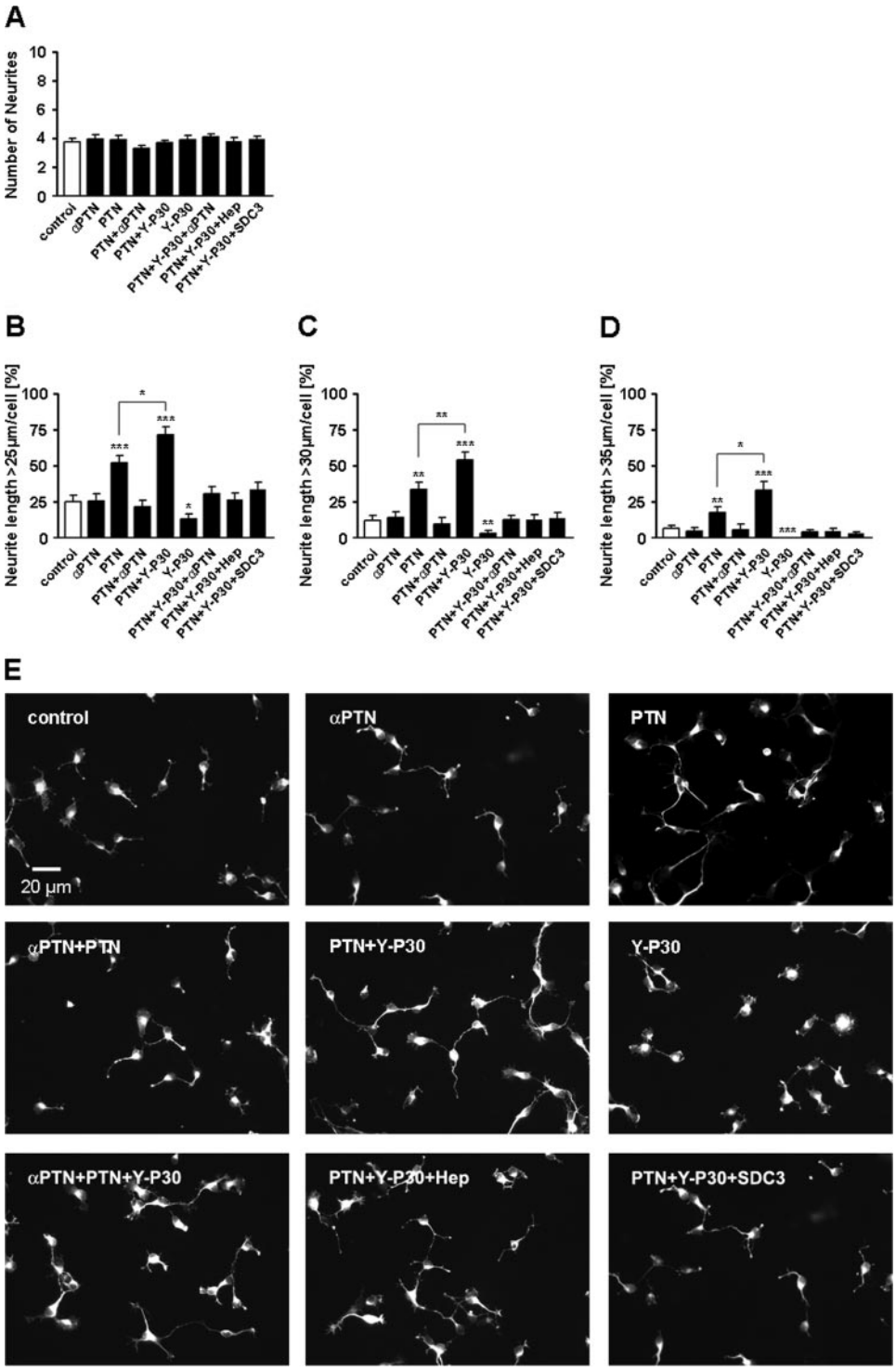

FIGURE 6. **Effects of PTN and Y-P30 on the neurite outgrowth of E16 primary thalamic neurons.** The neurite number (*A*) and length (*B–D*) of MAP2-stained neurons was determined with Sholl analysis 18 h after plating the neurons. Neurite length is depicted as the percentage of neurites/cell that reach a length >25 μm (*B*), 30 μm (*C*), or 35 μm (*D*). The *graphs* in *A–D* represent the mean of 30 cells from 6 different coverslips and 3 independent experiments for each group. ***, $p < 0{,}001$; **, $p < 0{,}01$; and *, $p < 0{,}05$. *E*, representative micrographs of MAP2 immunofluorescence for each experimental condition. Treatment conditions are indicated in each *panel*.

