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Insulin-like growth factor I in cultured rat astrocytes: expression of the gene, and receptor tyrosine kinase

Robert Ballotti, Finn C.Nielsen[1], Nigel Pringle[2], Aline Kowalski, William D.Richardson[2], Emmanuel Van Obberghen and Steen Gammeltoft[1]

INSERM U 145, Faculté de Médicine, Université de Nice, France, [1]Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark, and [2]Department of Zoology and Cell Biology, University College London, London WC1E 6BT, UK

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Gene expression, receptor binding and growth-promoting activity of insulin-like growth factor I (IGF I) was studied in cultured astrocytes from developing rat brain. Northern blot analysis of poly(A)$^+$ RNAs from astrocytes revealed an IGF I mRNA of 1.9 kb. Competitive binding and receptor labelling techniques revealed two types of IGF receptor in astroglial cells. Type I IGF receptors consist of α-subunits (M_r 130 000) which bind IGF I with significantly higher affinity than IGF II, and β-subunits (M_r 94 000) which show IGF I-sensitive tyrosine kinase activity. Type II IGF receptors are monomers (M_r 250 000) which bind IGF II with three times higher affinity than IGF I. Both types of IGF receptor recognize insulin weakly. DNA synthesis measured by cellular thymidine incorporation was stimulated 2-fold by IGF I and IGF II. IGF I was more potent than IGF II, and both were significantly more potent than insulin. Our findings suggest that IGF I is synthesized in fetal rat astrocytes and acts as a growth promoter for the same cells by activation of the type I IGF receptor tyrosine kinase. We propose that IGF I acts through autocrine or paracrine mechanisms to stimulate astroglial cell growth during normal brain development.

Key words: insulin-like growth factor I/gene expression/receptor tyrosine kinase/cultured astrocytes/brain development

Introduction

It is now recognized that polypeptide growth factors are essential for growth and survival of a variety of cell types (James and Bradshaw, 1984). Several growth factors and their specific receptors are expressed in the central nervous system (CNS) (for reviews see Gospodarowicz, 1984; Westermark *et al.*, 1985; Korsching, 1986; Gammeltoft *et al.*, 1987a), suggesting a role in regulation of cell growth and differentiation in the developing and mature brain, but in general the cell types which produce and respond to these growth factors are unknown.

Insulin-like growth factors I and II (IGF I and II) are related peptides which bear structural homology with proinsulin, and act as growth promoters for various cells of developing and adult animals (Humbel, 1984). One role of IGF I (also known as somatomedin C) is to induce skeletal growth under the control of growth hormone (Van Wyk, 1984). No clear physiological role for IGF II has yet been identified. The effects of IGF I and IGF II on cells are mediated by two types of receptor which differ in structure, binding specificity and the fact that the type I receptor but not the type II receptor is a tyrosine-specific protein kinase (Jacobs *et al.*, 1983; Nissley and Rechler, 1984; Ullrich *et al.*, 1986; Corvera *et al.*, 1986). The type I IGF receptor is composed of two α- and two β-subunits like the insulin receptor with which it shares sequence homology, notably in the β-subunit tyrosine kinase domain (Ullrich *et al.*, 1986). In contrast, the type II IGF receptor is a single 250 000 M_r protein devoid of intrinsic kinase activity, which may have a role in cellular uptake of IGF II (Czech, 1985; Corvera *et al.*, 1986). The recent description of the cDNA sequence of the human IGF II receptor reveals that it is identical to the cation-independent mannose-6-phosphate receptor (Morgan *et al.*, 1987).

Both IGF I and IGF II, and the two types of receptor are present in mammalian brain. IGF I is produced by fetal rat brain explants (D'Ercole *et al.*, 1980; Binoux *et al.*, 1981) and IGF II and its putative precursors are present in extracts of adult human brain (Haselbacher *et al.*, 1985). In fetal and adult brain of rat and man, IGF I and IGF II mRNAs have been found (Scott *et al.*, 1985; Brown *et al.*, 1986; Lund *et al.*, 1986; Hynes *et al.*, 1987). In adult rat and human brain the two types of receptor are present (Gammeltoft *et al.*, 1985). These observations raise questions about the cellular origin of IGFs in fetal and adult brain, the nature of their target cells in the CNS and their molecular mechanisms of action on brain cells, in particular the role of the type I IGF receptor tyrosine kinase.

Glial cells constitute a major cellular component of the CNS, and regulation of glial cell growth is clearly important in brain development (Raff *et al.*, 1983) and in repair of brain injury (Guilian *et al.*, 1986). In this study we describe the expression of the IGF I gene in cultured astrocytes, and the interaction of IGF I with the same cells. Our data suggest that astroglial cells

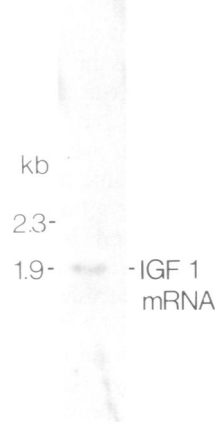

Fig. 1. Northern blot analysis of IGF I mRNA. Ten micrograms of poly(A)$^+$ RNA from astroglial cells were denatured and run on an agarose gel, blotted to a nylon membrane and hybridized to IGF I cDNA that was ^{32}P-labelled by random priming. After hybridization the blot was washed, air-dried and exposed for 1 week at −70°C with an intensifying screen. The sizes of PFGF A-chain transcripts on the same blot are indicated for comparison.

