Communication

The Core Protein of the Chondroitin Sulfate Proteoglycan Phosphacan Is a High-affinity Ligand of Fibroblast Growth Factor-2 and Potentiates Its Mitogenic Activity*

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Using a radioligand binding assay we have demonstrated that phosphacan, a chondroitin sulfate proteoglycan of nervous tissue that also represents the extracellular domain of a receptor-type protein tyrosine phosphatase, shows saturable, reversible, high-affinity binding (Kd ≈ 6 nM) to fibroblast growth factor-2 (FGF-2). Binding was reduced by only ∼56% following chondroitinase treatment of the proteoglycan, indicating that the interaction is mediated primarily through the core protein rather than the glycosaminoglycan chains. Immunocytochemical studies also showed an overlapping localization of FGF-2 and phosphacan in the developing central nervous system. At concentrations of 10 μg protein/ml, both native phosphacan and the core protein obtained by chondroitinase treatment potentiated the mitogenic effect of FGF-2 (5 ng/ml) on NIH/3T3 cells by 75–90%, which is nearly the same potentiation as that produced by heparin at an equivalent concentration.

Phosphacan and neurocan are nervous tissue-specific chondroitin sulfate proteoglycans that are high-affinity ligands of several immunoglobulin superfamily neural cell adhesion molecules and of the extracellular matrix proteins tenasin-C and tenascin-R (1–3). These interactions are variously mediated by the chondroitin sulfate chains, N-linked oligosaccharides present on the core glycoproteins, or by other structural features of the proteoglycans that have not yet been specifically identified. Phosphacan and neurocan both bind to neurons and have potent inhibitory effects on cell adhesion and neurite outgrowth, although in certain experimental situations phosphacan may also stimulate neurite growth. Neurocan is synthesized by neurons and is a member of the family of hyaluronan-binding chondroitin sulfate proteoglycans that also includes aggrecan, versican, and brevican, whereas phosphacan, which is produced by astrocytes, is an alternative splicing product representing the extracellular domain of a receptor-type protein tyrosine phosphatase. Phosphacan is also a ligand of the neural differentiation factor HB-GAM (3, 4), and both neurocan and phosphacan bind with high affinity to HB-GAM and to the related differentiation factor amphoterin, with which they co-localize in nervous tissue (3). The interactions with HB-GAM and amphoterin are largely mediated by the chondroitin sulfate chains of the proteoglycans.

In view of the finding that phosphacan and neurocan are high-affinity ligands of amphoterin and HB-GAM, we examined their interactions with FGF-2, a growth factor whose binding to the heparan sulfate chains of heparan sulfate proteoglycans has been much explored in relation to FGF-2 presentation to cell surface receptors. FGF-2, a heparin-binding protein that occurs in several isoforms of 16–25 kDa generated by alternative translation initiation sites and by proteolysis, is a mitogenic and/or differentiation factor for a variety of cell types of mesodermal and neuroectodermal origin, including fibroblasts, endothelial cells, and smooth muscle cells (5, 6). FGF-2 also has a wide distribution in nervous tissue, where it acts as a neurotrophic factor that promotes neuronal survival, stimulates neurite outgrowth, induces the synthesis of neurotransmitters, and is a mitogen and differentiation factor for glial cells (7, 8). The expression of FGF-2 is developmentally regulated from the late embryonic period to adulthood in the rat central nervous system, and this is reflected both in an increase in FGF-2 levels and a shift from lower to higher molecular weight forms (9, 10). Its biological effects are mediated through interactions with high-affinity transmembrane tyrosine kinase receptors and are modulated by its binding to low-affinity receptors in the form of cell surface heparan sulfate proteoglycans (6, 11).

