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Expression and Distribution of IGF-1 Receptors Containing a β-Subunit Variant (β_{gc}) in Developing Neurons

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β_{gc} is a β-subunit variant of the insulin-like growth factor-1 (IGF-1) receptor highly enriched in growth cone membranes prepared by subcellular fractionation of fetal rat brain (Quiroga et al., 1995). The present study is focused on the expression and on the cellular and subcellular distribution of β_{gc} in developing neurons and differentiating PC12 cells. In the developing cerebral cortex and, at least at early stages, in cultured primary neurons, β_{gc} expression was found to be correlated with neurite outgrowth. In PC12 cells β_{gc} expression was nerve growth factor (NGF)-dependent and also paralleled neurite outgrowth. In contrast, β-subunits of the insulin receptor and/or of other IGF-1 receptors ("β_{P5}"; detected with antibody AbP5) were downregulated as β_{gc} expression increased. Immunofluorescence studies confirmed the enrichment of β_{gc} at growth cones and demonstrated morphologically its spatial separation from β_{P5}, which is confined to the perikaryon. At the growth cone, β_{gc} colocalizes and associates in a proximal region with microtubules, but it seems independent of the more peripheral microfilaments. Some β_{gc} immunoreactivity is detected in the perinuclear region of PC12 cells, most likely the Golgi complex and its vicinity. β_{gc} seems to emerge from the periphery of this structure in an apparently vesicular compartment distinct from that carrying synaptophysin to the growth cones. The facts that (1) β_{gc} expression is correlated closely with neurite outgrowth, that (2) it is regulated in PC12 cells by a neurotrophin, NGF, and that (3) β_{gc} is concentrated in the proximal growth cone region raise new questions regarding a possible role of IGF-1 receptors containing β_{gc} in the regulation of neurite growth.

Key words: IGF-1 receptor; β-subunits; β_{gc}; growth cones; neurons; neurite outgrowth; neurotrophins; PC12 cells; development; tissue culture

Insulin-like growth factor-1 (IGF-1) is involved in the regulation of animal growth and tissue differentiation (Froesch et al., 1985; Daughaday and Rotwein, 1989), including that of the brain. Expression of the IGF-1 gene and its transcript is high in the developing brain but decreases in the adult (Rotwein et al., 1988; LeRoith et al., 1992). IGF-1 stimulates the growth and differentiation of fetal neurons in culture (DiCiccio-Bloom and Black, 1988), increases neuronal sprouting and outgrowth (Aizenman and De Vellis, 1987; Caroni and Grandes, 1990; Beck et al., 1993; Ishii et al., 1993), enhances neuronal protein synthesis (Heidenreich and Toledo, 1989), and regulates neuronal and glial function (Sara and Hall, 1990). In addition, IGF-1 has been implicated in the modulation of synaptic transmission (Schwartz et al., 1992). The receptor for IGF-1 resembles the insulin receptor and is a disulfide-linked heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein with extracellular ligand-binding (α) and intracellular tyrosine kinase (β) domains (Ullrich et al., 1986). The expression of this receptor in the CNS is high at late embryonic and early postnatal stages and declines significantly afterward (Ullrich et al., 1986; Werner et al., 1991), again suggesting an important role for this ligand–receptor system in brain development. This notion is supported further by the following observations: IGF-1 and its receptor are expressed permanently in the olfactory bulb, where neuronal remodeling and synaptogenesis continue throughout adult life (Bondy, 1991), and transgenic mice lacking IGF-1 receptors exhibit serious defects in CNS development (Liu et al., 1993).

We have reported previously the biochemical characterization of a β-subunit of the IGF-1 receptor, designated β_{gc}. It is immunochemically distinct from the described forms of this polypeptide, highly enriched in a growth cone fraction prepared from developing rat brain, and, within the neuron, spatially segregated from the insulin receptor (Quiroga et al., 1995). In the present study we provide new evidence on the cellular and subcellular localization of β_{gc} as well as on the regulation of its expression.

Three different systems were analyzed: developing rat cerebral cortex, primary neurons in culture, and PC12 cells differentiating *in vitro*. The rat pheochromocytoma cell line, PC12, is an excellent model system for studying growth cone formation, neurite outgrowth, and the expression of structural and membrane proteins involved in nerve cell morphogenesis (Greene and Tischler, 1976; Drubin et al., 1985; Greene et al., 1987; Bearer, 1992; Esmaeli-Azad et al., 1994). In the absence of nerve growth factor (NGF), the cells are round or polygonal. On stimulation with NGF, they extend several neurites tipped by well defined growth cones. To study the relationship between β_{gc} expression and nerve cell

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development, we took advantage of this cell system. We analyzed the levels and distribution patterns of β_{gc} during NGF-induced neurite outgrowth. Our results indicate that β_{gc} expression is controlled developmentally, increasing in parallel with process extension, and that it is regulated by NGF in PC12 cells. Our studies also provide morphological evidence that β_{gc} is a prominent growth cone component—in contrast to other β-subunits of the insulin or other IGF-1 receptors (Garofalo and Rosen, 1989), which are essentially restricted to cell perikarya. In addition, we show that, within growth cones, β_{gc} is associated spatially with microtubules, but not microfilaments, and segregated from synaptophysin.

MATERIALS AND METHODS

Cell cultures. PC12 cells (obtained from Dr. A. Ferreira, Harvard Medical School, Cambridge, MA) were grown in DMEM supplemented with 10% horse serum and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated onto poly-L-lysine-coated glass coverslips at densities ranging from 5000–10,000 cells/cm^2. After plating they were maintained for 2–3 d in serum-free medium supplemented with the additives of Bottenstein and Sato (1979). When appropriate, NGF (Boehringer Mannheim, Indianapolis, IN) was added at a concentration of 50 ng/ml. For some experiments PC12 cells were treated with cytochalasin D (5 μM) for 20 min or nocodazole (10 μg/ml) for 30 or 60 min. Primary neuronal cultures were prepared from the hippocampi or cerebral cortex of rat fetus (17–20 d gestation) as previously described (Cáceres et al., 1986, 1992; Kosik and Finch, 1987). Briefly, neurons were dissociated with trypsin in Ca- and Mg-free medium and plated onto poly-L-lysine-coated coverslips. Hippocampal neurons then were incubated for 2–3 hr in Eagle's Minimum Essential Medium (MEM) containing 10% horse serum to allow for attachment. Subsequently, these coverslips were transferred into dishes containing astroglial cells. The cultures were maintained in MEM supplemented with 0.1% ovalbumin and the additives of Bottenstein and Sato (1979).

Immunofluorescence. Cells were fixed before or after mild detergent extraction under microtubule-stabilizing conditions and processed for immunofluorescence as previously described (Cáceres et al., 1992; DiTella et al., 1994) (see also Black et al., 1994). The primary antibodies used were a monoclonal antibody (mAb) against tyrosinated α-tubulin (clone TUB-1A2, mouse IgG, Sigma, St. Louis, MO) diluted 1:1000; a mAb against acetylated α-tubulin (clone 6-11B-1; Ferreira and Cáceres, 1989); an affinity-purified rabbit antiserum against β_{gc} (Quiroga et al., 1995) diluted 1:50 or 1:100; and a rabbit antiserum designated AbP5, which recognizes previously described β-subunits of both the insulin and the IGF-1 receptors (Garofalo and Rosen, 1989). The double-antibody staining protocol consisted of labeling with a first primary antibody, washing with PBS, staining with fluorophore-labeled secondary antibody, washing again with PBS, and then repeating this procedure for the second primary antibody. Incubations with primary antibodies were for 1 or 3 hr at room temperature, whereas incubations with secondary antibodies (FITC or rhodamine-labeled, generated in the goat and obtained from Boehringer Mannheim) were performed for 1 hr at 37°C. The cells were observed with an inverted microscope (Zeiss Axiovert 35M) equipped with epifluorescence optics and photographed with 40× or 100× objectives (Zeiss, Oberkochen, Germany) and Tri X-Pan (400 ASA) or Kodak Gold Plus (400 ASA) film (Eastman Kodak, Rochester, NY).

In some experiments, the distribution of β_{gc} and synaptophysin was evaluated with high-resolution video microscopy and image processing as described (DiTella et al., 1994, 1996). So that images of labeled cells could be made, the epifluorescence illumination was attenuated with glass neutral density filters. Images were formed on the faceplate of a Silicon Intensified Target camera (SIT; Hamamatsu, Middlesex, NJ), set for manual high voltage, gain, and black level. They were digitized directly into a Metamorph/Metafluor image processor (Universal Imaging, West Chester, PA) controlled by a host IBM-AT computer. After digitization, images were corrected for shading distortion by dividing by a low-pass-filtered image of a featureless field and normalizing to the maximum intensity within that image. In some cases pseudocolor images were generated with the red/green overlay menu of the Metamorph/Metafluor system. For the purpose of presentation fluorescent images were photographed directly from a high-resolution video monitor with a 35 mm camera (automatic exposure setting). Film negatives were printed with equal exposure times.