interaction of PTN with syndecans by recording sensorgrams with increasing concentrations of syndecans in the running buffer (Fig. 4*A*). However, due to the limited amount of GFP-syndecans available we were not able to saturate binding and to subsequently determine the K_D. Sensorgrams with a GFP-control protein in the running buffer showed no measurable effect on resonance (Fig. 4*F*). Similarly, capturing MBP without PTN did not lead to a plasmon resonance response. In the next set of experiments we added increasing amounts of the untagged Y-P30 peptide. This had no effect on the recorded sensorgrams (Fig. 4, *A–D*), presumably due to the low mass of Y-P30. Moreover, preincubation of the syndecan analyte with increasing amounts of Y-P30 had also no effect on the PTN/syndecan as compared with PTN/syndecan/Y-P30 sensorgrams. Even at high concentrations of Y-P30 no competition or increase for binding to PTN/syndecan as evidenced by deflections in the sensorgram could be established (Fig. 4, *A–D*). Finally, preincubation of MBP-PTN with Y-P30 prior to adding syndecans to the running buffer did also not influence association and dissociation rates (Fig. 4, *A–D*). Thus, surface plasmon resonance studies suggest that the presence or absence of Y-P30 does not affect the affinity of PTN binding to syndecans and confirmed that both proteins interact with syndecans in a non-competitive manner.

Spontaneous Oligomerization of Y-P30 Results in SDS-resistant Complexes That Capture Large Amounts of PTN—To learn more about potential mechanisms, by which both peptides might bind to syndecans in a simultaneous manner we next analyzed whether Y-P30 and PTN might form oligomers spontaneously. In line with a previous report (3) we indeed found that Y-P30 in a Tris buffer under reducing conditions builds up SDS-resistant oligomers that migrate as a smear in the range of 22–47 kDa in SDS-PAGE (Fig. 5*A*). Moreover, these relatively large oligomers that potentially might contain up to 11 peptides as judged by the molecular weight of Y-P30 are capable of capturing PTN (Fig. 5*B*). In a glutaraldehyde fixation assay we found that PTN is associated with Y-P30 oligomers (Fig. 5*B*). Interestingly, when the assay was performed with equimolar amounts of pro-

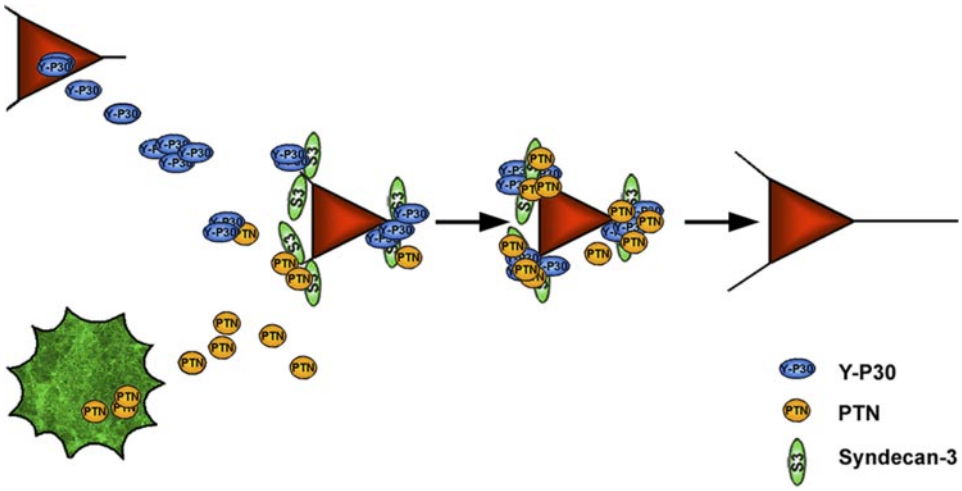

FIGURE 7. **Proposed model of Y-P30 neurite outgrowth-promoting activities.** Y-P30 accumulates in neurons during early brain development. The peptide has the capacity to form oligomers that in the extracellular space can capture larger amounts of PTN. Binding of both peptides to syndecan-3, which is at this stage of development the most abundant syndecan in neurons, will lead to the formation of larger macromolecular complexes. The high local concentration of all three components in the complex might then more efficiently stimulate neurite outgrowth via syndecan-3 signaling.

