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Insulin-like growth factor I receptors of fetal brain are enriched in nerve growth cones and contain a β-subunit variant

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ABSTRACT **Nerve growth cones isolated from fetal rat brain are highly enriched in a 97-kDa glycoprotein, termed β_{gc}, that comigrates with the β subunit of the IGF-I receptor upon two-dimensional PAGE and is disulfide-linked to this receptor's α subunit. Antibodies prepared to a conserved domain shared by the insulin and IGF-I receptor β subunits (AbP2) or to β_{gc} were used to study receptor distribution further. Subcellular fractionation of the fetal brain segregated most AbP2 immunoreactivity away from growth cones, whereas most β_{gc} immunoreactivity copurified with growth cones. Experiments involving ligand-activated receptor autophosphorylation confirmed the concentration of IGF-I but not of insulin receptors in growth cone fractions. These results indicate the enrichment of IGF-I receptors in (presumably axonal) growth cones of the differentiating neuron. Furthermore, the segregation of β_{gc} from AbP2 immunoreactivity suggests that such neurons express an immunochemically distinct variant of the IGF-I receptor β subunit at the growth cone.**

Substantial evidence indicates that insulin-like growth factors (IGFs) and insulin participate in the regulation of nervous system development. For example, the expression of IGF-I in the central nervous system is high during development but drops to low levels in the adult (1). IGF-I and insulin stimulate the growth and differentiation of fetal neurons in culture (2–5). They also regulate synapse formation in fetal cholinergic neurons (6), stimulate neuronal protein synthesis (7), and increase neuronal sprouting and outgrowth (8–10). The biological effects of IGF-I require the activation of specific cell-surface receptors. IGF-I interacts primarily with the heterotetrameric ($\alpha_2\beta_2$) IGF-I receptor, a transmembrane protein-tyrosine kinase that is structurally related to the insulin receptor (11). The expression of this receptor is developmentally regulated in brain, reaching its highest level at late embryonic and early postnatal stages (12, 13). Transgenic mice deficient in IGF-I receptors exhibit defects in the central nervous system (14). However, the precise functional roles and the differential effects of insulin and IGF-I in the developing brain remain unclear. In this communication we report the enrichment of IGF-I receptors in nerve growth cones isolated from developing rat brain. The IGF-I receptors of the growth cone appear to contain an immunologically distinct variant of the β subunit. We call this subunit β_{gc}.

MATERIALS AND METHODS

Subcellular Fractionation. Fetal Sprague–Dawley rat brains (18 days of gestation) were fractionated as described (15), with the following modifications. The low-speed supernatant of fetal homogenate was loaded onto a discontinuous sucrose density gradient in which the 0.75 M and 1.0 M sucrose layers were replaced with a single 0.83 M sucrose step. This facilitated collection of the interface material and increased growth-cone yield without decreasing purity (16). The 0.32/0.83 M sucrose interface (fraction A) was collected, diluted with 0.32 M sucrose, and pelleted to yield the growth cone particle fraction (17). The 0.83/2.66 M sucrose interface (BC) also was collected from the gradients. Crude membranes were prepared from the various fractions by lysing them with 6 mM Tris, pH 8.1/1 mM EDTA (lysis buffer) and pelleting for 1 hr at 200,000 × *g*. The membranes were washed with 300 mM Na_2SO_4 containing saponin (20 μg/ml) and repelleted at 200,000 × *g* for 1 hr (17).

