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Insulin-Like Growth Factor Binding Protein-2 Binds to Cell Surface Proteoglycans in the Rat Brain Olfactory Bulb*

V. C. RUSSO, L. A. BACH, A. J. FOSANG, N. L. BAKER, AND G. A. WERTHER

From The Centre for Hormone Research (V.C.R., N.L.B., G.A.W.), Royal Children's Hospital, Parkville 3052, Victoria, Australia; University of Melbourne Department of Medicine (L.A.B.), Austin and Repatriation Medical Centre, Heidelberg 3084 and Department of Paediatrics (A.J.F.), Orthopaedic Molecular Biology Research Unit, Royal Children's Hospital, Parkville 3052, Victoria, Australia

ABSTRACT

A family of six insulin-like growth factor binding proteins (IGFBPs) bind IGF-I and modulate its biological activity. IGFBPs may bind to macromolecules on the cell surface or pericellular extracellular matrix, and this interaction may modulate their effect on IGF activity. To date, little is known about the specificity of IGFBPs in the regulation of IGF action in the brain. We therefore explored whether IGFBPs were associated with cell membrane or extracellular matrix components in the rat brain. IGF-I binding sites with the characteristics of an IGFBP were found in the olfactory bulb mitral cell layer. This IGFBP was identified as IGFBP-2 by immunoprecipitation of both solubilized membrane preparations and cross-linked ^{125}I-IGF:IGFBP complexes. While binding of IGFBP-2 to cell membranes was unaffected by RGD-containing peptide, it was inhibited by high salt concentration, suggesting interaction with proteoglycans. IGFBP-2 bound *in vitro* to the glycosaminoglycans chondroitin-4 and -6-sulfate, keratan sulfate, and heparin. IGFBP-2 also bound the proteoglycan aggrecan, an effect reduced by digestion of its glycosaminoglycans. Binding of IGFBP-2 to chondroitin-6-sulfate decreased the binding affinity of IGFBP-2 for IGF-I approximately 3-fold. Finally, an IGFBP-2 antibody coimmunoprecipitated IGFBP-2 and an approximately 200 kDa proteoglycan containing chondroitin-sulfate side chains from the rat olfactory bulb, providing definitive evidence for IGFBP-2 binding to olfactory bulb proteoglycans. These findings indicate that IGFBP-2 binds to proteoglycans in cell membranes of the rat olfactory bulb. Because we have previously shown that IGFs are highly expressed in the rat olfactory bulb, cell associated IGFBP-2 may have an important role in directing IGFs to specific sites in this brain region. (*Endocrinology* **138:** 4858–4867, 1997)

THE INSULIN-LIKE growth factors (IGFs) are peptides that regulate growth and differentiation. They are synthesized in most tissues including the developing central nervous system and may act in an endocrine, autocrine, or paracrine manner (1, 2). Cellular responses to IGFs are modulated by a family of six insulin-like growth factor binding proteins (IGFBPs) (1, 2). Such modulation is the result of IGF/IGFBP interactions that occur in the pericellular and/or extracellular space, possibly involving components of the extracellular matrix (ECM) (2).

Recent studies have demonstrated association of some IGFBPs with the ECM or cell surface via glycoproteins, collagens, integrins (3, 4), and glycosaminoglycans (5–9). These studies showed that IGFBP-3 when cell-associated (7) or IGFBP-5 when ECM-bound (3) had lowered binding affinity for IGF-I. Additionally, the formation of IGF-I/IGFBP-5 complexes can be inhibited by heparin by altering the binding affinity of IGFBP-5 for IGF-I (5). More recently, it has been shown that IGFBP-2/IGF complexes can bind to heparin and extracellular matrix (10). These *in vitro* findings support the hypothesis that IGFBPs, in addition to stabilizing and regulating levels of diffusible IGFs, might regulate IGF-I cellular responses by facilitating receptor targeting of IGF-I or modulating IGF-I bioavilability in the pericellular space *in vivo* (2, 9).

The sites of expression of messenger RNA for IGFs and IGFBPs have been precisely located in rat brain (11–14). The olfactory bulb, a brain region undergoing postnatal differentiation and remodeling, is rich in locally expressed components of the IGF system including IGF-I, IGF-I receptor, and IGFBPs, most abundantly IGFBP-2 (15, 16, 14), which we have further characterized *in vitro* (14, 17).

Although brain-derived IGFBPs have also been characterized (14, 18), very little is known about their functional role in the nervous system. For example, it is not known whether interactions between IGFs and IGFBPs occurring in the extracellular space are further modulated by other biomolecules in the extracellular environment. We therefore explored whether any of the IGFBPs were associated with cell membrane or extracellular matrix components in the rat brain.

Materials and Methods

Reagents

Recombinant human insulin-like growth factor-I and II (IGF-I, IGF-II) were generous gifts from Dr. A. Sköttner (KabiPharmacia, Peptide Hormones, Sweden). Des(1–3) IGF-I was a gift from Dr. C. Williams (Uni-

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Address all correspondence and requests for reprints to: Assoc. Prof. George A. Werther, Centre for Hormone Research, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia. E-mail: Werther@cryptic.rch.unimelb.edu.au.
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versity of Auckland, New Zealand). Insulin was purchased from Novo Nordisk Pharmaceuticals Pty Ltd (North Rocks, New South Wales, Australia). Rabbit antibovine-IGFBP-2 antiserum that recognizes rat-IGFBP-2 (cross-reactivity < 0.5% with IGFBP-1, -3, -4, -5) (19) and rabbit antihuman-IGFBP-5 antiserum that recognizes rat IGFBP-5 (cross-reactivity < 0.5% with IGFBP-1, -2, -3, -4) (20) were purchased from UBI (Lake Placid, NY). Antichondroitin-sulfate monoclonal antibodies 3B3 and 2B6 were gifts from Prof. B. Caterson (Cardiff, UK) (21). Antimouse-IgM-HPR was obtained from Silenus (Silenus Labs., Hawthorn, Australia). Immunohistochemical staining was performed with Vectastain elite ABC kit (Vector Labs, Burlingame, CA). IGFBP-2 was purified as previously described by Bach *et al.* (22). The proteoglycan aggrecan was extracted from pig laryngeal cartilage as previously described (23). Purified keratan sulfate and glycosaminoglycans enriched in chondroitin-4-sulfate and chondroitin-6-sulfate were a kind gift from Prof. Dennis Lowther (Monash University, Melbourne, Australia). Chondroitin ABC lyase (*Proteus vulgaris*) and keratanase (*Pseudomonas sp.*) were obtained from Seikagaku Kogyo (Japan). ^{125}I-IGF-I (~2000 Ci/mmol) and ^{14}C protein molecular weight markers were bought from Amersham (North Ryde, New South Wales, Australia). Disuccinimidyl Suberate (DSS) was obtained from Pierce (Rockford, IL). Chemical reagents (Analar and HPLC grade) were purchased from BDH-Merck Pty Ltd (Kilsyth, Victoria, Australia). ECL immunodetection kit, phenylmethylsulfonylfluoride (PMSF), Triton X-100, proteinase inhibitors E-64 and pepstatin were purchased from Boehringer (Mannheim, Germany). Heparin, aminoalkilsilane, aprotinin, RIA grade BSA, and protein A-Sepharose CL-4B were purchased from Sigma Chemical Co. (St. Louis, MO). GRGDSP and GRGESP peptides were purchased from Auspep (Parkville, Victoria, Australia). Nitrocellulose membranes were obtained from Schleicher and Schuell (Dassel, Germany). X-Omat AR and Biomax films were obtained from Kodak (Eastman Kodak Company, NY). Microtiter immunoassay plates (Immunolon-4) were purchased from Dynatech Laboratories Inc. (Sullyfield Circle, Chantilly, VA). [4-(2-aminoethyl)benzene]sulfonyl-fluoride (AEBSF) was from Calbiochem-Novabiochem Pty Ltd (Alexandria, New South Wales, Australia). PBS was from Histo Labs (New South Wales, Australia) and Ilford K5 nuclear emulsion was from Ilford Limited (Mobberley, UK).

Brain tissue samples

One day postnatal Sprague-Dawley rats were killed by decapitation and whole brains or olfactory bulbs (OB) were obtained as previously described (24). Dissected whole brains were immediately snap frozen in dry ice/isopentane and stored at −70 C until tissue sectioning by cryostat. All procedures involving dissection of animals were approved by the Royal Children's Hospital Animal Experimentation Ethics Committee.