In this study we have shown that the phosphacan core protein binds with high affinity to FGF-2, is localized in regions of the central nervous system where FGF-2 is also present, and potentiates the mitogenic effects of FGF-2 on NIH/3T3 cells. These results suggest that FGF-2 interactions with chondroitin sulfate proteoglycans may play a previously unsuspected role in mediating their biological actions and that at least in certain tissues this process may be of equal importance to the well

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1 The abbreviations used are: HB-GAM, heparin-binding growth-associated molecule; FGF-2, fibroblast growth factor-2; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; M-CSF, macrophage colony-stimulating factor; TGF-β, transforming growth factor β.
established relationship of FGF-2 with heparan sulfate proteoglycans.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Phosphacan, phosphacan-KS, and neurocan (originally designated the 3F8, 3H1, and 1D1 proteoglycans, respectively) were isolated from rat brain by ion exchange chromatography, gel filtration, and immunoaffinity chromatography (12). Aggrecan and Ng-CAM were isolated as described by Milev et al. (13), and neurocan-C (the COOH-terminal half of neurocan) was isolated from adult rat brain by immunoaffinity chromatography using the 1D1 monoclonal antibody (12). Recombinant human FGF-2 was prepared as described previously (14) or obtained from R & D Systems (Minneapolis, MN).

**Binding Assays**—Binding assays were performed as described previously (13). Briefly, proteins were coated in removable Immulon-4 wells, and binding of $^{125}$I-labeled phosphacan and neurocan was measured in 20 mM Tris, pH 7.4, containing 150 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 0.02% NaN$_3$, and 1 mg/ml heat-treated BSA, following incubation with gentle shaking (45 rpm) for 3–12 h at room temperature. Reversibility of binding was demonstrated by incubation for varying periods in the presence of an excess of unlabeled proteoglycan (13). Scatchard plots were generated, and dissociation constants were determined using the Macintosh version of the Ligand program (15).

Phosphacan and neurocan were labeled to a specific activity of 1–$10^{18}$ cpm/mol with $^{125}$I by the lactoperoxidase/glucose oxidase method using Enzymobeads (Bio-Rad) according to the manufacturer’s instructions. Typically, 10–25 µg of protein were labeled per reaction, and free iodine was removed using a PD-10 column (Amersham Pharmacia Biotech).

To evaluate the effects on their binding properties of removing the chondroitin sulfate chains from phosphacan and neurocan, the labeled proteoglycans were treated for 1 h at 37°C with protease-free chondroitinase ABC (Seikagaku America, Rockville, MD) at a concentration of 0.5 unit/ml in 0.1 M Tris-HCl buffer, pH 8.0, and completeness of digestion was confirmed by SDS-PAGE on a 5% gel and autoradiography of the resulting core glycoproteins.

**Immunocytochemistry**—Double immunofluorescence localization of proteins was performed as described previously (16), using the 3F8 monoclonal antibody to phosphacan (12) and a rabbit antiserum to FGF-2 that was kindly provided by Dr. Daniel Rifkin (New York University Medical Center).

**Mitosogenesis Assay**—NIH/3T3 cells grown in DMEM supplemented with 10% fetal bovine serum (FBS) were seeded in 96-well microtiter plates (Falcon) at a density of 4$	imes$10$^3$ cells/well. After the cells became confluent (4–5 days) they were transferred overnight to DMEM containing 0.5% FBS. FGF-2 (5 ng/ml) was added in DMEM, 0.5% FBS, 1% BSA either alone or together with proteoglycan, or heparin, or medium alone. After 17 h the cells were counted (4–10 replicates) in a Coulter counter.

**RESULTS**

**Interactions of Phosphacan and Neurocan with Fibroblast Growth Factor-2**—In a radioligand binding assay, phosphacan and neurocan bound to FGF-2 to the extent of 46–67%, indicating that these interactions are mediated primarily through the core proteins rather than the glycosaminoglycan chains (Fig. 1). The purity of the proteoglycans and the effectiveness of the chondroitinase digestion were demonstrated by the shift of all $^{125}$I-labeled proteoglycan from the stacking gel to core proteins of the expected size in the separating gel following SDS-PAGE and autoradiography (Fig. 1). Binding of the COOH-terminal half of neurocan (designated neurocan-C) containing the EGF-, lectin-, and complement regulatory-like domains was 20% less than that of the full-length proteoglycan and was also reduced by one-third after chondroitinase treatment, whereas there was very little binding of other proteins tested, such as the neural cell adhesion molecule Ng-CAM (8%), the 3G2 monoclonal antibody to Ng-CAM (3.5%), or cartilage aggrecan (11%), whose binding was not affected by chondroitinase treatment (Fig. 1). Binding was reversible and inhibited to the extent of 90% or greater by unlabeled ligand (phosphacan or neurocan, 30–90 nM) as well as by heparin or heparan sulfate, but only by 20% by aggrecan or chondroitin sulfate (data not shown). $^{125}$I-Labeled FGF-2 also bound to immobilized native or chondroitinase-treated phosphacan and neurocan, although the percent bound was somewhat lower (i.e. 18–20% for phosphacan and 11–14% for neurocan, data not shown). This difference in extent of binding is probably attributable to partial hindrance of FGF-2 access to its binding site(s) as a result of immobilizing the large proteoglycans by adsorption to plastic.