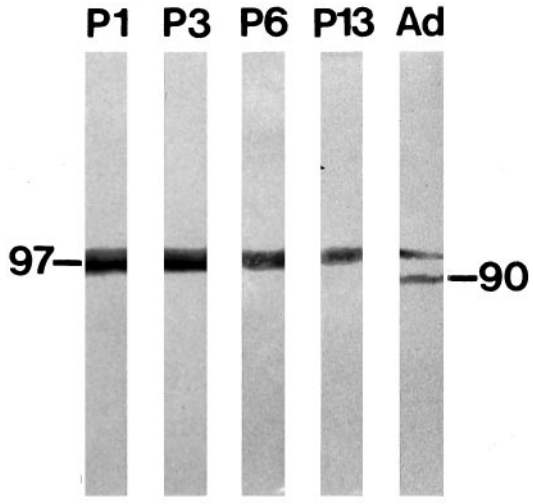

Figure 1. The expression of β_{gc} in the developing rat cerebral cortex as revealed by immunoblot analysis of whole-tissue extracts. *P1–P13*, Postnatal days 1–13; *Ad*, adult. β_{gc} is highly expressed in the developing cerebral cortex. A second and faster migrating band is present in the homogenates prepared from the adult cerebral cortex. Protein (20 μg) was loaded in each lane; the immunoblots were revealed by a rabbit ProtoBlot staining kit. The blots shown are representative of three independent experiments, all of which generated essentially identical results.

Morphometric analysis. For some experiments the neurite lengths of NGF-treated PC12 cells were measured with the morphometric menu of the Metamorph system as described (Cáceres et al., 1992).

Polyacrylamide gel electrophoresis and Western blotting. Whole-cell homogenates from brain tissue (cerebral cortex) or from cultured cells were prepared as described previously (Cáceres et al., 1988, 1992), and polypeptides were resolved by polyacrylamide gel electrophoresis (7.5% acrylamide; Laemmli, 1970). Polypeptides were electro-transferred to nitrocellulose and then probed with the β_{gc} or the AbP5 antibodies using an alkaline phosphatase detection kit (Promega ProtoBlot Detection Kits, Madison, WI) or iodinated protein A (see also Quiroga et al., 1995). For some experiments β_{gc} protein levels were quantitated in whole-cell homogenates from PC12 cells with a dot immunobinding assay as previously described (Cáceres et al., 1992).

Autophosphorylation and immunoprecipitation of β_{gc}. Membranes from PC12 cells cultured for 72 hr in the presence of NGF were resuspended in phosphorylation buffer (50 mM HEPES, pH 7.8, and 2.5 mM $MnCl_2$) with or without 10 nM IGF-1 (final reaction volume, 50 μl), and kinase reactions were performed as described (Garofalo and Rosen, 1989). Then β_{gc} antibody was added and allowed to bind overnight at 4°C. Protein G-agarose was added, and the incubation was continued for 120 min at

Table 1. Changes in β_{gc} and β_{P5} protein levels during NGF-induced neurite outgrowth in PC12 cells

Treatment	Total neurite length (μm)	β_{gc} (cpm)	β_{P5} (cpm)
None	0.25 ± 0.2	93 ± 5	1236 ± 50
NGF (1 d)	5 ± 1	102 ± 8	844 ± 38
NGF (2 d)	25 ± 4	142 ± 11	655 ± 29
NGF (3 d)	97 ± 13	208 ± 17	730 ± 42
NGF (4 d)	280 ± 15	573 ± 31	588 ± 24
NGF (4 d) + 6 hr without NGF	31 ± 6	255 ± 23	568 ± 31

β_{gc} and β_{P5} protein levels were determined by quantitative dot immunobinding assay using ^{125}I-protein A. Each value represents the mean ± SEM of measurements of five PC12 cultures grown on 18 mm glass coverslips. Values are expressed in cpm ^{125}I-protein A bound per microgram of total cellular protein.

Length values represent the means ± SEM and are expressed in micrometers. In total, 500 cells were measured for each time point.

room temperature. Immune complexes were collected by centrifugation, and the beads were washed as described (Garofalo and Rosen, 1989). Autophosphorylated receptors were eluted by addition of Laemmli sample buffer (Laemmli, 1970). They were analyzed by electrophoresis in 7.5% polyacrylamide gels and autoradiography.

RESULTS

Expression of β_{gc} in neurons from developing brain

Our previous study (Quiroga et al., 1995) showed that growth cone membranes isolated from fetal brain are enriched in β_{gc}, suggesting that the expression of this protein is correlated with process formation in developing neurons. To test this hypothesis, we have examined the time course of expression and the relative levels of β_{gc} during postnatal development of rat cerebral cortex. In addition, we analyzed its expression and distribution in primary cultures of neurons from fetal rat cerebral cortex (Kosik and Finch, 1987) and hippocampus (Cáceres et al., 1986; Dotti et al., 1988). For the first type of experiment, whole homogenates of cerebral cortex prepared on postnatal days 1, 3, 6, and 13 (P1–P13) and from adult rats were probed with the rabbit antiserum against β_{gc} by Western blotting. The β_{gc} antibody recognized a single, somewhat broad and heterogenous, immunoreactive band of ~97 kDa in the tissue extracts obtained at P1, P3, P6 and P13, whereas a second and faster migrating band (90 kDa) was present in the homogenates prepared from adult cerebral cortex (Fig. 1). β_{gc} expression was high at early postnatal stages but declined gradually and considerably with increasing age. In the adult brain the lowest levels were detected and the 97 kDa band appeared sharpened, as compared with earlier stages.

We next studied the distribution of β_{gc} in primary neurons sprouting in culture. After 12 hr in culture, most cortical and hippocampal pyramidal neurons had extended several short undifferentiated neurites, designated as minor processes; at this stage β_{gc} immunolabeling was concentrated heavily in the growth cones (Fig. 2*A,B*). Several hours later, the neurons differentiated one of their minor processes into an axon. In this type of culture any neurite that exceeded the other processes of the same neuron in length by 10 μm or more was considered to be an axon (Craig and Banker, 1994). The growth cones of these axons also displayed heavy staining for β_{gc}. An example of such a growth cone, labeled with the β_{gc} antibody, is shown in Figure 2*D* (tubulin staining of the same axon is shown in Fig. 2*C*). This particular neurite was over 100 μm long, exceeding in length the minor neurites from the same cell by >70 μm. Comparisons of β_{gc} fluorescence intensities of axonal growth cones versus those of minor processes revealed no significant differences. Our results also showed that the expression of β_{gc} in cortical and hippocampal pyramidal neurons was transient, declining significantly when cells began dendritic differentiation. Thus, in older cultures (>4 d *in vitro*) when the (nonaxonal) minor processes had become dendrites and were elongating, β_{gc} immunolabeling decreased con-

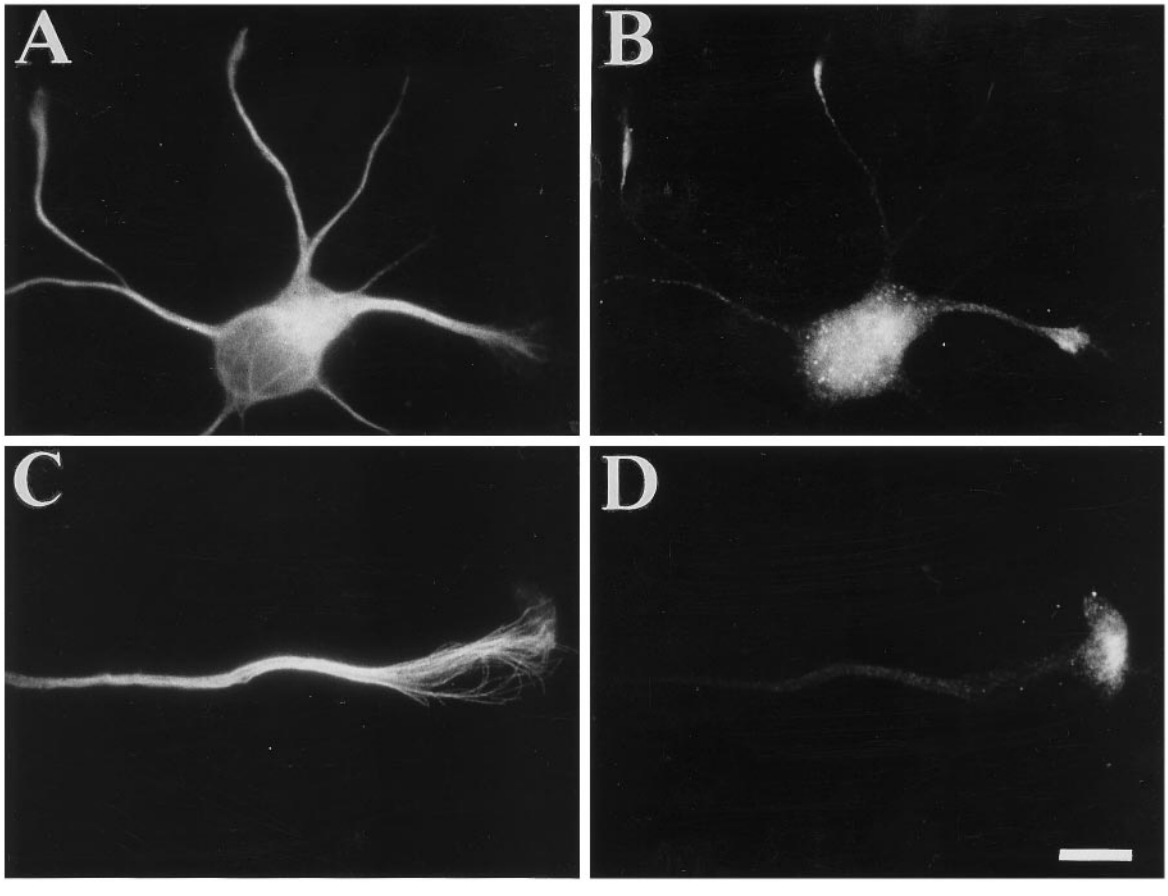

Figure 2. The distribution of β_{gc} in primary neurons. Double-immunofluorescence micrographs of a hippocampal pyramidal neuron maintained in culture for 12 hr show the distribution of tyrosinated α-tubulin (*A*) and β_{gc} (*B*). Note the intense and selective labeling of neurite tips with the β_{gc} antiserum. Double-immunofluorescence micrographs also show the distribution of microtubules (*C*) and β_{gc} (*D*) in an axon-like process from a hippocampal pyramidal neuron maintained in culture for 24 hr. Note that β_{gc} immunolabeling is restricted to the axonal growth cone. Calibration bar, 10 μm.