^{125}I-IGF-I in vitro autoradiography

The *in vitro* autoradiography protocol was adapted from that previously described by Werther *et al.* (15). Briefly, 20 μm parasaggittal whole brain and coronal OB frozen sections were cut by cryostat, allowed to dry, and stored at −70 C until used. Slides were rehydrated at RT for 1 h with 2 ml of 10 mM Tris/HCl, pH 7.4, 0.1% BSA, 2 mM $MgCl_2$, 2 mM $CaCl_2$, changing the buffer every 10 min. Sections were then incubated at 4 C for 16 h with the same buffer containing ^{125}I IGF-I (~ 30000 cpm) in the presence or absence of 1 μg/ml of IGF-I or des(1–3)IGF-I. Sections were washed four times for 5 min with 2 ml of ice-cold buffer. Slides were air dried and exposed to x-ray film for 1–5 days and then dipped in Ilford K5 nuclear emulsion and stored with dessicant at 4 C. Emulsion was developed in Kodak D19, fixed in Ilford Hypam, and the sections stained with hematoxylin.

OB membrane preparation

Olfactory bulb membranes were obtained by modification of a previously described method (25). Olfactory bulbs, fresh or from a frozen stock stored at −70 C, were resuspended in ice-cold buffer (10 mM Tris/HCl, 2 mM PMSF, 1 TIU/ml of Aprotinin) and mechanically disaggregated through 19 gauge and 23 gauge needle syringes. The tissue suspension was centrifuged at 500 rpm for 5 min at 4 C, and the supernatant was centrifuged at 14,000 rpm for 1 h at 4 C. The pellet, called the membrane fraction (MF), was resuspended in ice-cold 10 mM Tris-HCl, pH 7.4. Aliquots of MF were adjusted to a total protein concentration of 100 μg/80 μl, followed by addition of 0.1% BSA and storage at −20 C until used.

^{125}I-IGF-I binding and cross-linking to OB membranes

OB membranes (100 μg/80 μl) were incubated in a final volume of 100 μl with ^{125}I- IGF-I (~40,000 cpm/tube) in the presence or absence of the following competitors: 1 μg/ml IGF-I, IGF-II, des(1–3)-IGF-I or 10 μg/ml of insulin for 2 h at 37 C with rotation. Samples were then incubated on ice for 10 min followed by addition of DSS to a final concentration of 1 mM and further incubated for 15 min at 4 C. The cross-linking reaction was quenched by the addition of 45 μl of ice-cold 100 mM Tris, pH 7.4/10 mM EDTA. Samples were analyzed by denaturing SDS-PAGE (3–16% gradient gel) under reducing conditions where indicated. Gels were fixed (50% methanol/10% acetic acid), stained with Coomassie blue and dried before exposure to x-ray film for 3–10 days.

Western ligand blot (WLB) analysis

Olfactory bulb membranes were solubilized in nonreducing sample buffer and electrophoresed by SDS-PAGE. Adult rat serum (5 μl) and 5 μl of nonreduced ^{14}C protein MW markers were run in parallel lanes as necessary. Separated proteins were transferred to nitrocellulose filters and WLB of the transferred protein was carried out according to the method of Hossenlopp *et al.* (26) using ^{125}I-IGF-I (1.5×10^6 cpm/50 ml). Dried filters were exposed to x-ray film for 3–10 days.

Immunoprecipitation

Immunoprecipitation of IGFBP-2 and IGFBP–5 from fresh OB membranes or OB membranes cross-linked with ^{125}I-IGF-I was performed as previously described (14). Anti-IGFBP-2, anti-IGFBP-5, or normal rabbit serum were used at a final dilution of 1:100. The immunoprecipitated samples derived from fresh OB membranes were dissolved in Laemmli sample buffer, boiled for 5 min, run on 12% SDS-PAGE or 3–16% gradient gels, and WLB performed as described above. The immunoprecipitated samples derived from cross-linked OB membranes were processed as described for the cross-linking analysis. Pure IGFBP-2 (10 ng) or cross-linked ^{125}I-IGF-I/IGFBP-2 complex were also immunoprecipitated with both anti-IGFBP-2 and IGFBP-5 antisera and used as controls.

Immunohistochemistry

Olfactory bulbs, obtained as above, were fixed in 4% paraformaldehyde in PBS then processed through graded ethanol and xylene and infiltrated with paraffin wax. Sections (10 μm) were cut and mounted on aminoalkylsilane-coated slides. Dewaxed and PBS equilibrated sections were incubated at 4 C overnight with a 1:400 dilution of the rabbit anti-IGFBP-2 antiserum or with normal rabbit serum (1:400) as a control. IGFBP-2 immunoreactivity was detected with a Vectastain ABC kit according to the manufacturer's instruction. Sections were counter stained with hematoxylin and coverslipped.

Dissociation of endogenous IGFBP-2 from OB membranes by RGD/RGE peptides or ionic strength

OB membranes (100 μg/80 μl) were incubated in 120 μl of 10 mM Tris-HCl, pH 7.4, 0.1% BSA with or without: GRGDSP (10–100 μg/ml), GRGESP (10–100 μg/ml) or NaCl (150–500 mM). Samples were incubated for 16 h at 4 C with gentle rotation. Tubes were centrifuged at 15,000 rpm for 30 min at 4 C and the pellet was washed, spun, and resuspended in 80 μl of 10 mM Tris-HCl, pH 7.4, 0.1% BSA before incubation with ^{125}I-IGF-I in the presence or absence of 1 μg/ml of IGF-I (for 120 min at 37 C). Samples were cross-linked and electrophoresed as described above. Gels were fixed, stained, dried, and exposed to x-ray film for 3–10 days.

Binding of IGFBP-2 to proteoglycan and glycosaminoglycans

The procedure for binding of IGFBP-2 to proteoglycan and glycosaminoglycans was adapted from Bonaldo *et al.* (27). Immulon-4 96 well-

plates were coated with 500 ng of aggrecan (a proteoglycan containing both chondroitin sulfate and keratan sulfate) or with the glycosaminoglycans chondroitin-4-sulfate, chondroitin-6-sulfate, keratan sulfate or heparin in 200 μl of PBS (16 h, 37 C). To remove excess salt and unbound reagents, wells were washed 4 times for 5 min with 250 μl of binding buffer (BB) [16 mM Tris-HCl, pH 7.2, 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mg/ml BSA, 0.02% Tween 20]. Wells were then blocked with BSA (250 μl of 1% BSA in 16 mM Tris-HCl, pH 7.2, 50 mM NaCl) for 1 h at 37 C, washed four times with BB, then incubated at 37 C for 60 min with 200 μl of BB in the presence or absence of 10 ng of purified IGFBP-2. IGFBP-2 was also bound to aggrecan previously digested with chondroitinase ABC (0.25 U/mg) and keratanase (0.03 U/mg) in the presence of protease inhibitors (10 mM EDTA, 20 μg/ml E-64, 1 mM AEBSF and 2 μM pepstatin) at 37 C for 3 h. Unbound IGFBP-2 was removed by washing, and wells were incubated at 4 C for 16 h with ^{125}I-IGF-I ($\sim 3 \times 10^4$ cpm/200 μl or $\sim 6 \times 10^4$ cpm/200 μl as indicated) in the presence or absence of unlabeled IGF-I (0.5 μg/ml). Wells were then washed four times with BB. BSA coated wells were used as a control. Bound cpm were solubilized with 250 μl of 200 mM NaOH/0.1% Triton X-100 and radioactivity was determined by γ-counting. Experiments were performed three times, and each point was measured in triplicate or quadruplicate as indicated.

Determination of the binding affinities of IGF-I for glycosaminoglycan associated and soluble IGFBP-2

Glycosaminoglycan associated. Microtiter 96-well plates were coated with 500 ng/200 μl of chondroitin-6-sulfate(C-6S), saturated with 1% BSA, and incubated with IGFBP-2 (10 ng/well) as described above. Following removal of unbound IGFBP-2, wells were incubated for 16 h at 4 C with ^{125}I-IGF-I (3×10^4 cpm) in the presence or absence of increasing concentrations (0.006–6 nM) of unlabeled IGF-I. Nonspecific binding was measured in wells coated with C-6S without added IGFBP-2, and wells coated with BSA with (10 ng/well) or without IGFBP-2. Nonspecific binding was less than 1.5% of the added radioactivity for all these conditions. All wells were then washed four times with BB and bound radioactivity was measured in a γ-counter. Each point was measured in quadruplicates in each of three experiments. Data were analyzed by the LIGAND program (28).