tein most of the PTN immunoreactivity co-migrated with the Y-P30 complex (Fig. 5*B*). In subsequent gel-filtration experiments we could show that Y-P30-PTN associate *in vitro* to macromolecular complexes of about 450–700 kDa (Fig. 5*C*). Although, as reported previously (23) PTN alone appears to form dimers, this complex was much larger than the homooligomeric complex of PTN alone (data not shown). Thus, Y-P30 and PTN together are capable of extensive heterooligomerization.

PTN and Syndecans Are Essential for the Neuritogenic Activity of Y-P30—Y-P30 was identified as a survival promoting and sprouting enhancing peptide for thalamic neurons (1). Previous studies have shown that PTN has strong neurite-outgrowth promoting activities for thalamic neurons during development that require syndecan binding (13). To prove the idea that PTN- and syndecan binding is involved in the neuritogenic activity of Y-P30 we used E16 thalamic primary neuronal cultures as model system. Neurons were plated on coverslips coated with or without PTN and fixed 24 h later. Neurites were counterstained with MAP2, and quantification was done by Sholl analysis. In accord with previous reports (13) we found a significantly increased neurite length in cultures plated on a PTN coat (Fig. 6, *A–E*), whereas the total number of neurites was not affected by PTN treatment (Fig. 6, *A–E*). Surprisingly, Y-P30 application to the culture medium without PTN coating reduced neurite length significantly without affecting neurite number (Fig. 6, *A–E*). However, a combined treatment with PTN and Y-P30 significantly enhanced neurite length in E16 thalamic cultures as compared with controls (Fig. 6, *A–E*). Moreover, the combined application of PTN-Y-P30 was more effective than the PTN coat without Y-P30 (Fig. 6, *A–E*). In control experiments we could show that a PTN blocking antibody as well as bath application with heparin completely abolished the outgrowth promoting activity of PTN and PTN-Y-P30 (Fig. 6, *A–E*). Similar results were obtained when solubilized GFP-syndecan-3 was added to the culture. Neither PTN nor PTN-Y-P30 had any significant effect on neurite length under this condition (Fig. 6, *A–E*). Interestingly, no reduction in neurite length was also observed when heparin, PTN blocking antibody, or GFP-syndecan-3 was added alone to the media (Fig. 6, *A–E*). This probably reflects the low PTN levels directly after plating of the neurons.

DISCUSSION

In the present study, we have identified PTN-syndecan-2 and -3 complexes as molecular targets for the neurite outgrowth-promoting activity of Y-P30. Furthermore, binding of Y-P30 to these partners is a plausible mechanism for its neuritogenic actions. We therefore propose that most of the previously reported sprouting-promoting activity of the peptide might be related to these interactions. The proposed model derived from the present and a previous study (1) is summarized schematically in Fig. 7. Y-P30 accumulates particularly in cortical neurons during early development and appears to be released from these cells via yet unknown mechanisms during the first postnatal weeks (1). In the extracellular space, the released peptide can subsequently associate with PTN and its neuronal receptors syndecan-2 and -3. In the first two postnatal weeks this will be primarily syndecan-3 that is localized prominently in neurites and later mainly on axonal membranes (24). The potential of Y-P30 for spontaneous oligomerization can then lead to a Y-P30 polypeptide network that might help to trap larger amounts of PTN (Fig. 7). Concomitant binding of both, PTN and Y-P30, at the heparan sulfate and chondroitin sulfate side chain of syndecan-3 can hook-up the complex to neuronal membranes. Via such a mechanism the local concentration of PTN at syndecan-3 can be increased, and the local PTN-Y-P30 network will eventually cluster several syndecan-3 molecules in close proximity to each other (Fig. 7). This in turn will improve the efficiency of syndecan-3 signaling and thereby explain the increase in neurite outgrowth after combined PTN and Y-P30 application.