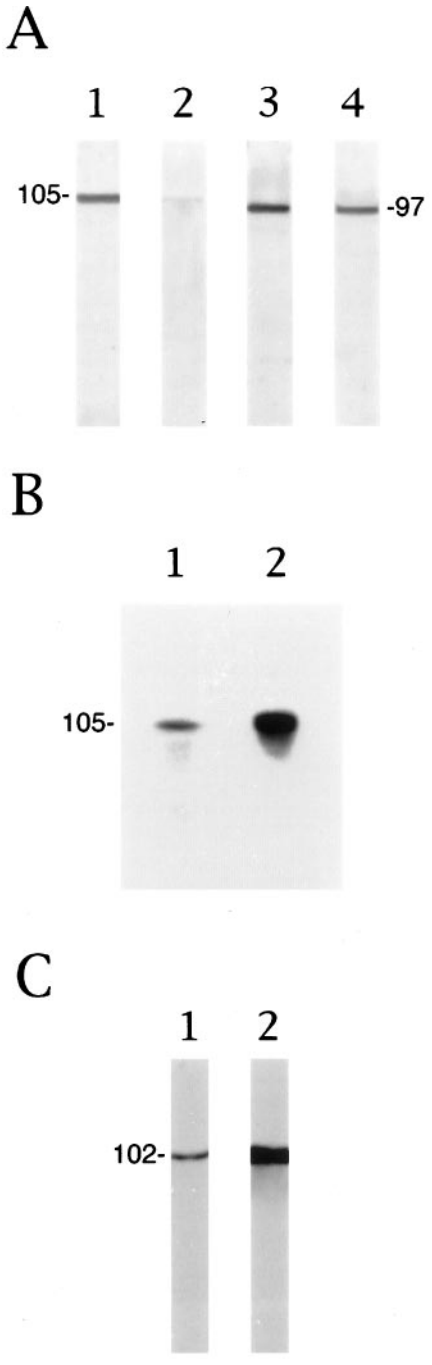

Figure 3. The expression of β_{gc} and β_{P5} in PC12 cells. *A*, Immunoblot analysis of whole-cell extracts from NGF-treated (*lane 1*) or nontreated (*lane 2*) PC12 cells reacted with the β_{gc} antiserum. NGF induces the expression of a single β_{gc}-immunoreactive protein species with an apparent molecular mass of 105 kDa. β_{gc}-immunoreactive polypeptides were not detected in undifferentiated PC12 cells under these conditions. A single β_{gc}-immunoreactive species of 97 kDa (see also Fig. 1 and Quiroga et al., 1995) is detected in tissue extracts obtained from 3-d-old rat cerebral cortex (*lane 3*) or in whole-cell extracts from hippocampal pyramidal cells differentiated in culture for 48 hr (*lane 4*). *B*, Autophosphorylation of IGF-1 receptor in PC12 membranes immunoprecipitated with the β_{gc} antibody. The addition of 10 nM IGF-1 (*lane 2*) dramatically stimulates the phosphorylation of the immunoprecipitated ~105 kDa polypeptide, as compared with the control experiment (*lane 1*). *C*, Immunoblot analysis of whole-cell extracts from NGF-treated (*lane 1*) or nontreated (*lane 2*) PC12 cells reacted with the AbP5 antiserum (β_{P5}). The 102 kDa β_{P5}-immunoreactive band is considerably less abundant in NGF-treated than in nontreated PC12 cells (NGF treatment was for 3 d). Protein (20 μg) was loaded in each lane, and the immunoblots were processed with a rabbit ProtoBlot staining kit. The blots and autoradiogram shown are representative of at last three independent experiments, all of which generated essentially identical results.

siderably, disappeared eventually from dendritic growth cones, and became diffusely distributed throughout the cell (data not shown).

Expression and distribution of β_{gc} in PC12 cells

In whole PC12 cell extracts the anti-β_{gc} antibody recognized a single polypeptide of ~105 kDa (Fig. 3*A*, *lane 1*). Thus, the β_{gc}-immunoreactive polypeptide from PC12 cells exhibited a higher apparent molecular mass than the single β_{gc}-immunoreactive protein species detected in tissue extracts obtained from the developing rat cerebral cortex (~97 kDa; Fig. 3*A*, *lane 3*). For the Western blot shown in Figure 3*A*, *lane 1*, extracts were prepared from PC12 cells cultured for 48 hr in the presence of NGF, i.e., from differentiating PC12 cells that had begun to form neurites. However, the β_{gc} antigen was not or only very weakly detectable by Western blot in undifferentiated cells grown without NGF, even when two- to threefold higher protein amounts were loaded on the gels (Fig. 3*A*, *lane 2*).

To ascertain that the 105 kDa immunoreactive band detected in PC12 cells was indeed the β_{gc} subunit of the IGF-1 receptor, we performed an autophosphorylation experiment with membranes of PC12 cells (grown with NGF) incubated in the presence or absence of IGF-1. Figure 3*B* shows autoradiograms of the immunoprecipitates obtained with the β_{gc} antibody and resolved by SDS-polyacrylamide gel electrophoresis. A single radiolabeled band was detected at 105 kDa, and its intensity was greatly enhanced when the autophosphorylation was performed in the presence of IGF-1 (Fig. 3*B*, *lane 2*). This established the identity of the 105 kDa band as β_{gc}.

The expression of β_{gc} in differentiating PC12 cells was compared with that of other β-subunits of the insulin and IGF-1 receptors with antibody AbP5. This polyclonal antibody was raised against a synthetic peptide representing part of the human insulin receptor β-subunit (amino acids 1328–1343; Garofalo and Rosen, 1989). The antibody cross-reacts with the β-subunit of the rat insulin receptor as well as with β-subunits of the IGF-1 receptor that are distinct from the β_{gc}-immunoreactive species (Garofalo and Rosen, 1989; S. Quiroga, unpublished observations). Figure 3*C* shows that AbP5 recognizes a single polypeptide at 102 kDa in PC12 cells. In contrast to β_{gc}, the AbP5-immunoreactive band (termed β_{P5}) is relatively faint in extracts of NGF-differentiated PC12 cells and much stronger in the nondifferentiated cells (Fig. 3*C*, *lane 1* vs *lane 2*, respectively).

To correlate β_{gc} and β_{P5} immunoreactivity quantitatively with differentiation, we determined average neurite lengths and β-subunit expression (by dot immunobinding assay of whole-cell extracts; Cáceres et al., 1988) in PC12 cells treated for up to 4 d with NGF. Table 1 shows the results. The dramatic increase of NGF-induced neurite length observed over 4 d was paralleled by a more than sixfold increase of β_{gc}, whereas expression of β_{P5} decreased to less than one-half the control value in the presence of NGF. If NGF-differentiated (4 d) PC12 cells were deprived of the neurotrophin for 6 hr, neurite length decreased dramatically, and so did β_{gc} expression. β_{P5} expression did not change during this short deprivation period, however. These results showed in PC12 cells that β_{gc} expression was tightly controlled by NGF,