Polyacrylamide Gel Electrophoresis and Western Blotting. Samples were solubilized in the appropriate sample buffer and resolved by either one-dimensional SDS/PAGE (18, 19) or two-dimensional PAGE [isoelectric focusing followed by SDS/PAGE (20)]. The resolved polypeptides were electrotransferred onto Immobilon-P (Millipore) (21, 22). The blots were stained with Ponceau S (23) and blocked in Tris-buffered saline (TBS: 150 mM NaCl/50 mM Tris, pH 7.4) with 5% dry milk and 0.2% Tween 20 (blotto T) for 2 hr at room temperature or overnight at 4°C. The blots were incubated with a 1:100 dilution of primary antibody in the same buffer for 2 hr at room temperature or overnight at 4°C. After three 10-min washes with blotto T, some of the blots (Figs. 1 and 4) were incubated for 2 hr at room temperature with 1 μCi (37 kBq) of ^{125}I-labeled protein A in 10 ml of blotto T. After three 10-min washes, the blots were dried and autoradiography was performed. For Figs. 2 and 3, 1:5000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase was used, followed by Renaissance detection reagents (DuPont). Chemiluminescence was recorded by contact exposure with x-ray film.

Purification of β_{gc} and Preparation of the Polyclonal Antibody. Lysed and salt-washed growth cone membranes prepared as described were solubilized in 20 ml of 0.5% Nonidet P-40 (NP-40) in TBS. The solubilized sample was applied to a 10-ml affinity column of wheat germ agglutinin linked to Sepharose, equilibrated in the same buffer (flow rate, 4 ml/hr). After washing with 30 ml of 20 mM *N*-acetylglucosamine in TBS containing 0.5% NP-40, 6 ml of 300 mM *N*-acetylglucosamine in the same buffer was allowed to enter the column and the flow was stopped for 1 hr. After this time 20 ml was eluted with the same buffer and the proteins were precipitated with methanol/chloroform/water (24). The protein pellet was resuspended in 3× sample buffer (18) and loaded on an 8–12% acrylamide gradient gel. The appropriate band (97 kDa and binding wheat germ agglutinin with high

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Abbreviations: AbP2, antibody P2; IGF, insulin-like growth factor; NP-40, Nonidet P-40.

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affinity, corresponding to spot 7 in ref. 25) was located by cutting one strip from each edge of the gel: one of the strips was stained with Coomassie brilliant blue; the other one was electroblotted onto Immobilon-P and probed with ^{125}I-labeled WGA. The identified band was excised from the remaining gel and cut into small pieces. These pieces were homogenized in the presence of Freund's adjuvant by passing them back and forth several times between two interconnected syringes and then injected subcutaneously into a rabbit. Booster injections were given every 4 weeks. The serum used in the experiments described here was obtained after five injections. Before use, the serum was preabsorbed with 50 μg of human keratin spotted onto nitrocellulose in order to remove antibodies to this protein.

Insulin-Sepharose Affinity Chromatography. Two hundred fifty micrograms of growth cone membranes was solubilized in 50 mM Hepes, pH 7.4/100 mM NaCl/containing 0.5% NP-40/4 mM EDTA/4 mM EGTA. This solution was incubated with 1 ml of insulin-Sepharose for 2 hr at 4°C under agitation and packed into a column. The column was washed with 5 ml of the initial buffer, at which point A_{280} had reached background. This was followed by elution with 5 ml of 50 mM Hepes, pH 7.4/500 mM NaCl/0.1% NP-40/4 mM EDTA/4 mM EGTA. A second high-salt elution step was performed at acid pH, with 5 ml of 50 mM sodium acetate, pH 5/500 mM NaCl/0.1% NP-40.

Autophosphorylation and Immunoprecipitation of Receptors. Membranes from homogenate and growth cone fractions were solubilized with 2% (vol/vol) Triton X-100 and clarified by centrifugation at 100,000 × *g* for 15 min in a TLA-45 rotor in a TL-100 ultracentrifuge (Beckman). Solubilized membranes (10 μg of protein per reaction) were diluted in phosphorylation buffer (50 mM Hepes, pH 7.8/2.5 mM $MnCl_2$) with the indicated concentrations of insulin or IGF-I (final reaction volume, 50 μl), and kinase reactions were performed as described (13). Antibody P2 (AbP2) was then added and allowed to bind for 15 hr at 4°C. Protein A-Sepharose was added and the incubation was continued for 60 min at 4°C. Immune complexes were collected by centrifugation and the beads were washed (13). Autophosphorylated receptors were eluted by addition of Laemmli sample buffer (18) and analyzed by electrophoresis in 6.5% polyacrylamide gels and autoradiography.