Solution binding assays (29). Rat IGFBP-2 (2 ng) was incubated with ^{125}I-IGF-I (~13 000 cpm) with and without increasing concentrations of unlabeled IGF-I (0.006–6 nM) for 16 h at 4 C in binding buffer as described above (final volume 0.4 ml). Bound and free ^{125}I-IGF-I were separated by incubation with 0.5 ml ice-cold 5% charcoal/2% fatty-acid free BSA in Dulbecco's PBS for 10 min on ice and centrifugation at 1300 × g (4 C for 30 min). Bound radioactivity in supernatants was quantitated in a γ-counter. Each point was measured in triplicate in each of two experiments. Binding affinities were calculated using the LIGAND program (28).

Western immunoblot analysis with antichondroitin sulfate antibodies

The monoclonal antibodies 3-B-3 and 2-B-6 recognize chondroitin sulfate chains containing a nonreducing terminal saturated or Δ-4–5-unsaturated glucuronic acid residue adjacent to *N*-acetylgalactosamine-6-sulfate or *N*-acetylgalactosamine-4-sulfate, respectively (21, 30). Because very few chondroitin sulfate chains terminate with these structures, pretreatment of the membrane with chondroitinase-ABC (0.01 U/ml for 3 h at 23 C in 50 mM sodium acetate pH 7.4) is required to remove the terminal saccharides and expose internal epitopes. Monoclonal antibodies 3-B-3 and 2-B-6 together, each diluted 1:2,000 in 1% skim milk powder in PBS were incubated on the membranes overnight at 4 C. Antimouse IgM/HRP was used at a dilution of 1:7,500. Immunoreactivity was detected using the ECL kit according to the manufacturer's instructions.

Results

^{125}I-IGF-I binds to both receptor and nonreceptor-binding sites in rat brain

We used *in vitro* autoradiography to localize ^{125}I-IGF-I binding in the rat brain and found widespread binding displaceable by IGF-I (Fig. 1c). In contrast, des(1–3)IGF-I, an IGF-I analog which has conserved binding affinity for the IGF-I receptor (29) but has greatly reduced affinity for all the IGFBPs (31) displaced binding throughout the brain except in the OB, the cerebellum and possibly hippocampus (Fig. 1b). In the OB, this noncompetible binding was localized to the mitral cell layer (MI), which is a layer of high density binding for ^{125}I-IGF-I (15). Because IGFBPs, particularly IGFBP-2 and IGFBP-5, are also synthesized in this region (11–14), we hypothesized that cell-associated IGFBPs could account for the nonreceptor binding of IGF-I in this brain region.

A 38-kDa IGF/IGFBP complex is found in OB membrane preparations

To further characterize these nonreceptor-IGF-I binding sites, we performed ^{125}I-IGF-I binding to OB membrane in the presence or absence of an excess of cold IGF-I, II, des(1–3)IGF-I (1 μg/ml), or insulin (10 μg/ml), followed by cross-linking with DSS. In addition to bands consistent with the IGF-I receptor (bands at ~110 and >200 kDa), autoradiog-

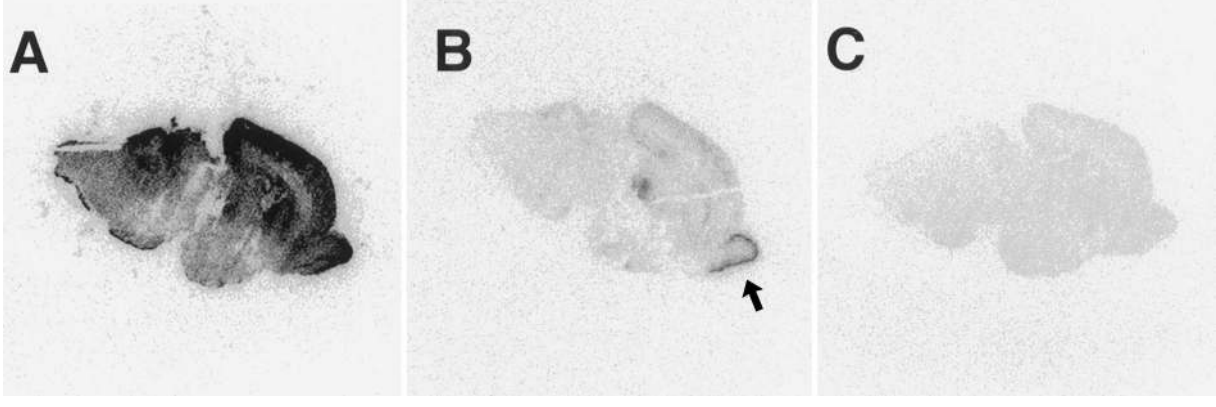

FIG. 1. ^{125}I-IGF-I binds to both receptor and nonreceptor-binding sites in the rat brain. Parasagittal whole brain sections were incubated at 4 C for 16 h with ^{125} IGF-I (~30000 cpm) in the presence of 1 μg/ml of IGF-I (C) or des(1–3)IGF-I (B), or in the absence of growth factor (A). Autoradiography in panel B shows the residual ring of nonreceptor binding located in the plexiform layer of the OB (*arrow*). Exposure to x-ray film was for 5 days.

raphy also revealed a band with apparent M_r of 38 kDa (Fig. 2, lane a). Although des(1–3)-IGF-I was equipotent to IGF-I and IGF-II in competing for ^{125}I-IGF-I binding to the IGF-I receptor (bands at ~100 kDa and >200 kDa) (lanes b, c, e), the 38 kDa band was abolished by coincubation with an excess of cold IGF-I or II (lanes b and c, respectively) but not by des(1–3)IGF-I (lanes e) or insulin (lanes d). These findings are consistent with the presence of a membrane-associated IGFBP/IGF-I complex.

IGFBP-2 is the IGFBP associated with OB membranes

To further characterize the IGFBPs in OB tissue, we performed WLB on solubilized OB membranes. The results showed a single band at about 32 kDa (Fig. 3, lane 2). This band was barely detectable by WLB on membranes obtained from whole brain (data not shown). The size of this band is consistent with either IGFBP-2 or IGFBP-5, both of which are expressed in this region of the brain (14). However, an antiserum against IGFBP-2 (Fig. 4A, lane b) but not IGFBP-5 (Fig. 4A, lane c) immunoprecipitated a 32-kDa band from solubilized OB membranes. Similarly, a 38-kDa complex was immunoprecipitated from cross-linked OB membrane (Fig. 4B, lane b) or cross-linked purified IGFBP-2 (Fig. 4B, lane d) by the IGFBP-2 antiserum but not by anti-IGFBP-5 antiserum (Fig. 4B, lane c).

Our results obtained by cross-linking of ^{125}IGF-I to OB membrane (Fig. 2), WLB (Fig. 3), and immunoprecipitation (Fig. 4) show that IGFBP-2 is cell membrane associated in the OB and supports the hypothesis that it may account for the

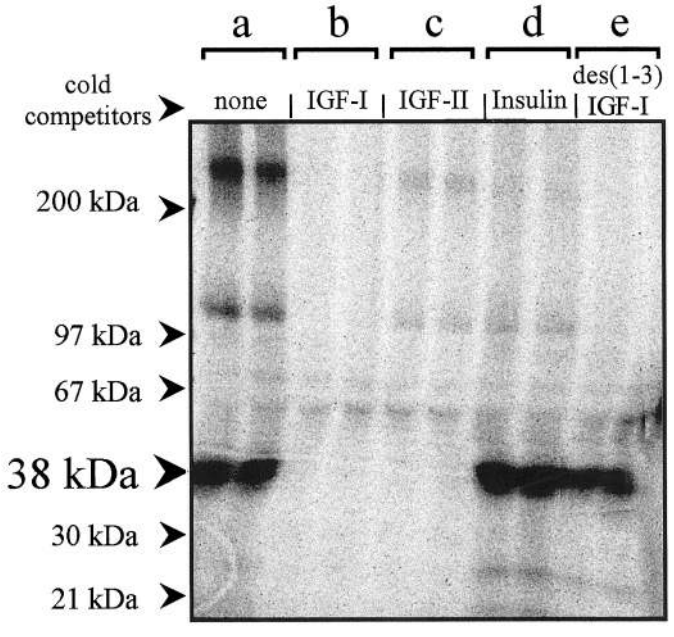

FIG. 2. A 38-kDa IGF/IGBP complex is found in OB membranes. OB membranes were incubated with ^{125}I-IGF-I (~40000 cpm) in the presence (lanes b–e) or absence (lane a) of 1 μg/ml of IGF-I (lane b), IGF-II (lane c), des(1–3)IGF-I (lane e) or 10 μg/μl of insulin (lane d). Samples were then cross-linked by 1 mM DSS and subjected to 3–16% SDS-PAGE under reducing conditions. The 38-kDa band (lanes a, d, e) represents an IGFBP/^{125}I-IGF-I complex. The bands at ~110 and >200 kDa (a–e) represent the IGF-I receptors. Molecular weight markers are indicated. Autoradiography was for 5 days.