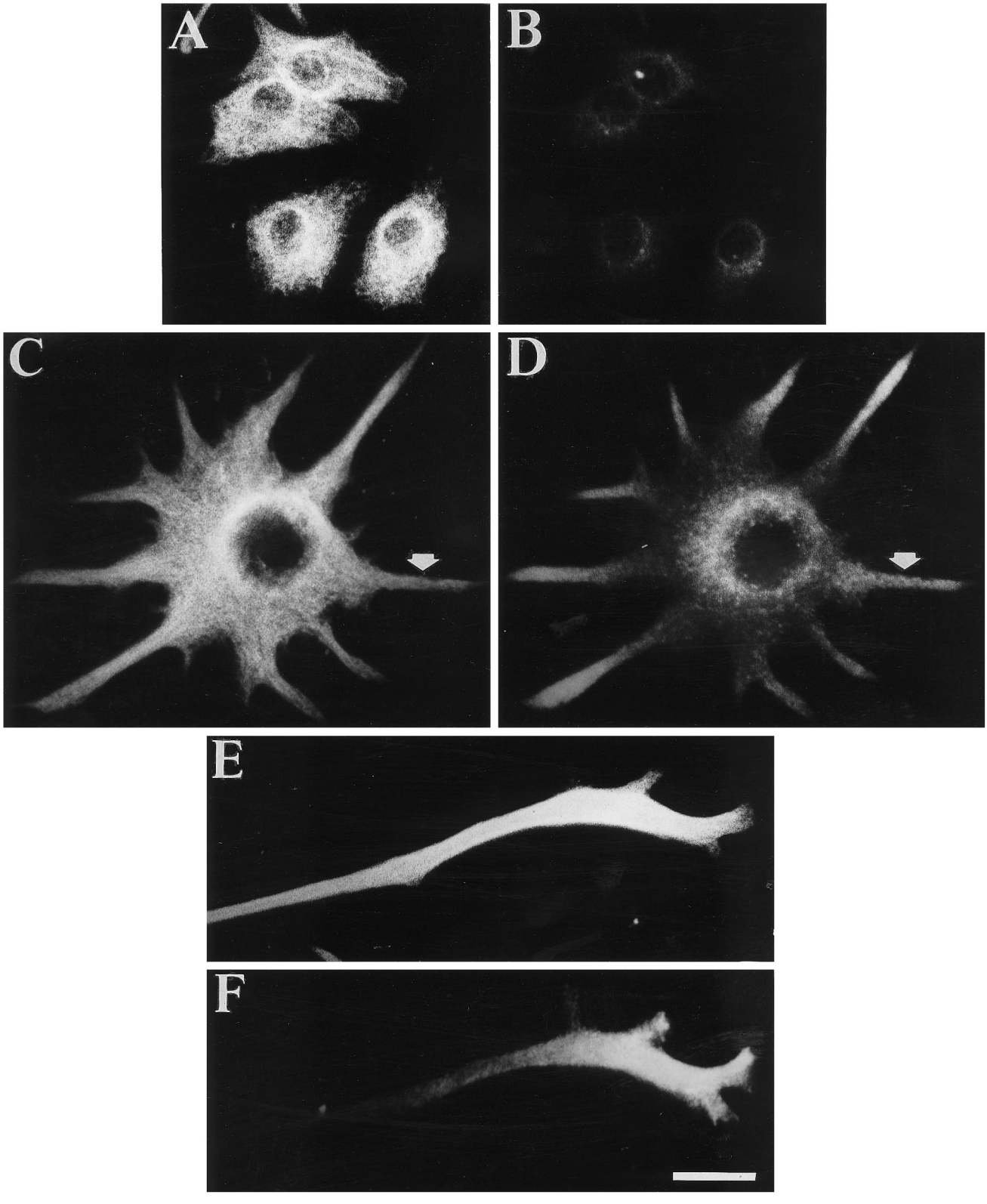

Figure 4. β_{gc} becomes localized to growth cones in differentiated PC12 cells. Double-immunofluorescence micrographs show the distribution of tyrosinated α-tubulin (*A*, *C*, *E*) and β_{gc} (*B*, *D*, *F*) in PC12 cells. PC12 cells cultured in the absence of NGF display low positive immunofluorescence for the β_{gc} antibody (*A*, *B*). A dramatic increase in β_{gc} immunofluorescence is detected in PC12 cells treated with NGF for 2 (*C*, *D*) or 4 (*E*, *F*) d. In these cells β_{gc} is localized preferentially in the perinuclear region and at neurite tips. In young NGF-treated PC12 cells, a few neurites display a granular type of staining that extends from the cell body to the tip (*arrow* in *D*); however, in further differentiated PC12 cells, the cell body and neuritic shafts are devoid of labeling (*E*, *F*). Calibration bar, 10 μm.

together with differentiation, but that β_{P5} obeyed an inverse regulation pattern.

Immunolocalization of β_{gc} in PC12 cells

In the next series of experiments the spatial distribution of β_{gc} was studied in PC12 cells by double immunolabeling with anti-β_{gc} and a mAb that recognized tyrosinated α-tubulin (clone TUA 1.2). PC12 cells cultured in the absence of NGF had a round or polygonal morphology, as expected (Fig. 4*A*, tubulin antibody), and exhibited very weak immunofluorescence when incubated with the β_{gc} antibody (Fig. 4*B*). When used at very high concentrations (dilution 1:5–1:10), the β_{gc} antibody labeled a small perinuclear area of the cell cytoplasm that resembled the Golgi complex (data not shown). As expected, a dramatic increase in β_{gc} immunofluorescence was evident when PC12 cells were cultured in the presence of NGF. This phenomenon was detected as early as 24 hr after the addition of NGF, when PC12 cells began to acquire a neuron-like morphology. At that stage the cells had several short neurites tipped with small growth cones. β_{gc} immunofluorescence was localized preferentially to the perinuclear region and to the growth cones (Fig. 4*D*; compare with tubulin staining in 4*C*), whereas most of the cell body and of the neuritic shafts was devoid of β_{gc} immunolabeling. An exception was occasional short neurites that contained a continuous band of granular staining between the perinuclear region and the growth cones (see *arrow*, Fig. 4*D*). After 72 hr in the presence of NGF, PC12 cells had extended several long neurites that ended in prominent growth cones. At this stage β_{gc} immunostaining had become very intense within the growth cone area but had disappeared completely from neuritic shafts (Fig. 4*F*). A similar pattern was detected in PC12 cells cultured with NGF for longer periods of time (3–7 d).

Our subcellular fractionation data published earlier (Quiroga et al., 1995) indicated spatial separation of β_{gc} from other insulin and IGF-1 receptor β-subunits. Therefore, we performed immunolocalization studies with the AbP5 antibody on PC12 cells. Uniformly distributed AbP5 immunofluorescence was readily detectable in undifferentiated PC12 cells (data not shown). To demonstrate the localization of β_{P5} versus β_{gc} in differentiated PC12 cells, we performed double immunofluorescence. All cells were labeled with the tubulin antibody (secondarily tagged with the green FITC) and then with either AbP5 or anti-β_{gc} (secondarily tagged with the red rhodamine). Because permeabilization was necessary to reveal the β_{gc} antigen (presumably because the epitope is intracellular) and, of course, tubulin, we could not decide whether the β_{gc} staining was on the cell surface and/or in the cellular interior. However, at least part of the perinuclear label almost certainly was associated with sites of synthesis, especially distal regions of the Golgi complex (compare Fig. 7). At the growth cone, however, at least some of the β_{gc} label must have been associated with the cell surface, because the receptor could be activated by externally applied IGF-1 (Quiroga et al., 1995). Figure 5*A* shows the expected pattern for β_{gc}, i.e., intensely red-yellow staining of growth cones (primarily β_{gc}), green neurites (mostly tubulin), and yellow perikarya (overlap of tubulin and β_{gc}). Figure 5*B* illustrates the contrasting pattern for AbP5: absence of obvious growth cone labeling, green neurites stained with anti-tubulin only, and yellow perikarya doubly labeled with anti-tubulin and AbP5. (As before, these samples had to be permeabilized for immunolabeling so that it was difficult to discriminate between β-subunits exposed on the cell surface and those within the cell). These experiments indicate strikingly the differential distribution of β_{gc} versus the other β-subunit(s) (β_{P5}) in differentiated PC12 cells.

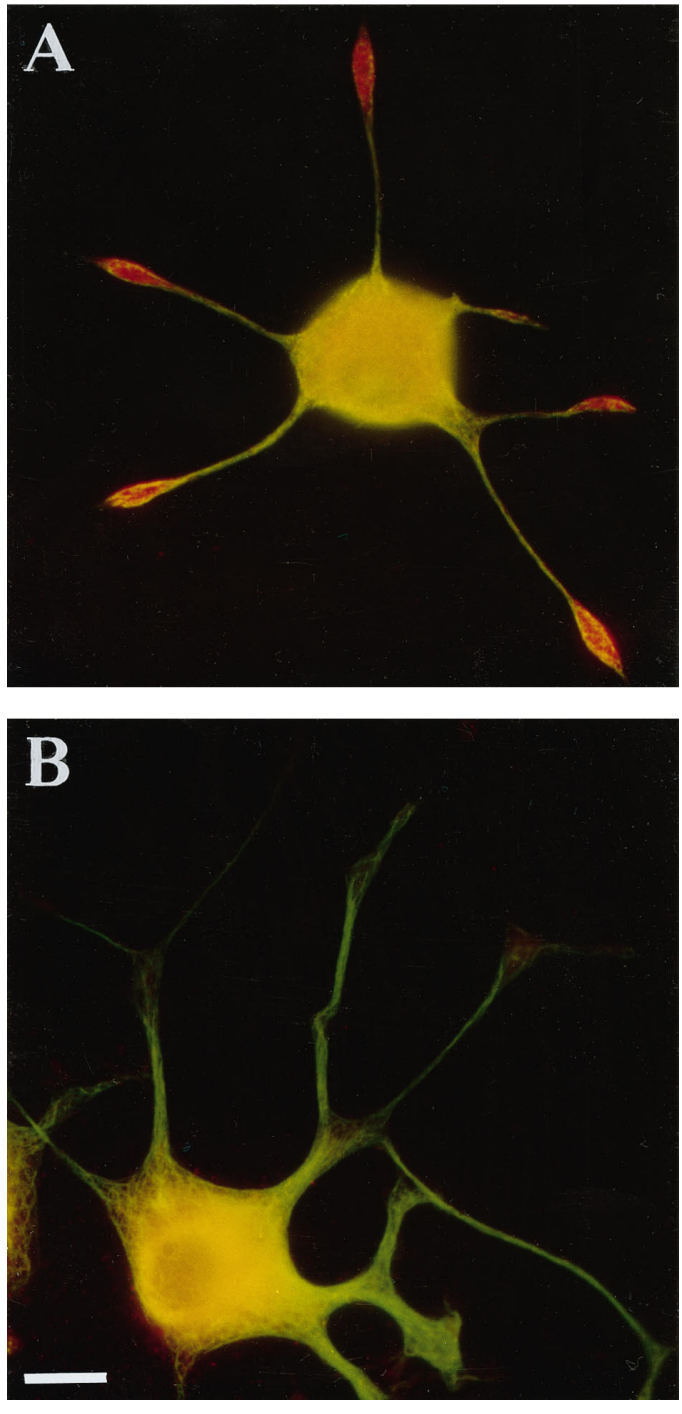

Figure 5. Differential distribution of β_{gc} and AbP5-immunoreactive insulin/IGF-1 β-subunits. *A*, Double-immunofluorescence micrograph shows the distribution of tyrosinated α-tubulin (*green*) and of β_{gc} (*red*). β_{gc} is highly enriched at neurite tips. *B*, Double-immunofluorescence micrograph shows the distribution of tyrosinated α-tubulin (*green*) and AbP5 immunofluorescence (*red*) in a differentiated PC12 cell. Overlapping label appears *yellow*. Note the absence of AbP5 immunolabeling within the neurites and growth cones. Calibration bar, 10 μm.