RESULTS AND DISCUSSION

Our studies on growth cone membranes revealed a consistent glycoprotein spot of 97 kDa with a pI of about 4.9 that binds WGA with high affinity (spot 7 in ref. 25). A monospecific polyclonal antiserum recognizing this membrane glycoprotein was prepared (Fig. 1*A*). A glycoprotein with similar characteristics had been described previously in growth cone membranes and was known to be phosphorylated on tyrosine upon stimulation with IGF-I (26, 27). To investigate whether our antigen (named β_{gc}) was indeed related to the β subunits of either IGF-I or insulin receptors, we compared its position in two-dimensional PAGE with the β subunits recognized by AbP2. AbP2 is a polyclonal antibody raised against a synthetic peptide representing a highly conserved region of the tyrosine kinase domain of the human insulin receptor β subunit (aa 1143–1162; ref. 28). Fig. 1 shows that there is precise comigration in 2D-PAGE of β_{gc} and the larger (97-kDa) β subunit recognized by AbP2. This suggests that β_{gc} may be part of the IGF-I receptor (13). To explore this possibility further we tested for association of β_{gc} with the IGF-I receptor α subunit by examining their migration in nonreducing SDS/PAGE. Fig 2 (lane 1) shows for comparison that in reducing SDS/PAGE β_{gc} migrated as a single band of about 97 kDa. Under nonreducing conditions, however, β_{gc} migrated as a much larger complex of about 430 kDa (Fig. 2, lane 2). This complex

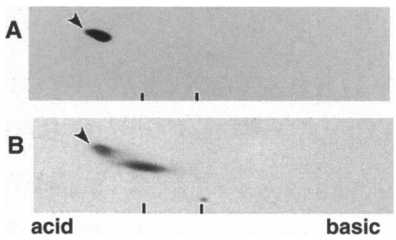

FIG. 1. Comigration of β_{gc} and the IGF-I receptor β-subunit in two-dimensional PAGE. Western blots (80- to 105-kDa region) of growth cone membrane proteins (100 μg) resolved by two-dimensional PAGE were probed with anti-β_{gc} (*A*) and AbP2 (*B*) antibodies. Arrowheads point to the comigrating spots recognized by both antibodies. Vertical dashes at the bottom of each panel indicate the position of endogenous β-tubulin (left, pI 5.2) and actin (right, pI 5.5), used as internal pI standards. Control blots with preimmune serum were clean (data not shown).

comigrated with a band recognized by AbP2 (Fig. 2, lane 3) and by an antibody to the α subunit of the IGF-I receptor (lane 4). (The anti-IGF-I receptor α subunit antibody, which does not crossreact with the insulin receptor α subunit, was the generous gift of Steven A. Rosenzweig, University of South Carolina.) This result shows that β_{gc} is part of a large, disulfide-linked complex with the molecular mass expected for the IGF-I receptor $\alpha_2\beta_2$ tetramer.

Affinity chromatography was performed to demonstrate linkage between β_{gc} and the IGF-I receptor α subunit. The IGF-I receptor (via its α subunits) binds insulin with relatively high affinity (K_d of 10^{-8} M vs. 10^{-10} M for IGF-I; ref. 1). If β_{gc} is indeed linked to the IGF-I receptor α subunit then it should be possible to recover β_{gc} from receptors purified on an insulin column. Most of the IGF-I receptor molecules can be eluted from an insulin column with high salt, whereas the insulin receptor can be released only by a combination of high salt and acid pH (29). Fig. 3 shows Western blots probed with anti-β_{gc} (*A*) and with the antibody to the α subunit of the IGF-I receptor (*B*), of proteins eluted in the wash (lane 1), by high salt (lane 2), and by acid plus high salt (lane 3). The initial wash was continued until the A_{280} dropped to background. Upon subsequent application of high salt and acid, the levels of protein eluted remained very low, not detectable by A_{280}.