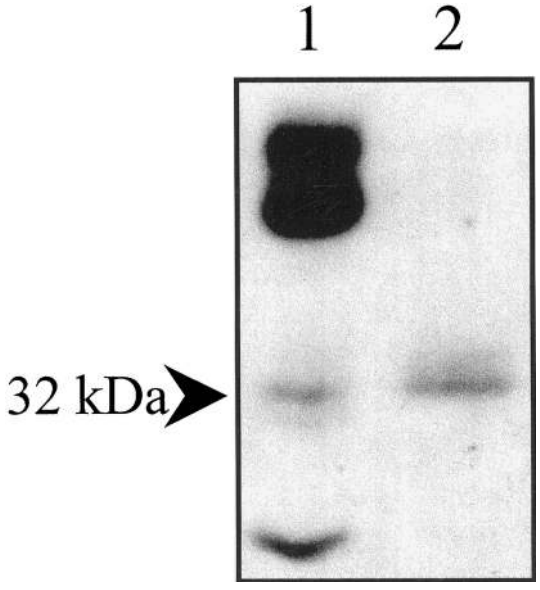

FIG. 3. A 32-kDa IGFBP is detected in OB membranes. Solubilized OB membranes were fractionated on a 12% SDS-PAGE, electroblotted to a nitrocellulose filter and processed for WLB. A single autoradiographic band at 32 kDa is detected in OB membrane (lane 2). Adult rat serum (5 μl) was run in lane 1 as control. Autoradiography was for 7 days.

nonreceptor-IGF-I binding shown by *in vitro* autoradiography (Fig. 1B). We therefore performed immunohistochemistry using the same anti-IGFBP-2 antiserum on OB paraffin sections and localized IGFBP-2 to cells in the mitral cell layer (MI) of the OB (Fig. 5B). When emulsion autoradiography was performed on the section shown in Fig. 1b following coincubation with des(1–3)IGF-I and ^{125}I-IGF-I, residual binding indicated by silver grains was similarly located in the mitral cell layer (Fig. 5A). These findings strongly suggest that the membrane-associated IGFBP in the OB *in vivo* is IGFBP-2.

IGFBP-2 binding to OB membranes is displaced by NaCl but not an RGD motif

Association of IGFBPs to the cell surface can occur through proteoglycans and their glycosaminoglycan side chains (6) or membrane proteins (32) including integrins (4). IGFBP-2, like IGFBP-1, contains an RGD sequence near the carboxy-terminal (1, 2) and IGFBP-1 binds to the $\alpha5\beta1$ integrin (4). A potential heparin binding motif (PKKLRP) is also present in the intercysteine rich domain of IGFBP-2 (aa 160–165), and a similar glycosaminoglycan binding motif is present in IGFBP-3 and -5 (33), which may mediate their association with heparin.

We therefore first investigated whether the RGD sequence of IGFBP-2 was involved in membrane binding. A GRGDSP hexa-peptide and a GRGESP hexa-peptide (used as control), both at 10–100 μg/ml, failed to dissociate IGFBP-2 from OB membranes (Fig. 6A), suggesting that the RGD sequence was not involved in cell membrane association of IGFBP-2 in this system. In contrast, NaCl dissociated IGFBP-2 from the OB membrane in a dose dependent manner (Fig. 6B), and dissociation was complete with 500 mM NaCl. These findings

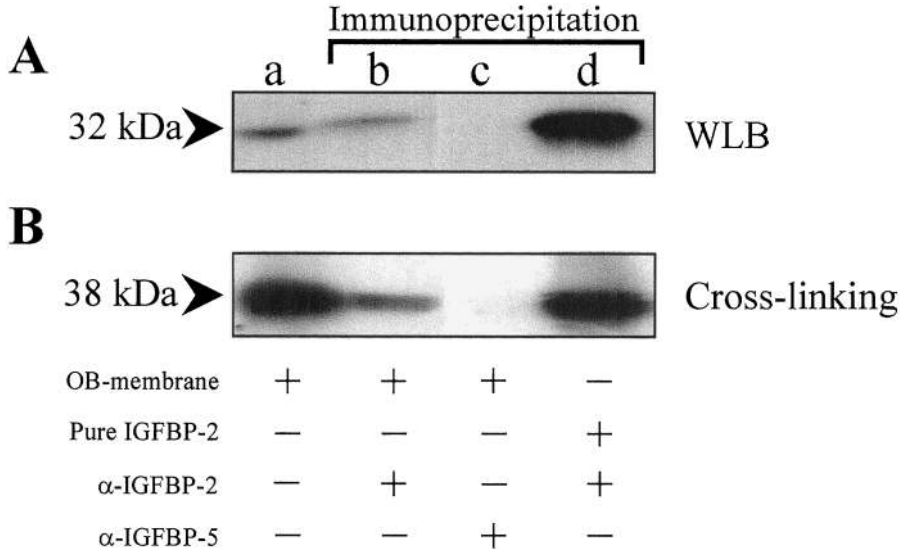

FIG. 4. IGFBP-2 is the IGFBP associated with OB membranes. Fresh OB membrane (panel A, lanes b–c) or purified IGFBP-2 (panel A, lane d) and cross-linked OB membrane (panel B, lanes b–c) or IGFBP-2/^{125}I-IGF-I complex (panel B, lane d) were incubated with the anti-IGFBP-2 (panels A and B, lanes b and d) or with the anti-IGFBP-5 antisera (panels A and B, lane c). A, Fresh OB membranes were analyzed by WLB. B, Cross-linked membranes were analyzed by autoradiography. Fresh OB membrane (panel A, lane a); cross-linked OB membrane (panel B, lane a). Molecular weight markers are indicated. Film exposures were 10 days for panel A; 3 days for panel B.

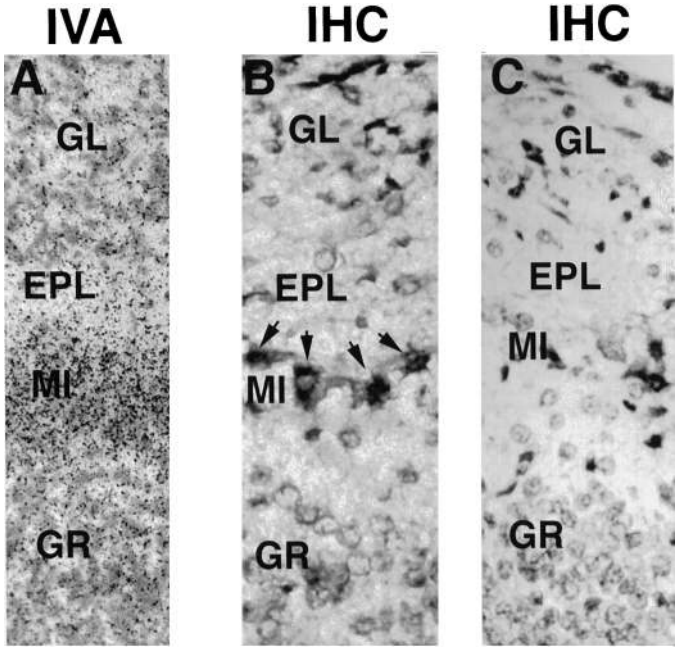

FIG. 5. IGFBP-2 protein colocalizes with nonreceptor-IGF-I binding sites. Photographic emulsion of ^{125}I-IGF-I incubated in the presence of des(1–3)IGF-I in panel A (bright field) shows silver grains (*black dots*) localized to the mitral cell layer (MI). OB sections were incubated with anti-IGFBP-2 antisera (panel B) or with normal rabbit serum as control (panel C). Immunoreactivity with IGFBP-2 in panel B (*arrows*) is similarly localized to the mitral cell layer (MI). Cell layers are indicated as follows: glomerular cell layer (GL), external plexiform layer (EPL), mitral cell layer (MI), granular cell layer (GR). Magnification in panels A, B, and C is 250×.

suggested that IGFBP-2 may bind to cell membranes via ionic interactions and raise the possibility that proteoglycans may be involved.