To investigate β_{gc} further, we used fluorescence microscopy combined with image processing to analyze its subcellular distribution in differentiating PC12 cells and to compare it with that of

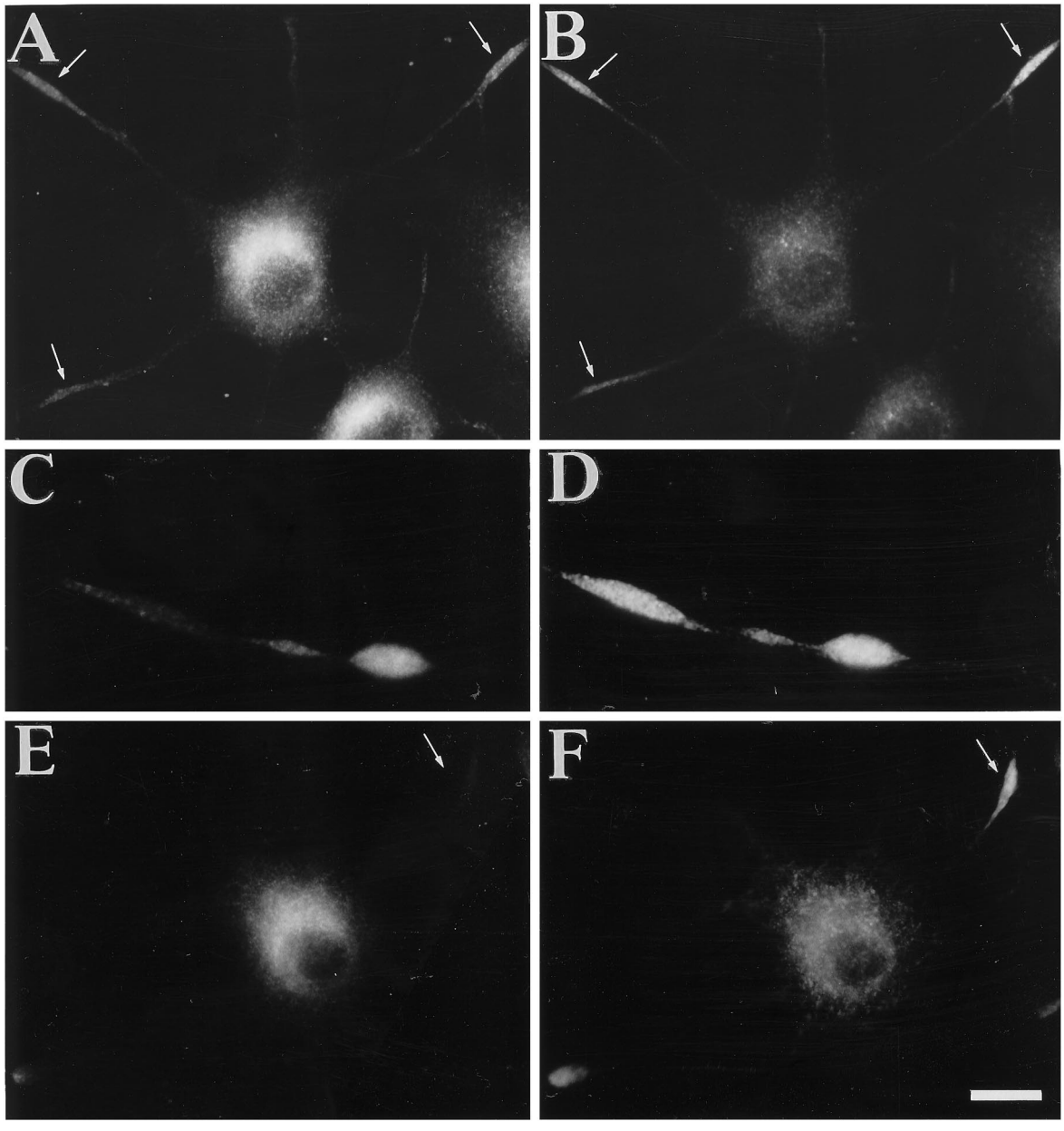

Figure 6. β_{gc} and synaptophysin exhibit similar, but not identical, distributions within growth cones. Double-immunofluorescence micrographs show the distribution of synaptophysin (*A*, *C*, *E*) and β_{gc} (*B*, *D*, *F*) in PC12 cells treated with NGF for 3 d. Both antigens are localized preferentially in the perinuclear region of the cell bodies and in growth cones (*arrows*), but there are clear differences in their staining patterns (see text). Calibration bar, 10 μm.

synaptophysin. Synaptophysin is a membrane protein associated with synaptic vesicles and enriched in the growth cones of developing neurons (Fletcher et al., 1990). Although the antibodies specific for β_{gc} and synaptophysin labeled essentially the same areas of the cell, namely the perinuclear region and the growth cones (Fig. 6*A,B*), we detected clear differences in their staining patterns. For example, as shown in Figure 6, the distribution of β_{gc} immunofluorescence (Fig. 6*D,F*) in growth cones exhibited in some cases little overlap with that of synaptophysin (Fig. 6*C,E*). Also, during the initial stages of neurite extension (24–48 hr after the addition of NGF) growth cones highly immunoreactive for β_{gc} commonly were stained only faintly or not at all with the synaptophysin antibody, although prominent immunolabeling was detected in the perinuclear region (Fig. 6*E,F*). Within the perikarya of differentiating PC12 cells, we were able to observe punctate fluorescent structures of variable size, vesicle-like structures clearly located in the cell interior and apparently emerging from the perinuclear region. With the synaptophysin antibody (Fig. 7*A*), these fluorescent dots were quite small and numerous and tended to form strands radiating from the perinuclear region (*arrows*). The β_{gc} antibody (Fig. 7*B*), however, labeled a heterogeneously sized, generally much larger and sparser, compartment. A pseudocolor superimposition image is shown in Figure 7*C*. There was substantial overlap between the staining patterns in the thicker region of the cell near the nucleus (near the top left-hand corner), as expected. However, in the thinner, more peripheral

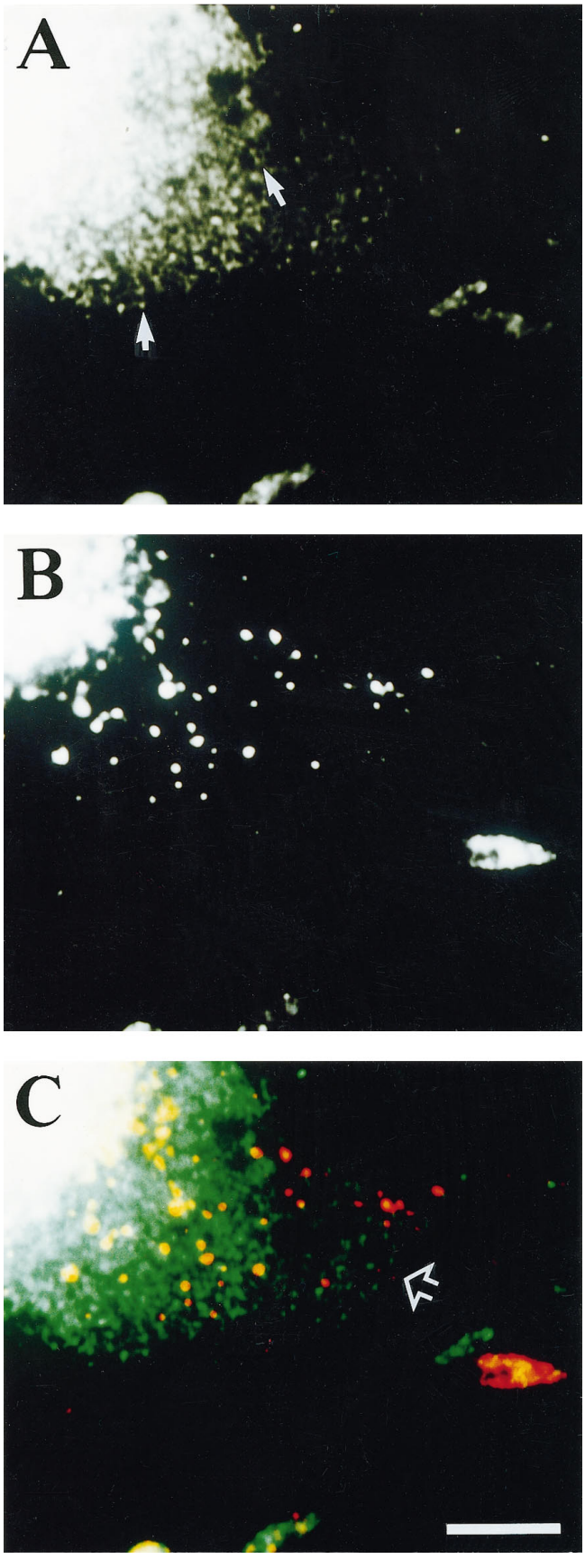

Figure 7. β_{gc} and synaptophysin appear to emerge from the perinuclear region in distinct vesicle-like structures. Double-immunofluorescence micrographs show the distribution of synaptophysin (*A*) and β_{gc} (*B*) in the perinuclear region of a PC12 cell treated with NGF for 3 d. The *arrows* in *A* point at apparent strands of small fluorescent *dots*. *C*, Overlay of digitized synaptophysin (*green*) and β_{gc} (*red*) images shown in *A* and *B*, respectively. The *open arrow* points at a thin region of the perikaryon where there is no overlap between labeled compartments. Calibration bar, 10 μm.

parts of the perikaryon (*arrow*) there was very little, if any, overlap between the staining patterns.