Saturation curves and Scatchard plots for the binding of chondroitinase-treated phosphacan and neurocan to FGF-2 gave dissociation constants of 6–12 nM (Fig. 2), and similar dissociation constants were obtained for binding of native phosphacan and neurocan and of chondroitinase-treated neurocan-C. Immunocytochemical studies showed an overlapping localization of FGF-2 with phosphacan (Fig. 3) and neurocan (data not shown) in the developing central nervous system, which is a necessary condition if their high affinity interactions
demonstrated in radioligand binding assays are to be biologically relevant.

Phosphacan Potentiates the Mitogenic Activity of FGF-2—To determine the significance of the high affinity interactions between FGF-2 and phosphacan and neurocan, we examined the possibility that these proteoglycans might affect the mitogenic activity of FGF-2. When tested at concentrations of 10 μg of protein/ml (\(1.5 \times 10^{15}\) cpm/mol), and FGF-2 was coated on wells using a concentration of 1.5 μg/ml. Points in the saturation curves are averages of duplicate determinations ± S.E.

**FIG. 2.** Saturation curves and Scatchard plots for the binding of chondroitinase-treated \(^{125}\)I-labeled phosphacan and neurocan to FGF-2. Binding values represent specific binding defined as total counts/min bound minus counts/min bound to wells coated with BSA. Phosphacan and neurocan were tested at 1.5–70 ng/well (1.5–3 \(\times\) \(10^{15}\) cpm/mol), and FGF-2 was coated on wells using a concentration of 1.5 μg/ml. Points in the saturation curves are averages of duplicate determinations ± S.E.

**FIG. 3.** Double immunofluorescence showing the co-localization of FGF-2 (A, C, E) and phosphacan (B, D, F) in the central nervous system. Both proteins are present in the ventricular zone (arrows) of E13 cerebrum (A/B), in the dorsal root entry zone (asterisk), mantle layer (ml), and dorsal root ganglia (dg) of E13 spinal cord (C/D), and in E19 cerebral cortex (E/F). Bars, 200 μm.

**FIG. 4.** Stimulation by phosphacan of FGF-2-induced \(^{3}\)H-thymidine incorporation into NIH/3T3 cells. Confluent cells were transferred overnight to medium containing 0.5% fetal bovine serum before measuring the mitogenic effects of 10% serum or FGF-2 (5 ng/ml) in the presence and absence of heparin (10 μg/ml) or proteoglycan (10 μg protein/ml). The bars show the percent increase in \(^{3}\)H-thymidine incorporation (average ± S.E. of triplicate samples from a typical experiment) in the presence of the indicated additions of serum, heparin, or proteoglycan. Phosphacan was also treated with chondroitinase (Chase) to compare the effects of the core protein with those of the native proteoglycan. Results similar to those shown above were obtained in three separate experiments. Incorporation of \(^{3}\)H-thymidine in the presence of 0.5% serum was 10,000–30,000 cpm/well, and base-line labeling in the presence of FGF-2 alone was usually in the range of 100,000–150,000 counts/min/well.