IGFBP-2 binds to glycosaminoglycans and aggrecan in vitro

Three major proteoglycans containing chondroitin-6-sulfate (C-6S) and a proteoglycan containing keratan sulfate glycosaminoglycans have been extracted and partially characterized from plexiform and mitral cell layers of the rat OB (34). The latter is the same anatomical layer in which we located the membrane-associated IGFBP-2 by immunohistochemistry, and it was thus of interest to determine whether IGFBP-2 could interact with glycosaminoglycans. To test this, we incubated purified IGFBP-2 in wells preadsorbed with purified glycosaminoglycans. The binding of IGFBP-2 to coated wells was detected by incubation with ^{125}I-IGF-I. IGFBP-2 bound to wells coated with chondroitin-4-sulfate, chondroitin-6-sulfate, keratan sulfate, and heparin (Fig. 7A). ^{125}I-IGF-I in the absence of IGFBP-2 bound minimally to glycosaminoglycans (Fig. 7A) and IGFBP-2 did not bind to wells coated with BSA. IGFBP-2 was also able to bind to the proteoglycan aggrecan (Fig. 7, A and B), which contains both chondroitin sulfate and keratan sulfate chains and shares homologies and functional domains with some of the brain chondroitin sulfate proteoglycans (CSPGs) (35). IGFBP-2 binding to aggrecan was reduced following digestion with chondroitinase ABC or keratanase (Fig. 7B), suggesting specific interaction of IGFBP-2 with the glycosaminoglycan side chains of this proteoglycan. Thus, IGFBP-2 binds glycosaminoglycans and, further, IGF-I efficiently binds to IGFBP-2 associated with glycosaminoglycans, suggesting that IGF-I might bind to both receptors and glycosaminoglycan-associated IGFBP-2 *in vivo*.

Binding of IGFBP-2 to chondroitin 6-sulfate reduces its binding affinity for IGF-I

Association of IGFBP-5 with heparin substantially decreases its binding affinity for IGF-I and heparin has a lesser effect on binding of IGF-I to IGFBP-3 (5–7). We therefore compared the binding affinities of soluble and C-6S-bound IGFBP-2 for IGF-I. The Ka for C-6S-bound IGFBP-2 (Fig. 8, *open squares*) was $5.8 \pm 1.1 \times 10^9$ M^{-1} (mean ± SEM), whereas that for soluble IGFBP-2 (Fig. 8, *filled squares*) was $15 \pm 2.0 \times 10^9$ M^{-1}, indicating that association of IGFBP-2 with glycosaminoglycans decreased the binding affinity of IGFBP-2 for IGF-I by approximately 3-fold.

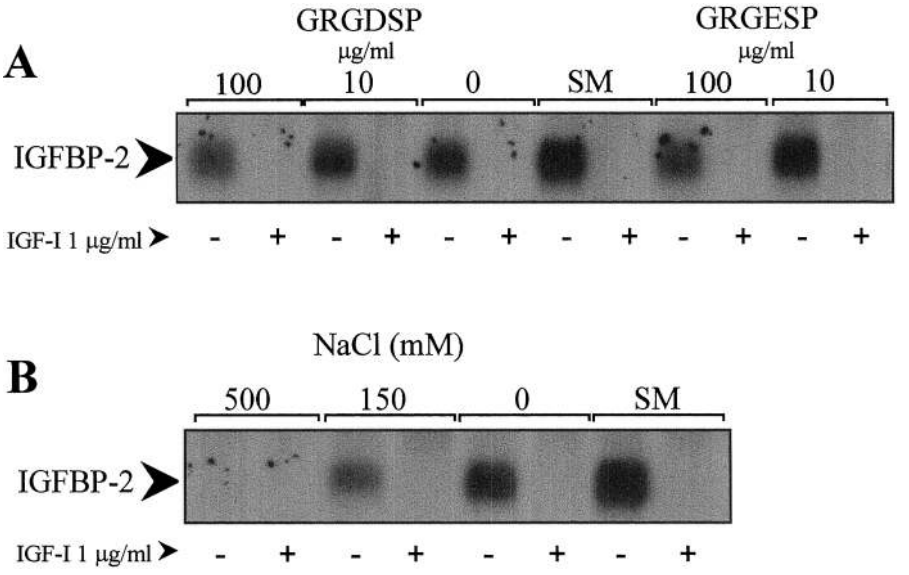

FIG. 6. IGFBP-2 membrane association in the OB involves ionic interations and not a RGD motif. OB membranes were incubated with GRGDSP or GRGESP peptides (A) or with NaCl (B). Residual membrane-associated IGFBP-2 was affinity cross-linked to ^{125}I-IGF-I with 1 mM DSS in the presence (+) or absence (-) of unlabeled IGF-I (1 μg/ml). Reduced samples were analyzed by 12% SDS-PAGE. Autoradiographs of the dried gel in Fig. 6A or 6B represent the effects on IGFBP-2 association to OB membranes by the RGD or RGE peptides (0–100 μg/ml) or NaCl (0–500 mM) respectively. Fresh cross-linked OB membranes (called starting material, SM) were run as control. Exposure was for 5 days.

IGFBP-2 binds to cell surface chondroitin sulfate proteoglycans in rat brain olfactory bulb

In preliminary experiments with rat OB membranes, we identified bands on Western immunoblots that were immunoreactive with the chondroitin sulfate-specific monoclonal antibodies 3B3/2B6 (Fig. 9). The apparent molecular size of these bands, before and after treatment with chondroitinase ABC, was greater than 200 kDa (Fig. 9). This finding is consistent with the studies of Gonzalez *et al.*, who showed that proteoglycans immunoreactive with 3B3 were prominent in extracts from OB tissue (34).

To determine whether IGFBP-2 was binding to chondroitin sulfate proteoglycans on rat OB membranes, we used anti-IGFBP-2 antiserum to immunoprecipitate IGFBP-2-containing complexes and detected chondroitin sulfate proteoglycan with specific antibodies. A single band of 3B3/2B6 immunoreactivity was detected with an apparent molecular size greater than 200 kDa (Fig. 10A). No immunoreactivity was present in the control lane which was immunoprecipitated with nonimmune serum. WLB analysis of the same samples confirmed that the anti-IGFBP-2 antiserum, but not the nonimmune antiserum, was able to specifically immunoprecipitate IGFBP-2 (Fig. 10B). This experiment shows unequivocally that IGFBP-2 binds to cell surface chondroitin sulfate proteoglycans in rat OB and so suggests a role for proteoglycans in modulation of IGF-I bioactivity *in vivo.*

Discussion

This study shows that IGFBP-2 is membrane-bound in the mitral cell layer of the rat brain olfactory bulb. Specifically, a 38-kDa cross-linked complex, consistent with ^{125}I-IGF-I: IGFBP, was identified in membranes prepared from olfactory bulbs, and this complex could be immunoprecipitated by an antiserum to IGFBP-2 but not IGFBP-5. Immunohistochemical distribution of IGFBP-2 in the mitral cell layer of the olfactory bulb colocalized with the distribution of nonreceptor IGF-I binding sites identified by *in vitro* autoradiography. Although membrane-associated IGFBP-2 has been identified in lung carcinoma cells (8) and lung smooth muscle cells (36), the mechanism of cell-association was not explored in these studies.

Like IGFBP-1, the amino acid sequence of IGFBP-2 contains an Arg-Gly-Asp sequence, which is implicated in binding of extracellular matrix components to integrins, near its carboxyl-terminus (1). IGFBP-1 binds to the $\alpha_5\beta_1$ integrin through this motif, and this interaction mediates IGF-independent migration of CHO cells (4). However, an Arg-Gly-Asp-containing peptide did not compete for binding of IGFBP-2 to membranes in the present study, making binding to integrins an unlikely mechanism for the membrane association of this IGFBP.

The findings in the present study indicate that IGFBP-2 binds to the glycosaminoglycan component of membrane proteoglycans. Binding of proteins to the glycosaminoglycan component of proteoglycans is often charge-dependent, although more specific, higher affinity interactions may also occur (37). Because charge-dependent binding to glycosaminoglycans is inhibited by increasing ionic strength, the inhibition of cell-association of IGFBP-2 in a dose-dependent manner by NaCl indirectly supports the hypothesis that IGFBP-2 was binding to glycosaminoglycans. *In vitro*, IGFBP-2 bound to chondroitin-4-sulfate, chondroitin-6-sulfate, keratan sulfate, heparin and the proteoglycan aggrecan, the latter of which was blunted by specific digestion of component glycosaminoglycans. Although the structural basis of the binding to glycosaminoglycans was not addressed in this study, it is possible that the putative heparin-binding motif (Pro-Lys-Lys-Leu-Arg-Pro) at positions 160–165 in the mid-region of IGFBP-2 (33) may be involved.