Interactions between β_{gc} and the cytoskeleton of growth cones

The distribution of several growth cone-associated surface antigens seems to depend on interactions with microtubules and/or microfilaments (Goslin et al., 1989; Bearer, 1992; DiTella et al., 1994). The highly polarized distribution of β_{gc} raised the question of whether this membrane protein also interacted with the cytoskeleton. To study such relationships, we examined first whether and where β_{gc} was detectable in association with the cytoskeletons remaining after detergent extraction of differentiated PC12 cells (prepared under microtubule-stabilizing conditions; see Material and Methods). The cytoskeletons were double-labeled with the antibody to tyrosinated α-tubulin and the β_{gc} antiserum and then analyzed by fluorescence microscopy. On detergent extraction most β_{gc} immunolabeling was lost from the perinuclear region. However, it remained unaffected within growth cones, where it colocalized extensively with tyrosinated microtubules (Fig. 8*A,B*). It is unlikely that this was the result of incomplete solubilization and extraction of membrane proteins because, under the same extraction conditions, the staining for synaptophysin was abolished completely (Fig. 8*C*, synaptophysin label, and 8*D*, β_{gc} label of the same cell). Therefore, our observation indicated that a significant proportion of β_{gc} of the growth cone was linked somehow to the cytoskeleton.

Next we investigated whether β_{gc} was linked to microtubules and/or to actin filaments. Differentiated PC12 cells were treated with the microtubule toxin, nocodazole, for 30 or 60 min before fixation, and the distribution of β_{gc} and microtubules was analyzed by immunofluorescence. As shown in Figure 9, this treatment depolymerized throughout the cell most of the microtubules containing tyrosinated α-tubulin (Fig. 9*B*), but it preserved the tubules enriched in acetylated α-tubulin (a marker of stable polymer); these were localized preferentially to neuritic shafts but absent from growth cones (Fig. 9*A*; Ferreira and Cáceres, 1989; Arregui et al., 1991). The distribution of β_{gc} (Fig. 9*D*) was altered dramatically in nocodazole-treated cells; the immunostaining disappeared from the growth cones while punctate and disperse labeling appeared along neuritic shafts (compare Fig. 9*C*, detyrosinated microtubules of the cell seen in 9*D*). Growth cones, however, remained attached, and their gross morphology essentially was unchanged in these experiments (see also Gonzalez-Agosti and Solomon, 1996). On removal of nocodazole, tyrosinated microtubules rapidly reassembled proximo-distally from the cell body toward the neurites as well as within growth cones. β_{gc} immunolabeling at neuritic tips closely paralleled the reappearance of tyrosinated microtubules within the growth cones (data not shown).

It is now well established that the organization of microfilaments is altered considerably after microtubule depolymerization (Goslin et al., 1989; DiTella et al., 1994) so that the observed nocodazole-induced redistribution of β_{gc} could be a secondary phenomenon dependent on actin. Therefore, we sought to determine whether β_{gc} distribution was dependent on the integrity of

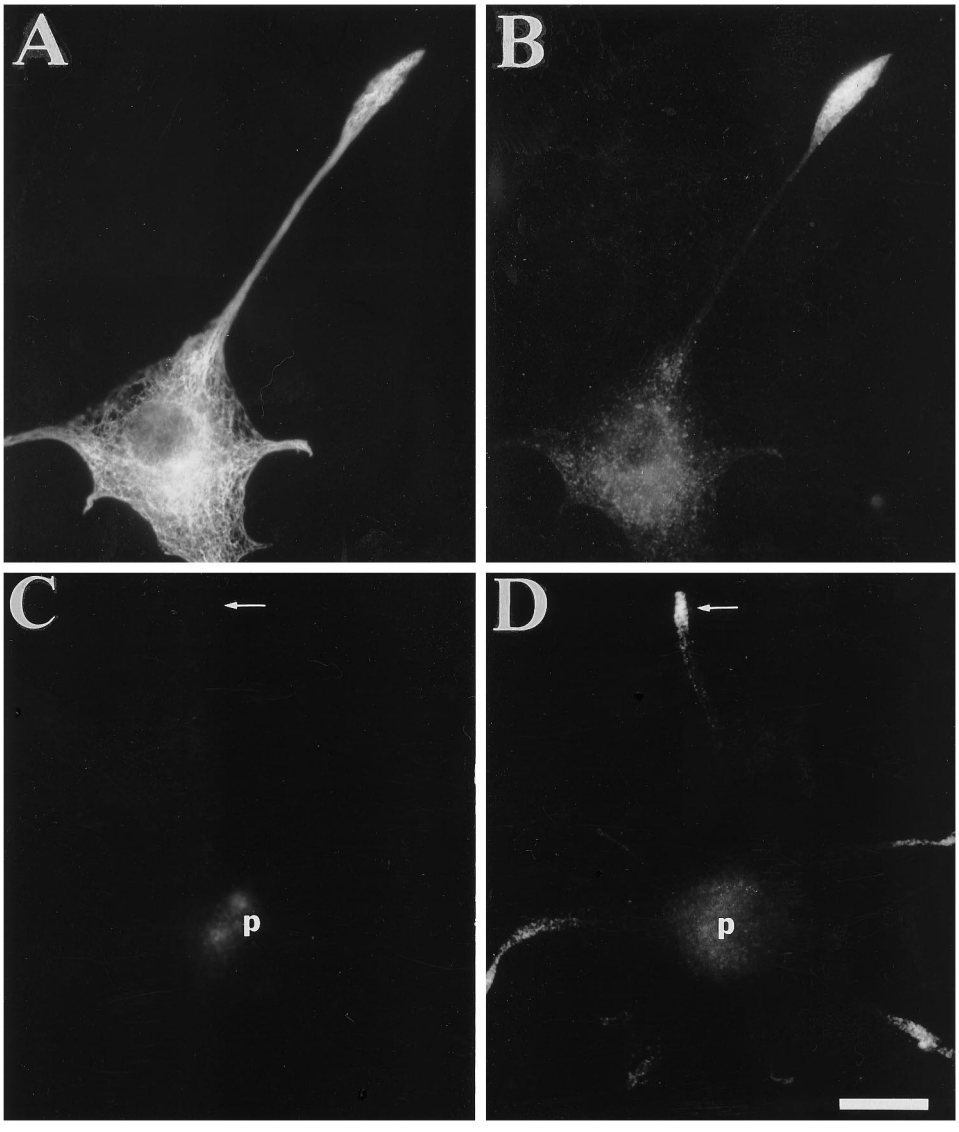

Figure 8. β_{gc} and dynamic microtubules colocalize within growth cones. Double-immunofluorescence micrographs show the distribution of tyrosinated α-tubulin (*A*) and β_{gc} (*B*) in a detergent-extracted cytoskeletal preparation from a differentiated PC12 cell. Note the colocalization of β_{gc} immunolabeling with tyrosinated microtubules at the neurite tip. Double-immunofluorescence micrographs also show the distribution of synaptophysin (*C*) and β_{gc} (*D*) in a detergent-extracted cytoskeletal preparation from a differentiated PC12 cell. Synaptophysin has disappeared completely from growth cones (*arrows*), although β_{gc} immunolabeling remains unaffected. *p*, Perinuclear region. Calibration bar, 10 μm.

actin filaments. Initially, we compared the patterns of β_{gc} and actin filaments in normal growth cones and then analyzed the distribution of these proteins in cells treated with cytochalasin D. Phalloidin staining of differentiated PC12 cells revealed actin filaments primarily within the peripheral growth cone structures, as expected, and in some cases, in veils and filopodia along neuritic shafts (Fig. 10*A*). These F-actin-rich peripheral growth cone structures were almost devoid of microtubules (Fig. 10*B*; cf. Forscher and Smith, 1988; Letourneau and Shattuck, 1989). Comparing the distributions of β_{gc} (Fig. 10*D*), F-actin (Fig. 10*A,C*), and tubulin (Fig. 10*B*) revealed that the β_{gc} staining pattern resembled more closely that of tubulin than that of F-actin (see also Fig. 8*A,B*). Although both phalloidin and the β_{gc} antiserum stained growth cones prominently, the F-actin-rich structures, the filopodia and lamellipodia, were in a position clearly distal to the region enriched in β_{gc}, with little overlap between them (Fig. 10*C,D*; compare position of *arrows*). In most cases, F-actin disassembly induced by cytochalasin D (Fig. 10*E,F*) did not alter growth cone morphology and did not result in changes in the distribution of β_{gc} immunofluorescence in growth cones. However, sometimes the cytochalasin treatment caused the collapse and/or detachment of growth cones and the loss of β_{gc} immunolabeling from neuritic tips.