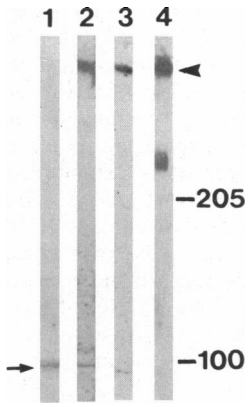

FIG. 2. Comigration of β_{gc}, the AbP2 antigen, and IGF-I receptor α subunit in nonreducing gels. Western blots of growth cone membrane proteins resolved by reducing SDS/PAGE (lane 1) and nonreducing SDS/PAGE (lanes 2–4) were probed with anti-β_{gc} (lanes 1 and 2), AbP2 (lane 3), and anti-IGF-I receptor α subunit (lane 4) antibodies. The gels were 3–10% polyacrylamide gradients (*N,N'*-methylenebisacrylamide/acrylamide weight ratio, 1:100). Arrow points to the 97-kDa band recognized by anti-β_{gc}. Arrowhead points to the nonreduced 430-kDa band recognized by anti-β_{gc}, AbP2, and the antibody to the α subunit of the IGF-I receptor. Size markers (205 and 100 kDa) are at right.

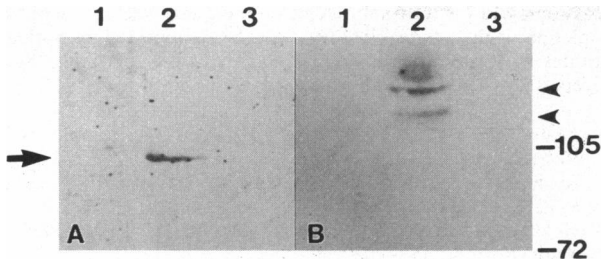

FIG. 3. Coelution of β_{gc} and the IGF-I receptor subunit from an insulin-Sepharose affinity column. Western blots of the fractions eluted from the insulin-Sepharose column with starting buffer at the end of the wash (lanes 1), high-salt buffer (lanes 2), and acidic high-salt buffer (lanes 3) were probed with anti-β_{gc} antibody (*A*) and anti-IGF-I receptor α subunit antibody (*B*). Protein levels in all three fractions were too low to be detectable by A_{280}. Note the coelution of both antigens in the fraction eluted with high salt. Arrow, β_{gc}; arrowheads, two α isoforms commonly observed in developing nerve tissue. The density just above the larger α band is a blotting artifact. Size markers (105 and 72 kDa) are at right.

However, β_{gc} (Fig. 3*A*, lane 2) as well as the α subunit of the IGF-I receptor (Fig. 3*B*, lane 2) were retained on the column during the wash and could then be eluted with high salt, indicating copurification and association of the subunits. Taken together, the results described above demonstrate that β_{gc} exhibits the electrophoretic properties of an IGF-I receptor β subunit and is linked by disulfide to this receptor's α subunit.

The subcellular distribution of β_{gc} in the developing nervous system was compared with that of the α subunit of the IGF-I receptor and the β subunits recognized by AbP2. Fig. 4*A* shows that β_{gc} was highly enriched in isolated growth cones (G), especially in growth cone membranes (GM), but not detectable at the same protein loading in homogenate (H), low-speed supernatant (L), and fraction A (A), the crude growth cone-containing fraction. Since the IGF-I receptor is an integral