Three proteoglycan core proteins each containing chondroitin-6-sulfate glycosaminoglycan have been extracted and partially characterized from extracellular matrix of the rat olfactory bulb, and immunologically localized to the mitral and plexiform layers (34). In the present study, we show, for the first time, that a large proteoglycan (>200 kDa) containing chondroitin sulfate chains, is able to bind IGFBP-2. We

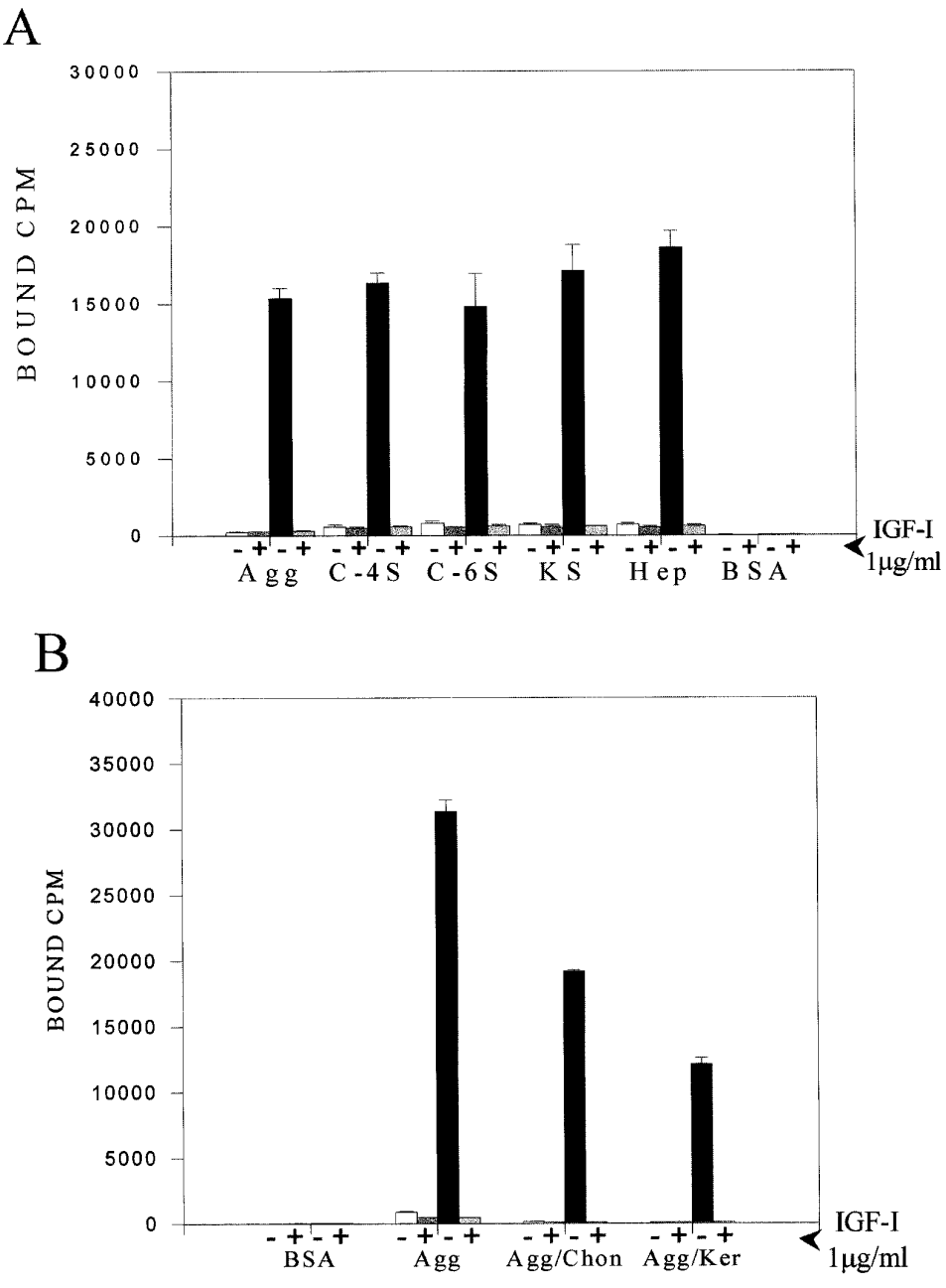

FIG. 7. A–B, IGFBP-2 binds to glycosaminoglycans and aggrecan *in vitro*. Microtiter 96-well plates were coated with: aggrecan (Agg); chondroitin-4-sulfate (C-4S); chondroitin-6-sulfate (C-6S); keratan sulfate (KS); heparin (Hep); aggrecan digested with chondroitinase ABC (Agg/Chon); or keratanase (Agg/Ker). IGFBP-2 was bound to the coated wells, followed by ^{125}I-IGF-I 30,000 cpm/well (A) or 60,000 cpm/well (B) in the presence (▨), or absence (■), of an excess of cold IGF-I. ^{125}I-IGF-I alone was also incubated in the presence (▨), or absence (□), of cold IGF-I. Wells coated with BSA were incubated in the same way and used as control. Results are shown as mean ±SD of three experiments, and each point was measured in quadruplicate.

provided definitive evidence of IGFBP-2 binding to a glycosaminoglycan-containing proteoglycan *in vivo* in the rat olfactory bulb by showing that a 3B3/2B6 immunoreactive proteoglycan of similar size (>200 kDa) was coimmunoprecipitated with IGFBP-2 by the anti-IGFBP-2 antiserum. Whether this is a matrix proteoglycan as described above by Gonzales *et al.* (34) or a distinct membrane proteoglycan such as the 280 kDa chondroitin sulfate proteoglycan identified in chicken brain neurons (38), is not known. However, the precise composition of proteoglycans in this region may underlie the specific localization of membrane-associated IGFBP-2 in rat brain.

Both IGFBP-3 and IGFBP-5 bind to membranes and/or ECM from different cells, and salt and heparin displacement suggest that binding to glycosaminoglycan may be involved (3, 5, 6, 39, 40). Whether these IGFBPs bind directly to glycosaminoglycans, however, is controversial because heparinase (which digests the carbohydrate component) or chlorate (which inhibits glycosaminoglycan sulfation) variably affect IGFBP-3 and IGFBP-5 binding (39, 41). Glycosaminoglycans compete for binding of IGFBP-3 and IGFBP-5 to cell monolayers (6, 42) and decrease the binding affinity of IGFBP-5 for IGF-I (5). The amino acid sequences of IGFBP-3 and IGFBP-5 both contain putative heparin binding sites, and peptides based on these binding sites are effective competitors of membrane binding by these IGFBPs (5, 39). The specific basic amino acids that are necessary for heparin binding by IGFBP-5 have recently been identified by site-directed mu-

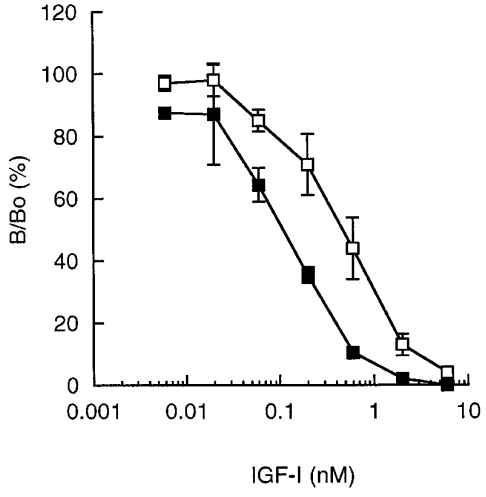

FIG. 8. Binding of IGFBP-2 to chondroitin-6-sulfate reduces its binding affinity for IGF-I. IGFBP-2 (10 ng/well) was bound to microplate wells coated with chondroitin-6-sulfate. Glycosaminoglycan-bound IGFBP-2 was incubated with ^{125}I-IGF-I ± unlabeled IGF-I for 16 h at 4 C. Similarly soluble IGFBP-2 (2 ng) was incubated with ^{125}I-IGF-I ± unlabeled IGF-I. Results are shown as percentage of specific binding in the absence of unlabeled IGF-I (%B/Bo). Results are shown as mean ± SEM of two experiments for soluble IGFBP-2 (■) and three experiments for bound IGFBP-2 (□).