Although appealing, the presented model leaves a variety of questions unanswered. This particularly concerns the nature of the PTN-Y-P30 triggered intracellular signal transduction. PTN contains two thrombospondin-like domains connected by a flexible linker and flanked by lysine rich domains at the N and C termini (Fig. 1). Interestingly, it was shown previously that the individual domains bind heparan sulfate weakly and fail to produce significant biological effects in neurite outgrowth assays (14). The sugar moieties responsible for differential binding have been recently identified (20), and it was observed that the interaction with syndecan-3 and its neurite-outgrowth-promoting activities crucially relies on the heparan sulfate side chains of syndecan (10). Accordingly, we found that syndecan binding as well as the neurite-outgrowth-promoting

activity of Y-P30 require glycosylation. At present, however, the intracellular signaling cascade involved is unknown. PTN stimulates neurite outgrowth in a cortactin-Src kinase-dependent manner (12), but the exact molecular mechanisms have not been deciphered yet.

Previous work has shown that the receptor biology of PTN is quite complex. Apart from syndecan the receptor protein-tyrosine phosphatase β (25) and anaplastic lymphoma kinase (26) have been reported to bind to PTN and induce intracellular signaling in a variety of cell types. It will be of interest in future studies to determine if Y-P30 also functionally interacts with these receptors rather than serving as an exclusive co-ligand for syndecan-binding. Noteworthy in this regard is a report that shows that Y-P30/PIF induces shedding of syndecan-1 and -2 from endothelial cells in culture (27). In light of the present study that identifies syndecan-2 and -3 as the first potential Y-P30 receptors it will be particularly interesting to address these questions. Additionally it is conceivable that Y-P30-binding triggers syndecan signaling on its own and not only provides a mechanism to induce clustering of PTN syndecan and/or PTN-syndecan complexes. Y-P30/PIF-related peptides reportedly affect several intracellular signal transduction cascades in different cell lines. For instance, Y-P30/PIF induces the phosphorylation of Akt kinase in a phosphoinositide 3-kinase-dependent manner (28), activates the NF-κB pathway (29–31), increases phospholipase A_2 (32), and also binds to calreticulin (33).

How does the interaction relate to other known functions of PTN and Y-P30 in the brain? Both proteins increase the survival of neurons in culture and after injury. It is unclear, however, which receptor mediates these neurotrophic effects. Particularly in cortical neurons and dopaminergic midbrain neurons, PTN has strong survival promoting effects (34), which resemble those observed for Y-P30. In addition to its neurotrophic role it was recently reported that PTN enhances regeneration of myelinated axons across a graft in the transected sciatic nerve of adult rats (35). It is therefore conceivable that administration of both peptides might provide previously unrecognized treatment options for neuronal diseases associated with defective neurite outgrowth and axon injury.

Moreover, both peptides might also be involved in other human disease processes. In cancer, a secreted glycosylated version of PIF is present in human urine, the amino acid sequence of which is almost identical to Y-P30 and which is supposed to be a cachexia factor (2). The synthesis of PIF/Y-P30 in tumor cells has been shown in independent reports (3, 36–38), and these findings are of potential clinical relevance, because PTN has been linked in many studies to tumor growth, probably due to its angiogenic effects (39). Noteworthy in this regard are also findings that point to a role of both PTN and YP30 in the pathogenesis of human breast cancer (6, 40). It has been suggested that PTN may be essential to the malignant phenotype of human breast cancers *in vivo*. Along these lines it was shown that expression of a dominant negative form of PTN inhibits growth and malignancy of tumor cell lines deriving from human breast cancer (41). Interestingly, this dominant negative form of PTN, which is able to heterodimerize with endogenous PTN, is most likely incapable of binding to YP30 (data not shown). Because cell motility and tissue invasion of tumor cells is also linked to the presence of syndecans on the cell surface (42), it will be of interest to investigate in future studies whether the newly established link to PTN and syndecans is related to the putative pathophysiological role of Y-P30 in cancer.

Acknowledgments—We gratefully acknowledge the professional technical assistance of C. Borutzki and M. Marunde. Finally we thank Dr. C. I. Seidenbecher for valuable comments on the manuscript.

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