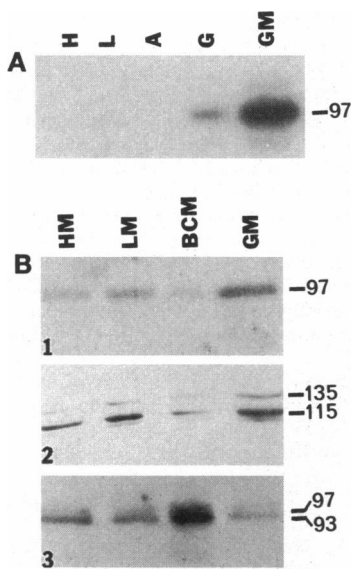

FIG. 4. Distribution of β_{gc}, IGF-I receptor α subunit, and AbP2-positive β subunits in different subcellular fractions of fetal brain. (*A*) Western blot of homogenate (H), low-speed supernatant (L), fraction (A), growth cones (G), and growth cone membranes (GM) probed with the anti-β_{gc} antibody. (*B*) Western blots of membrane proteins of homogenate (HM), low-speed supernatant (LM), fraction BC (BCM), and growth cones (GM). The blots were probed with anti-β_{gc} (blot 1), anti-α (blot 2), and AbP2 (blot 3). The apparent molecular masses (kDa) of the various bands are shown at the right. Note coenrichment of β_{gc} and IGF-I receptor α subunits. In contrast, AbP2-positive β subunits decrease in GM, with concomitant increase in BCM.

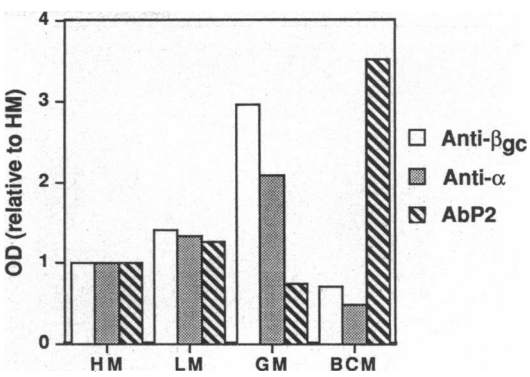

FIG. 5. Densitometric analysis of the subcellular distributions of β_{gc}, IGF-I receptor α subunit, and AbP2-positive β subunits in membrane preparations of fetal brain homogenate (HM), low-speed supernatant (LM), growth cones (GM), and BC fraction (BCM). Western blots identical to the ones shown in Fig. 4*B* (three for anti-β_{gc}; two each for anti-α and AbP2) were digitized on a Xerox Datacopy GS Plus attached to a Macintosh II computer using MACIMAGE version 2.30 software. Average optical densities (values were within 10% of one another) are shown relative to that of HM.

membrane protein, we also studied the distribution of β_{gc}, IGF-I receptor α subunit and AbP2 antigens in membrane preparations from homogenate (HM), low-speed supernatant (LM), growth cones (GM), and the growth cone-depleted subfraction of the low-speed supernatant, BC (BCM). β_{gc} was highly enriched in GM (Fig. 4*B*, blot 1). Of special interest was the difference in β_{gc} levels between GM and BCM, membranes derived from neuronal perikarya, neuritic shafts, dendrites, intracellular organelles, and glial fragments (15). Thus, β_{gc} was highly enriched in the isolated growth cones (which are primarily of axonal origin)¶, compared with the rest of the developing neuron and the glia. The distribution of the IGF-I receptor α subunit (Fig. 4*B*, blot 2) paralleled closely that of β_{gc}, as expected if these two polypeptides are subunits of the same receptor. Two isoforms of the IGF-I receptor α subunit have been observed before, with the 115-kDa species prevalent on neurons (30, 31). The distribution of insulin and IGF-I receptor β subunits detected with AbP2 (Fig. 4*B*, blot 3) was very different from that described for β_{gc} and α. The two AbP2 antigens (97 and 93 kDa) were enriched in BCM but relatively sparse in GM. Several such experiments (summarized in Fig. 5) again showed the parallel distributions and growth cone enrichment of β_{gc} and the IGF-I receptor α subunit. In contrast, AbP2 antigens were somewhat reduced in growth cone membranes but prevalent in the fractions that contained primarily non-growth cone membranes of the neuron and glial elements. Interestingly, IGF-I receptor α subunits were not enriched in fraction BC, despite the large amount of β subunits (Figs. 4 and 5). These β subunits must then be coupled to α subunits derived either from the insulin receptor or, possibly, IGF-I receptor subtypes not reactive with this antibody. Immunologically distinct IGF-I receptor α subunits have been described in other systems (32).