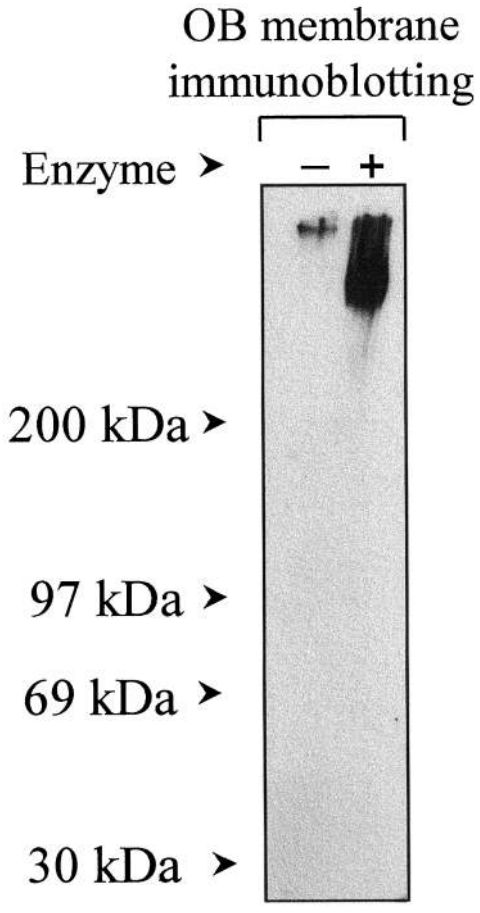

FIG. 9. A chondroitin-6-sulfate proteoglycan > 200 kDa is present in OB membranes. Undigested (−) or chondroitinase ABC digested OB membrane (+) were solubilized in nonreducing Laemmli buffer, fractionated onto a 3–16% gradient gel, and electroblotted. The membrane was treated with chondroitinase ABC and then incubated with the monoclonal antibodies 3B3/2B6.

tagenesis (41). It would therefore appear that IGFBP-3 and IGFBP-5 interact both with proteins and proteoglycans on the cell surface and in ECM. Whether binding to glycosaminoglycans is direct is unclear; this may depend upon cell type and specific conditions of study. Similar to these IGFBPs, IGFBP-2 binds to heparin-agarose (2), heparin sepharose, and extracellular matrix proteins (10) *in vitro*, but lower salt concentrations are needed to displace IGFBP-2 from heparin and ECM (2) and the presence of IGF-I or IGF-II is required to stabilize binding to heparin-sepharose and ECM proteins (10). There are differences in the interactions reported for IGFBP–2, -3, and -5 and those which we describe for IGFBP-2. In the present study, IGFBP-2 bound specifically to chondroitin 4-sulfate, chondroitin 6-sulfate, and keratan sulfate, glycosaminoglycans that interact minimally with IGFBP-5 or IGFBP-3 (39, 41). Furthermore, these glycosaminoglycans do not dissociate IGFBP-2/IGF-I complexes from heparin-sepharose (10). In addition, we found that pure rat IGFBP-2 binds *in vitro* to heparin and these glycosaminoglycans in the absence of IGF-I which was only added subsequently in radiolabeled form to detect glycosaminoglycan-bound IGFBP-2. These differences in our findings compared with those of Arai *et al.* (10) might reflect the inherent differences in our solid phase binding, compared with solution binding assay, or may be due to the different species of IGFBP-2 used. However, because the rat OB is a brain region rich in IGF-I (11, 14, 16), we cannot exclude the possibility that association of IGFBP-2 to an OB membrane chondroitin sulfate proteoglycan might require the presence of IGF-I.

Cell-association of IGFBPs is of particular interest because it may influence the way in which IGF action is modulated by IGFBPs (1, 2, 43). In many situations, IGFBPs inhibit IGF activity, but they may also enhance IGF activity. The mechanism of potentiation of IGF activity by IGFBPs is not completely understood, but much attention has focused recently on cell-association as a contributing factor. Some growth factors, notably basic fibroblast growth factor, bind to cell membrane and ECM heparan sulfate proteoglycans, and this interaction is important for subsequent binding to specific high affinity receptors (43). Additionally, basic FGF is protected from proteolysis while bound to proteoglycans. In contrast, IGFs do not substantially bind to proteoglycans or other ECM components. Cell-associated IGFBPs may therefore act as linker molecules allowing pericellular sequestration of IGFs.

The binding affinities of cell-associated and heparin-associated IGFBP-3 (5, 39) and IGFBP-5 (5, 44) for IGFs are lower than those of soluble IGFBPs. It has been postulated that this decrease in binding affinity facilitates release of the IGFs from IGFBPs for binding by receptors. Similarly, in the present study, glycosaminoglycan-bound IGFBP-2 had reduced (~3-fold) binding affinity for IGF-I compared with soluble IGFBP-2. In the case of IGFBP-3, cell association leads to a 10-fold decrease in affinity for IGF-I, related to processing of the IGFBP to lower molecular weight forms (7). Whether a similar process may occur with cell association of

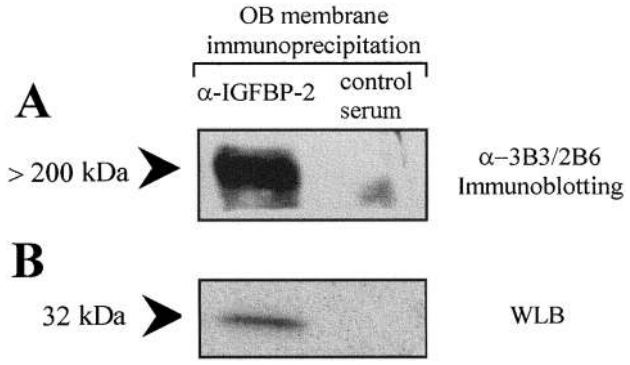

FIG. 10. A chondroitin sulfate proteoglycan of MW > 200 kDa co-immunoprecipitated with IGFBP-2. Fresh OB membranes were incubated with the anti-IGFBP-2 or control serum, as indicated, and immunoprecitated as described in *Materials and Methods*. Samples were then separated in a 3–16% gradient gel and transferred to a nitrocellulose filter. Membranes were then incubated with the 3B3/2B6 antibodies (A), or subjected to WLB (B) as described in *Materials and Methods*. In panel A, a 3B3/2B6 immunoreactive band was detectable in sample immunoprecipitated with the anti-IGFBP-2 but not by the contol serum. In panel B, WLB analysis revealed a 32-kDa band (IGFBP-2) only in lane 1. Film exposure was for 3 days for the lower panel.

IGFBP-2 *in vivo*, further reducing its affinity for IGF-I, was not examined in the current study.

In conclusion, we have shown that IGFBP-2 is membrane-associated in the mitral cell layer of rat olfactory bulb, at least in part by interacting with the glycosaminoglycan component of membrane proteoglycans. The specificity of the binding is unique amongst IGFBPs so far described in that IGFBP-2 binds to chondroitin sulfate and keratan sulfate, glycosaminoglycans that are abundant in proteoglycans from that part of the rat brain. In addition, glycosaminoglycan association lowered the binding affinity of IGFBP-2 for IGF-I. Although the structural basis of these effects remains to be determined, this may represent an additional mechanism for spatial localization of IGFBPs and targeting of IGF actions to specific sites in the developing brain.