**Phosphacan Interactions with Fibroblast Growth Factor-2**

Phosphacan Potentiates the Mitogenic Activity of FGF-2—To determine the significance of the high affinity interactions between FGF-2 and phosphacan and neurocan, we examined the possibility that these proteoglycans might affect the mitogenic activity of FGF-2. When tested at concentrations of 10 μg of protein/ml (\(~60\) nM), phosphacan and phosphacan-KS (which contains both chondroitin sulfate and keratan sulfate chains on the phosphacan core protein; Refs. 12 and 17) potentiated the mitogenic activity of FGF-2 on NIH/3T3 cells to approximately the same extent (i.e. by \(~85–90\)% as heparin at a concentration of 10 μg/ml, although on a molar basis this concentration of heparin (\(~670\) nM based on an molecular size of 15,000) is over 10 times that required for phosphacan. Neurocan had no potentiating activity, and the effect of phosphacan was only slightly reduced by chondroitinase treatment of the proteoglycan (Fig. 4). Similar effects (data not shown) were observed using (at 10 μg of protein/ml) the unfractionated native or chondroitinase-treated PBS-soluble chondroitin sulfate proteoglycans of rat brain, from which phosphacan, phosphacan-KS, and neurocan were isolated by immunoaffinity chromatography (12) and of which they constitute the major components. When added by themselves in the absence of FGF-2, neither phosphacan, phosphacan-KS, nor the unfractionated proteoglycans showed any mitogenic activity, demonstrating that like heparin and heparan sulfate they specifically modulate the
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activity of FGF-2 in the absence of any intrinsic growth stimulatory properties and that the effects observed were not due to contaminating growth factors that co-purified with the proteoglycans.

DISCUSSION

Our studies have demonstrated that phosphacan and neurocan are high-affinity ligands of FGF-2 and that both native phosphacan and its core protein potentiated the mitogenic activity of FGF-2 on NIH/3T3 cells to an extent essentially equivalent to that of heparin. Although neurocan did not show similar potentiating effects, it is possible that neurocan may also modulate the mitogenic activity of FGF-2 for particular cell types in the central nervous system but that this was not evident in our assays due to different properties of the FGF-2 receptors in 3T3 cells. Alternatively, neurocan may not be involved in the presentation of FGF-2 to its receptors but rather modulate its actions in other ways, such as by serving as a reservoir for FGF-2 in the extracellular space.

Neither the binding affinities nor the extent of mitogenic potentiating activity were greatly affected by chondroitinase treatment of the proteoglycans, and the relatively small decreases observed following removal of chondroitin sulfate (20–30%) may be largely related to effects of the glycosaminoglycan chains on the core protein conformation, rather than implying a direct participation in the binding and presentation of FGF-2. These interactions therefore appear to be largely mediated by the proteoglycan core (glyco)proteins and in this respect differ both from the interaction of FGF-2 with heparan sulfate proteoglycans (which is mediated by the heparan sulfate chains) and from the binding of phosphacan and neurocan to differentiation factors such as amphoterin and the heparin-binding growth-associated molecule (HB-GAM)/pleiotrophin. However, the interactions of phosphacan and neurocan with FGF-2 resemble its binding to a carboxyl-terminal peptide sequence present in a chondroitin sulfate proteoglycan form of macrophage colony-stimulating factor (M-CSF), which serves as a precursor for the 85-kDa M-CSF that is generated by proteolytic processing (18). The binding of two other chondroitin sulfate proteoglycans, betaglycan and decorin, to transforming growth factor β (TGF-β), is also mediated by their core proteins rather than the glycosaminoglycan chains (19). Whereas betaglycan is considered to serve as a co-receptor for TGF-β, both decorin and the proteoglycan precursor of M-CSF neutralize rather than potentiate the mitogenic activities of their growth factor ligands. The high-affinity binding of the phosphacan core protein to FGF-2 and its ability to potentiate the mitogenic effects of FGF-2 to an extent comparable with that of heparin and heparan sulfate on a weight basis (and with 10 times greater potency on a molar basis) suggest that FGF-2 interactions with chondroitin sulfate proteoglycans may play a previously unsuspected role in mediating their biological actions and that at least in certain tissues this mechanism may be of equal importance to the well established relationship of FGF-2 with heparan sulfate proteoglycans. The specific localization and developmental regulation of phosphacan and neurocan in the central nervous system (12, 16, 20) suggests that their interactions with FGF-2 may partially regulate growth factor availability and activity during nervous tissue histogenesis. Because phosphacan is an mRNA splicing product that contains the entire extracellular domain of a receptor-type protein tyrosine phosphatase (17), binding of FGF-2 to phosphacan may also compete with or otherwise modulate similarly high-affinity FGF-2 interactions with the transmembrane phosphatase and thus provide an additional mechanism for tyrosine kinase/phosphatase-mediated FGF-2 signaling across the plasma membrane.

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