The data shown in Fig. 1 suggested that AbP2 and anti-β_{gc} recognized the same β subunit. However, this is not consistent with the results of the fractionation experiments (Fig. 4 and 5): during growth cone isolation most of the AbP2 immunoreactivity at 97 kDa became segregated from the bulk of the immunoreactivity detected by anti-β_{gc}. Even if we assume

¶The growth cone fraction is highly enriched in the microtubule-associated protein tau and in the growth-associated protein GAP43/pp46, both markers of growing axons, but not in MAP2, the microtubule-associated protein found in growing dendrites (K. Lohse, S. Helmke, M. Wood, S. Quiroga, B. dela Houssaye, P. Nègre-Aminou, K. Pfenninger, unpublished work).

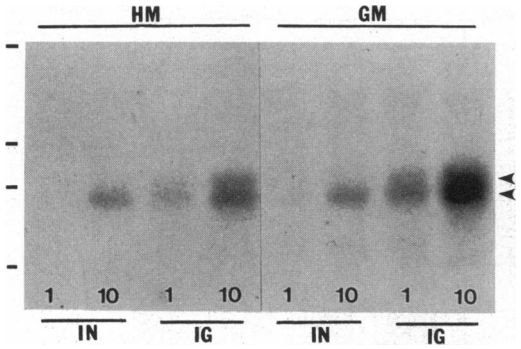

FIG. 6. Insulin and IGF-I receptor autophosphorylation in homogenate and growth cone membranes. Solubilized membranes of homogenate (HM) and growth cones (GM) were incubated with insulin (IN) or IGF-I (IG) at either 1 or 10 nM (as indicated), and autophosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP. Immunoprecipitation was carried out with AbP2 (1:100 dilution). The 97- and 93-kDa β subunits are indicated by the upper and lower arrowheads, respectively. Size standards are indicated by the bars at left (from top to bottom, 205, 116, 97, and 68 kDa).

different affinities for the two antibodies, this result suggests that β_{gc} may represent a subpopulation, an immunochemical variant, of the 97-kDa β subunits recognized by AbP2. The distinguishing epitope could be the result of differences in amino acid sequence and/or posttranslational modification, a subject for further investigation.

Our data also suggested that the 93-kDa receptor β subunit recognized by AbP2, presumably belonging to the insulin receptor (13), was enriched in BC membranes but not in growth cones. Therefore, the relative enrichment of insulin and IGF-I receptors in growth cones was examined by measuring ligand-induced autophosphorylation. Membranes of fetal brain homogenate and growth cones were incubated with insulin or IGF-I and allowed to autophosphorylate in the presence of [γ-^{32}P]ATP, and receptors were immunoprecipitated with AbP2 (Fig. 6). Insulin at 10 nM stimulated autophosphorylation of its receptor β subunit to about the same levels in homogenate and growth cone membranes [63 $\pm$ 13 (n = 4) vs. 96 $\pm$ 16 (n = 5) fmol/mg, respectively]. However, at 1 nM insulin, no autophosphorylation above basal was detected. In contrast, IGF-I incubation resulted in a nearly 3-fold increase of β-subunit phosphorylation in growth cones compared with homogenate [465 $\pm$ 40 (n = 7) vs. 165 $\pm$ 16 (n = 6) fmol/mg, respectively]; in addition, significant stimulation was evident at 1 nM (Fig. 6). This provides functional evidence for the selective enrichment of IGF-I receptors in growth cones from the developing brain.

Overall, our data indicate a remarkable differential distribution pattern of insulin and IGF-I receptors in the developing brain. While insulin receptors seem to be distributed uniformly, IGF-I receptors are highly concentrated at the growth cone, most likely the axonal growth cone. Furthermore, our results suggest that differentiating neurons synthesize an immunochemical variant of the IGF-I receptor β subunit, β_{gc}, which is found primarily in these growth cones. These observations are consistent with the hypothesis that IGF-I and its receptor play a specific role in axonal growth promotion in the developing brain.

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