DISCUSSION

The molecular identity of β_{gc} remains unclear. We have not been able to establish so far whether it is, e.g., the product of a separate gene or the result of a specific post-translational modification of a "conventional" IGF-1 receptor β-subunit. Clear, however, are the distinctive immunochemical properties of β_{gc} (Quiroga et al., 1995).

Our Western blots identified β_{gc} in developing cerebral cortex as a heterogeneous band of 97 kDa, as expected (see Quiroga et al., 1995). The observed heterogeneity of this band most likely is the result of different phosphorylation states of the polypeptide, but differential glycosylation may be involved as well. In PC12 membranes the β_{gc} antibody recognized a larger 105 kDa band. IGF-1-stimulated autophosphorylation of the immunoprecipitated polypeptide identified it as β_{gc}. The higher M_r of β_{gc} in PC12 cells, as compared with brain, is consistent with previous studies showing that IGF-1 receptor β-subunits present in cells outside the CNS exhibit a higher apparent molecular weight, a phenomenon resulting apparently from differential glycosylation (Ocrant

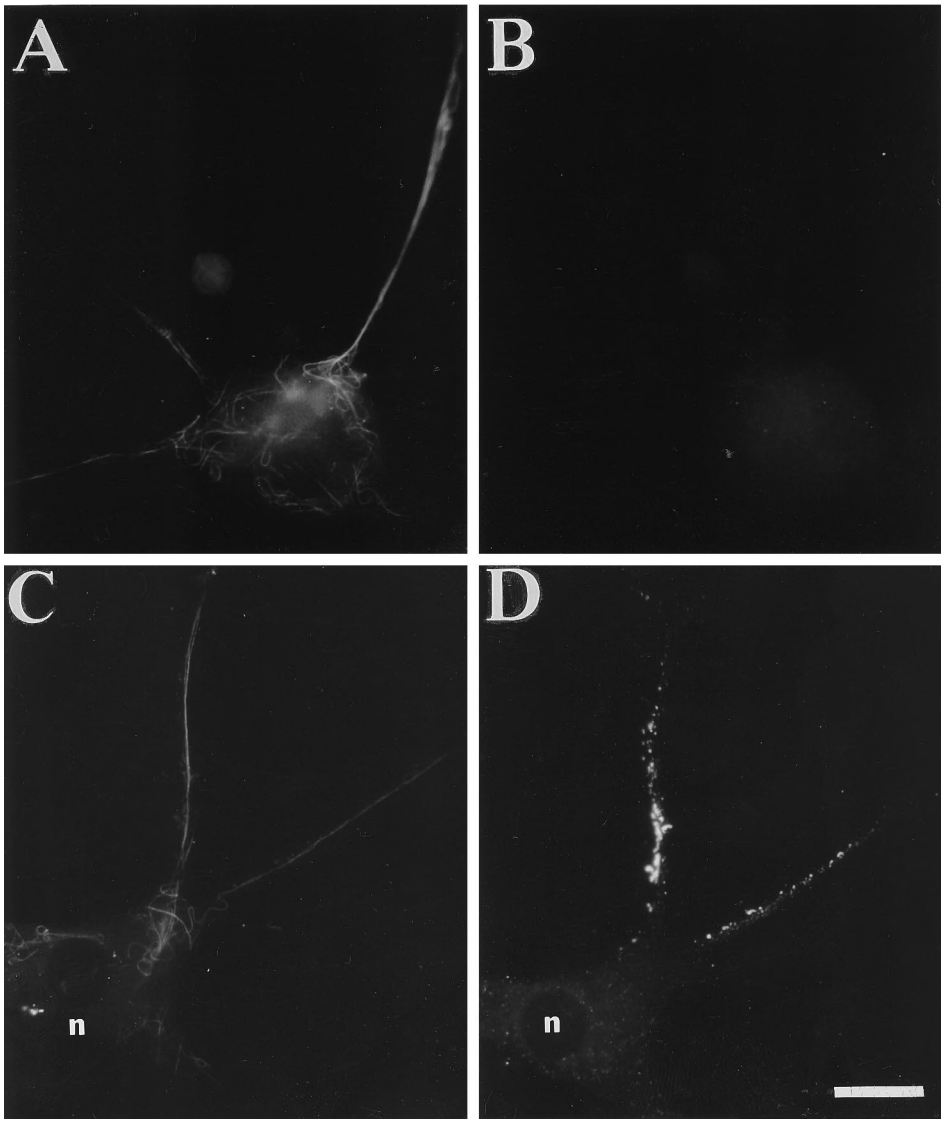

Figure 9. The growth cone localization of β_{gc} depends on the integrity of dynamic microtubules. Double-immunofluorescence micrographs show the distribution of detyrosinated (*A*) and tyrosinated (*B*) α-tubulin in a detergent-extracted cytoskeletal preparation from a differentiated PC12 cell treated with nocodazole for 30 min. Note the complete disappearance of tyrosinated microtubules. Double-immunofluorescence micrographs also show the distribution of detyrosinated microtubules (*C*) and β_{gc} (*D*) in a detergent-extracted cytoskeletal preparation from a differentiated PC12 cell treated with nocodazole for 30 min. Note the retraction of β_{gc} immunolabeling from neurite tips and the presence of immunostaining along neurite shafts. *n*, Nucleus. Calibration bar, 10 μm.

et al., 1988). That greater glycosylation accounts for the higher M_r of β_{gc} in PC12 cells thus is likely but has not been established.

Spatial and temporal distribution of β_{gc} in development

The previous fractionation studies (Quiroga et al., 1995) demonstrated that β_{gc} immunoreactivity in fetal brain is membrane-associated. Our immunofluorescence analysis of primary neuron cultures presented here shows morphologically that β_{gc} is highly enriched in growth cones, whereas there is little immunoreactivity associated with neuritic shafts. The *quasi*-solid staining of neuronal or PC12 growth cones can be explained by the abundance of immunoreactivity in the plasma membrane, combined with the flattened configuration of the growth cone in culture and the staining of intracellular compartments carrying β_{gc} to the distal neurite (see below). Based on the staining pattern in the cell body, i.e., its diffuse distribution excluding the nucleus and the enhanced perinuclear labeling, β_{gc} immunoreactivity in the perikaryon most likely can be attributed to sites of synthesis, especially the Golgi complex (compare with below), rather than plasmalemmal receptors (cells were permeabilized before labeling). During the early phase of differentiation of cultured hippocampal pyramidal cells β_{gc} is present in the growth cones of all types of neurite, including minor processes as well as axons. However, after 2–3 d in culture, when axons have elongated considerably and minor processes begin to differentiate into dendrites, β_{gc} immunoreactivity becomes redistributed throughout the cell while declining significantly overall. It follows that, in cultured neurons, β_{gc} (unlike synaptophysin, synapsin I, and GAP43; Fletcher et al., 1990) is not targeted specifically to axonal growth cones, and its expression is transient. The former observation is surprising considering the high enrichment of β_{gc} in the growth cone fraction from fetal brain, which is predominantly axonal in origin (Saito et al., 1992; Lohse et al., 1996). However, β_{gc} distribution in the developing neuron may be related to the potential of all processes to differentiate into axons, at least during the early stages (Dotti and Banker, 1987). Conceivably, our observations in culture may reflect the (initial) lack of a mechanism sorting certain types of membrane protein to the axon during the establishment of neuronal polarity (Dotti and Simons, 1990). Finally, the different environmental conditions of the cultured neurons may explain the apparent discrepancy between these findings and those obtained by subcellular fractionation of fetal brain.