Acknowledgments

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References

1. **Bach LA, Rechler MM** 1995 Insulin like growth factor binding proteins. Diabete Rev 3:38–61
2. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding protein: biological actions. Endocr Reviews 16:3–34
3. **Jones JI, Gockerman A, Busby Jr WH, Camacho-Hubner C, Clemmons DR** 1993 Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol 121:679–687
4. **Jones JI, Gockerman A, Busby Jr WH, Wright G, Clemmons DR** 1993 Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha 5\beta 1$ integrin by means of its Arg-Gly-Asp sequence. Proc Natl Acad Sci USA 90:10553–10557
5. **Arai T, Parker A, Busby Jr W, Clemmons DR** 1994 Heparin, heparan sulfate, and dermatan sulfate regulate formation of the insulin-like growth factor-I and insulin-like growth factor-binding protein complexes. J Biol Chem 269:20388–20393
6. **Smith EP, Lu L, Chernausek SD, Klein DJ** 1994 Insulin-like growth factor binding protein-3 (IGFBP-3) concentration in rat Sertoli cell-conditioned medium is regulated by a pathway involving association of IGFBP-3 with cell surface proteoglycans. Endocrinology 135:359–364
7. **Conover, CA** 1991 Glycosylation of insulin-like growth factor binding protein-3 (IGFBP-3) is not required for potentiation of IGF-I action: evidence of processing bound IGFBP-3. Endocrinology 129:3259–3268
8. **Reeve JG, Morgan J, Shwander J, Bleehen NM** 1993 Role for membrane or secreted insulin-like growth factor binding protein-2 in the regulation of insulin-like growth factor action in lung tumors. Cancer Res 53:4680–4685
9. **McCusker RH Camacho-Hubner C, Bayne ML, Cascieri MA, Clemmons DR** 1990 Insulin-like growth factor (IGF) binding to human fibroblast and glioblastoma cells: the modulating effect of cell released IGF binding proteins (IGFBPs). J Cell Physiol 144:244–253
10. **Arai T, Busby W, Clemmons DR** 1996 Binding of insulin-like growth factor (IGF)-I or II to IGF-binding protein-2 enables it to bind to heparin and extracellular matrix. Endocrinology 137:4571–4575
11. **Bondy C, Lee WH** 1993 Correlation between Insulin-like growth factor (IGF)-binding protein 5 and IGF-I expression during brain development. J Neurosci 13:5092–5104
12. **Lee WH, Michels KM, Bondy CA** 1993 Localization of insulin-like growth factor binding protein-2 messenger RNA during post-natal brain development: correlation with insulin-like growth factors I and II. Neuroscience 53:251–265
13. **Brar AK, Chernausek SD** 1993 Localization of insulin-like growth factor binding protein-4 expression in the developing and adult rat brain: analysis by *in situ* hybridization. J Neurosci Res 35:103–114
14. **Russo VC, Edmondson SR, Mercuri FA, Buchanan CR, and Werther GA** 1994 Identification, localization and regulation of insulin-like growth factor binding proteins (IGFBPs) and their messenger RNAs in new born rat olfactory bulb. Endocrinology 135:1437–1446
15. **Werther GA, Hogg A, McKinley M, Oldfield B, Figdor R, Mendelsohn FAO** 1989 Localization and characterization of insulin-like growth factor-I receptor in rat brain and pituitary, using *in vitro* autoradiography and computerized densitometry J. Neuroendocrinol 1:370–377
16. **Werther GA, Abate M, Hogg A, Cheesman H, Oldfield B, Hards D, Hudson P, Power B, Freed K, Herington AC** 1990 Localization of insulin-like growth factor-I mRNA in rat brain by *in situ* hybridization - relationship to IGF-I receptors. Mol Endocrinol 4:773–778
17. **Werther GA, Cheesman H, Russo VC** 1993 Olfactory bulb organ culture is supported by combined insulin-like growth factor-I and basic fibroblast growth factor. Brain Res 617:339–342
18. **Ocrant I, Fay CT, Parmelee JT** 1990 Characterization of insulin-like growth factor binding proteins produced in the rat central nervous system. Endocrinology 127:1260–1267
19. **Bourner MJ, Busby Jr WH, Siegelm NR, Krivi GG, McCusker RH, Clemmons DR** 1992 Cloning and sequence determination of bovine insulin-like growth factor-binding protein 2 (IGFBP-2): comparison of its structural and functional properties with IGFBP-1. J Cell Biochem 48:215–226
20. **Camacho-Hubner C, Busby Jr WH, McCusker RH, Wright G, Clemmons DR** 1992 Identification of the forms of insulin-like growth factor-binding protein produced by human fibroblast and the mechanisms that regulates their secretion. J Biol Chem 267:11949–11956
21. **Caterson B, Calabro T, Hapton A** 1987 In: Wright T, Mecham R (eds) Biology of Proteoglycans. Monoclonal Antibodies as Probes for Elucidating Proteoglycan Structure and Function. Academic Press, New York, pp 1–26
22. **Bach LA, Hsieh S, Sakano K, Fujiwara H, Perdue JF, Rechler MM** 1993 Binding of mutants of human insulin-like growth factor II to insulin-like growth factor binding proteins 1–6. J Biol Chem 268:9246–9254
23. **Hardingham TE** 1979 The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochem J 177:237–247
24. **Russo VC, Cheesman H, Werther GA** 1994 Organ culture: an *in vitro* system for the sustained growth of neonatal olfactory bulb. Neurosci Protocols 3:1–11
25. **Goodyer CG, De Stephano L, Hsien Lai W, Guyda HJ, Posner BI** 1984 Characterization of insulin-like growth factor receptors in rat anterior pituitary, hypothalamus and brain. Endocrinology 114:187–1195
26. **Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M** 1986 Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal Biochem 154:138–143
27. **Bonaldo P, Russo V, Bucciotti F, Doliana R, Colombatti A** 1990 Structural and functional features of the alpha-3 chain indicate a bridging role for collagen type VI in connective tissue. Biochemistry 29:1245–1254
28. **Munson PJ, Rodbard D** 1984 Computerized Analysis of Ligand Binding Data: Basic Principles and Recent Development. In: Rodbard D, Forti G (eds) Computers in Endocrinology. Raven Press, New York, pp 117–145
29. **Ballard FJ, Ross M, Upton FM, Francis GL** 1988 Specific binding of insulin-like growth factors 1 and 2 to the type 1 and type 2 receptors, respectively. Biochem J 249:721–726
30. **Hascall VC, Midura RJ, Sorrell JM, Plaas AH** 1995 Immunology of chondroitin/dermatan sulfate. Adv Exp Med Biol 376:205–216
31. **Oh Y, Muller HL, Lee D, Fielder PJ, Rosenfeld RG** 1993 Characterization of the affinities of insulin-like growth factor (IGF)-binding proteins 1–4 for IGF-I, IGF-II, IGF-I/insulin hybrid, and IGF-I analogs. Endocrinology 132:1337–1344

32. **Oh Y, Muller HL, Pham H, Rosenfeld RG** 1993 Demostration of receptor for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J Biol Chem 268:26045–26048
33. **Hodgkinson SC, Napier JR, Spencer GS, Bass JJ** 1994 Glycosaminoglycan binding characteristics of the insulin-like growth factor-binding proteins. J Mol Endocrinol 13:105–112
34. **Gonzalez ML, Malemud C, Silver J** 1993 Role of astroglial extracellular matrix in the formation of rat olfactory bulb glomeruli. Exp Neurol 123:91–105
35. **Fosang AJ, Hardingham TE** 1996 In: Comper WD (ed) Extracellular Matrix: Molecular Components and Interactions. Matrix Proteoglycans. Hardwood Academic Publishing, Singapore, 2:200–229
36. **Cohen P, Noveral JP, Bhala A, Nunn SE, Herrick DJ, Grunstein MM** 1995 Leukotriene D4 facilitates airway smooth muscle cell proliferation via modulation of the IGF axis. Am J Physiol 269:L151–L157
37. **Kjellen L, Lindahl U** 1991 Proteoglycans: structures and interactions. Annu Rev Biochem 60:443–475
38. **Hoffman S, Edelman GM** 1987 A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. Proc Natl Acad Sci USA 84:2523–2527
39. **Martin JL, Ballesteros M, Baxter RC** 1992 Insulin-like growth factor-I (IGF-I) and transforming growth factor-beta1 release IGF-binding protein-3 from human fibroblasts by different mechanisms. Endocrinology 131:1703–1710
40. **Hodgkinson S, Fowke P, Al Somai N, McQuoid M** 1995 Proteins in tissue extracts which bind insulin-like growth factor binding protein-3 (IGFBP-3). J Endocrinol 145:R1–R6
41. **Arai T, Clarke J, Parker A, Busby J, W., Nam T, Clemmons DR** 1996 Substitution of specific amino acids in insulin-like growth factor (IGF) binding protein 5 alters heparin binding and its change in affinity for IGF-I in response to heparin. J Biol Chem 271:6099–6106
42. **Booth BA, Boes M, Andress DL, Dake BL, Kiefer MC, Maack C, Linhardt RJ, Bar K, Caldwell EE, Weiler J** 1995 IGFBP-3 and IGFBP-5 association with endothelial cells; role of C-terminal heparin binding domain. Growth Regul 5:1–17
43. **Rechler MM** 1995 Non-receptor binding proteins for insulin-like growth factors and other cytokines: modulators of peptide action. In: Weintraub BD (ed) Molecular endocrinology: basic concepts and clinical correlations. Raven Press, New York, pp 155–181
44. **Andress DL** 1995 Heparin modulates the binding of insulin-like growth factor (IGF) binding protein-5 to a membrane protein in osteoblastic cells. J Biol Chem 270:28289–28296