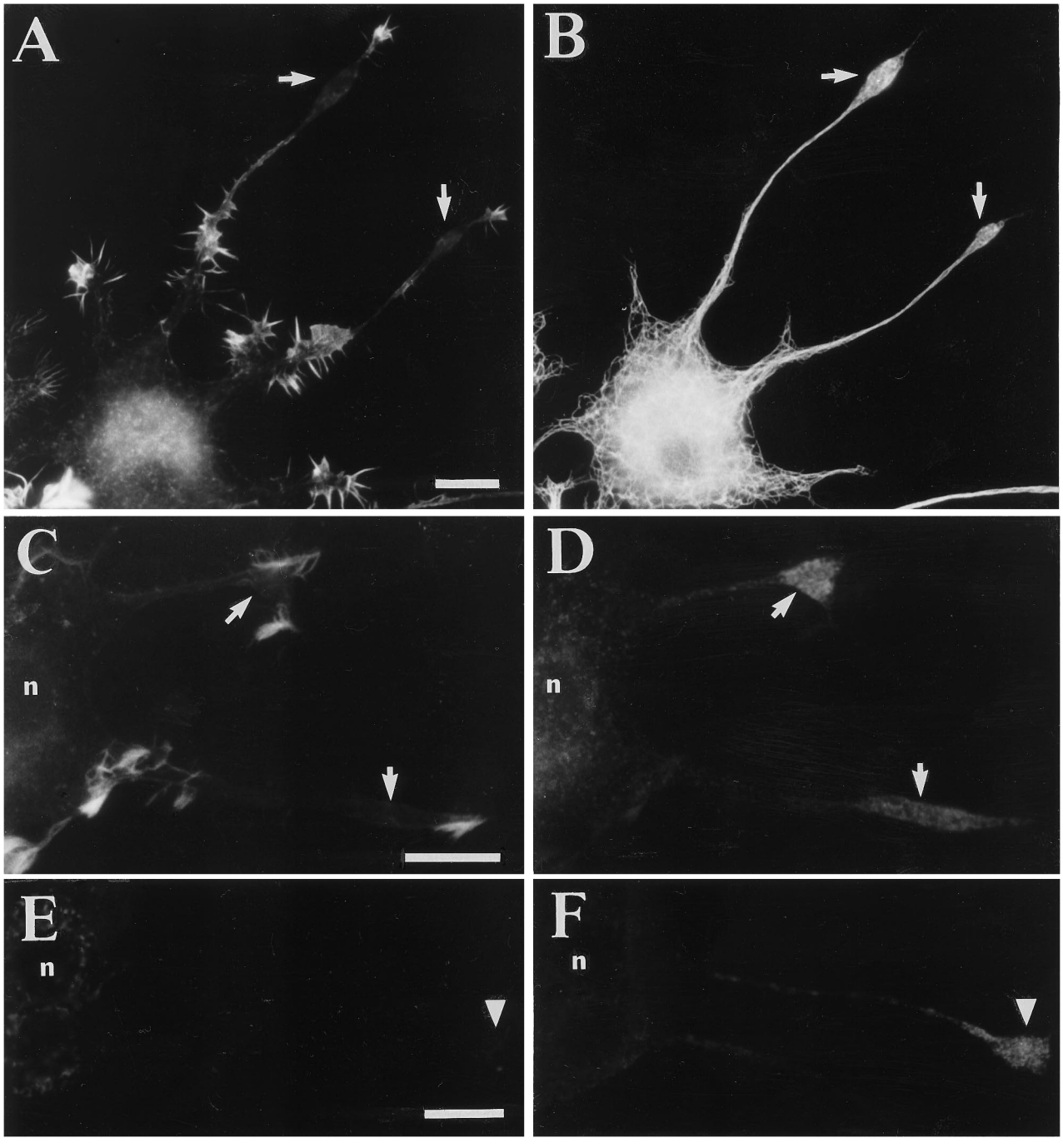

Figure 10. β_{gc} does not colocalize with F-actin. Double-immunofluorescence micrographs show the distribution of F-actin (*A*) and tyrosinated microtubules (*B*) in a differentiated PC12 cell. Note that F-actin, visualized by phalloidin staining, is detected primarily in regions distal or peripheral to, and almost devoid of, tyrosinated microtubules. *Arrows* point at corresponding proximal growth cone structures. Double-immunofluorescence micrographs also show the distribution of F-actin (*C*) and β_{gc} (*D*) in a differentiating PC12 cell. Phalloidin and anti-β_{gc} stain the tips of neurites prominently, but F-actin localizes to a position distal to that enriched in β_{gc}. *Arrows* point at corresponding proximal growth cone structures. *n*, Nucleus. Finally, double-immunofluorescence micrographs show the distribution of F-actin (*E*) and β_{gc} (*F*) in differentiating PC12 cells treated with cytochalasin D for 30 min. Note the complete disappearance of phalloidin staining from neurite tips, whereas β_{gc} immunolabeling remains highly enriched within growth cones (*arrowheads*). *n*, Nucleus. Calibration bars, 10 μm.

Growth cone enrichment of β_{gc} in the brain, in cultured neurons, and in PC12 cells suggests a correlation between neurite outgrowth and β_{gc} expression. Although this correlation is less clear in primary neurons in culture (initial increase followed by decline after the first few days in culture), it holds quantitatively for NGF-stimulated PC12 cells as well as for the developing brain: the β_{gc} polypeptide is abundant during the late fetal and early postnatal days of development when neurite formation is prevalent. [Our more recent data indicate that brain-derived neurotrophic factor (BDNF) also increases β_{gc} expression, concomitant with neurite outgrowth, in primary cultures of hippocampal neurons; studies in progress.] During maturation of the brain and in

the adult, expression of the 97 kDa β_{gc} subunit is much reduced. However, a clearly detectable amount of immunoreactivity remains. This may be analogous to the reduced but continued expression in the adult brain of other growth-regulated and growth-cone-enriched proteins, such as GAP43; this phenomenon has been attributed to continued sprouting activity (see Pfenninger et al., 1991). The appearance after P13 of a second 90 kDa polypeptide reacting with the β_{gc} antibody is not understood at this time. This polypeptide may be the product of oligodendrocytes, which appear relatively late in development.

Establishment and maintenance of β_{gc} distribution

Our immunofluorescence data on cultures of primary neurons and differentiated PC12 cells indicate that β_{gc} labeling does not stain the very periphery of the growth cone, the microfilament-filled filopodia, and lamellipodia. Instead, labeling is coextensive with the distal ends of dynamic (tyrosinated) microtubules, which stop in the proximal region of the growth cones (Forscher and Smith, 1988). This suggests that β_{gc} may be anchored to these microtubules to maintain its spatial distribution. Indeed, a substantial proportion of β_{gc} (unlike synaptophysin) is resistant to detergent extraction and remains codistributed with the distal regions of tyrosinated microtubules. Furthermore, actin depolymerization has little effect on β_{gc} distribution as long as neurites remain attached; in contrast, depolymerization of dynamic microtubules with nocodazole results in β_{gc} spreading to more proximal segments of neurites, where stable microtubules (detyrosinated or acetylated α-tubulin) are present. Based on these data, β_{gc} falls into a category of proteins, more specifically receptors, capable of interacting directly or indirectly with polymerized tubulin. For example, there are two receptors at the synapse that are linked to microtubules. One of these is the γ-aminobutyric acid receptor A, which seems to interact directly with tubulin (Item and Sieghart, 1994); the other is the glycine receptor, which is linked to microtubules by gephyrin, a putative microtubule-associated protein involved in membrane–cytoskeleton interactions (Prior et al., 1992). The β_{gc}-microtubule association raises the interesting possibility of functional effects of the β_{gc}-containing IGF-1 receptor on the cytoskeleton. One of the proteins that is known to be phosphorylated by activation of the IGF-1 receptor is the microtubule-associated protein-2, MAP2 (Pillion et al., 1992). Therefore, β_{gc} could be positioned at the growth cone to control neurite outgrowth by modulating the phosphorylation status of proteins known to be actively involved in cytoskeletal assembly, such as MAP2 or tau (Cáceres and Kosik, 1990; Cáceres et al., 1992; Harada et al., 1994).

To establish its distal, polarized distribution, β_{gc} must be transported from perikaryal sites of synthesis to the growth cones. Synaptophysin, although not nearly so abundant in axonal growth cones as in synaptic endings (Lohse et al., 1996), is an example of a membrane protein shuttled to, and concentrated in, growth cones. Our micrographs of PC12 cells show very small synaptophysin-positive dots in the vicinity of heavily stained perinuclear regions, consistent with the antigen being sequestered into small (putatively synaptic) vesicles and about to be exported into the growing neurites. When cells are labeled with anti-β_{gc}, one finds in the same region sparser, irregularly sized, and generally larger fluorescent dots. These images are compatible with the presence of a larger and distinct vesicular compartment, presumably also destined for export to growth cones. This is of particular interest because plasmalemmal growth of the developing neurite is known to occur primarily at the growth cone, and the plasmalemmal precursor is believed to be the large, clear, irregularly sized vesicles typically found in growth cones (Pfenninger and Maylié-Pfenninger, 1981; Lockerbie et al., 1991; Pfenninger and Friedman, 1993).

Regulation of β_{gc} expression and functional implications

As discussed already, the expression of β_{gc}-containing IGF-1 receptors is correlated, at least at the early stages, with growth cone formation and neurite outgrowth. Particularly striking, however, is the effect of NGF on the expression of β_{gc} and of β-subunits of other IGF-1 and insulin receptors (β_{P5}, recognized by AbP5) in PC12 cells. Undifferentiated PC12 cells express β_{P5} almost exclusively, whereas β_{gc} is essentially undetectable with our methods. In contrast, under the influence of NGF β_{P5} expression declines and remains confined to the perikaryon, whereas that of β_{gc} increases several-fold. Furthermore, NGF withdrawal causes not only neurite retraction but also a precipitous drop in β_{gc} levels. It follows that IGF-1 receptors containing β_{gc} are strictly regulated by NGF and thus are a differentiation product of PC12 cells—in contrast to β_{P5}-containing receptors, which seem to be more important for trophic effects in the proliferating cells (they are also abundant in developing brain). The observed differences in regulation and distribution between β_{gc} and β_{P5} may be particularly significant because the IGF-1 and insulin receptors do not seem to have mainly redundant functions *in vivo*, despite a high degree of similarity in protein sequence and substrate specificity (Ullrich et al., 1986; Steele-Perkins et al., 1988; Gronborg et al., 1993). The predominant physiological actions of insulin seem to involve glucose, protein, and lipid metabolism. In contrast, IGF-1 seems to function in most cells primarily as a mitogenic peptide and, in the particular case of the developing nervous system, as a neurotrophic factor promoting neurite outgrowth (see introductory remarks). Although the reasons for these functional differences are poorly understood (Le Roith et al., 1992), the contrasting regulations and distributions of β_{gc}-containing IGF-1 versus β_{P5}-containing insulin/IGF-1 receptors may be essential for their apparently different functional roles in the neuron.

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