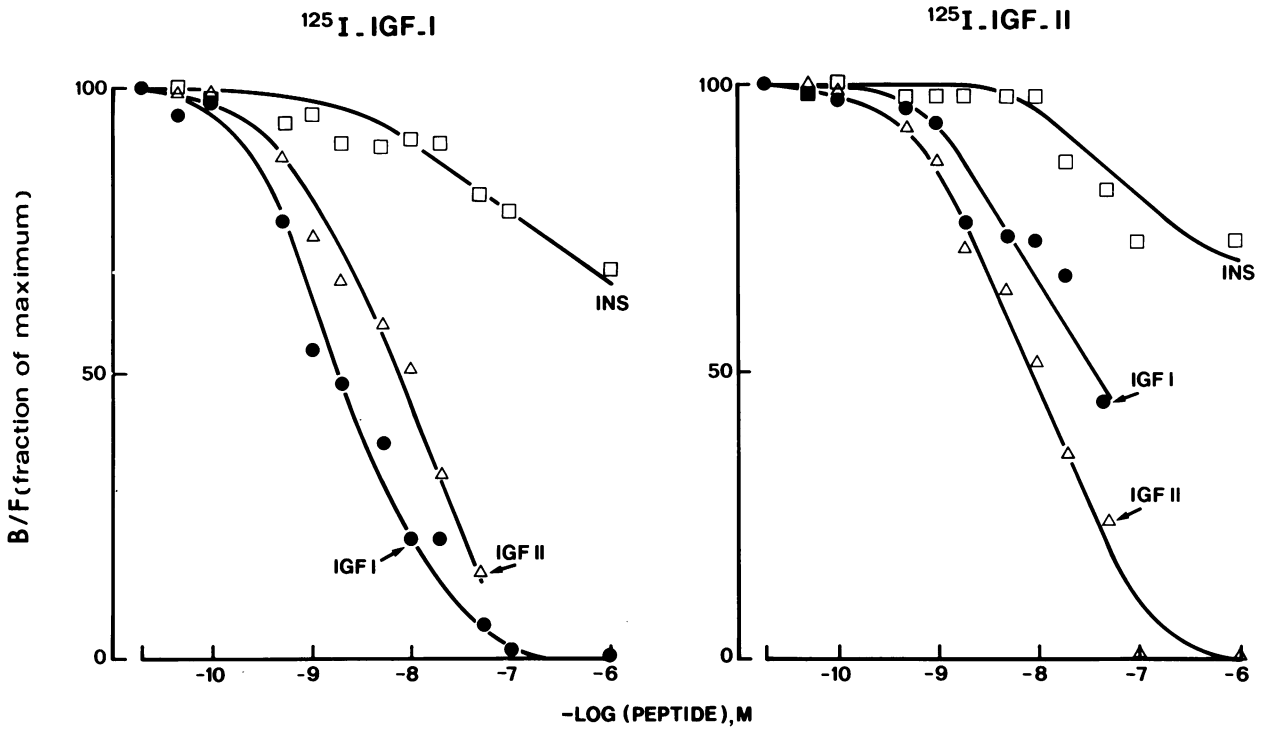

Fig. 2. Binding of ^{125}I-labelled IGF I and IGF II to solubilized receptors. Purified glycoproteins from rat astroglial cells were incubated 15 h at 4°C with either [^{125}I]IGF I (**left panel**) or [^{125}I]IGF II (**right panel**) in concentrations of 150 pM with the addition of IGF I (●), IGF II (△) or insulin (□) at the indicated concentrations. The receptor binding was expressed as the ratio between bound and free tracer in per cent of maximum (the value with tracer alone). Points are the mean of three experiments.

both make and respond to IGF I, and that the type I receptor tyrosine kinase is involved in the mechanism of IGF I-induced astroglial cell growth.

Results

Expression of the IGF I gene in cultured astrocytes

Poly(A)$^+$ RNAs purified from cultured neonatal rat astrocytes were electrophoresed on a denaturing gel and subjected to Northern blot analysis, using a ^{32}P-labelled human IGF I cDNA probe. As shown in Figure 1, the IGF I probe hybridized with one transcript of ~1.9 kb, estimated by comparison with the position of a 1.9-kb platelet-derived growth factor A-chain transcript (Betsholtz *et al.*, 1986) on the same blot (not shown). An IGF I transcript of similar size was also found in cultured meningeal cells from newborn rats, and in whole brain from fetal and adult rats (data not shown). We have been unable to detect any IGF II transcripts in the same or different preparations of astrocyte poly(A)$^+$ RNA using a human IGF II cDNA probe. In contrast, we found a major IGF II transcript of ~4.0 kb and a minor one of 2.7 kb in fetal and adult rat brain (data not shown). Our major transcript probably corresponds to the 3.9-kb transcript described by Lund *et al.* (1986). A further IGF II transcript of ~5.0 kb was apparent in rat brain when blots were washed at low, but not high stringency. This may represent cross-hybridization of the IGF II probe with 28S rRNA.

Characterization of two types of IGF receptor

Receptor binding of IGF I and IGF II was measured by a competitive binding assay on solubilized wheat-germ agglutinin (WGA)-purified glycoproteins from fetal rat astroglial cells in secondary culture. Binding of [^{125}I]IGF I was inhibited by unlabelled IGF I with an apparent K_d of ~2 nM and by IGF II with ~5 times lower potency (Figure 2, left panel). [^{125}I]IGF

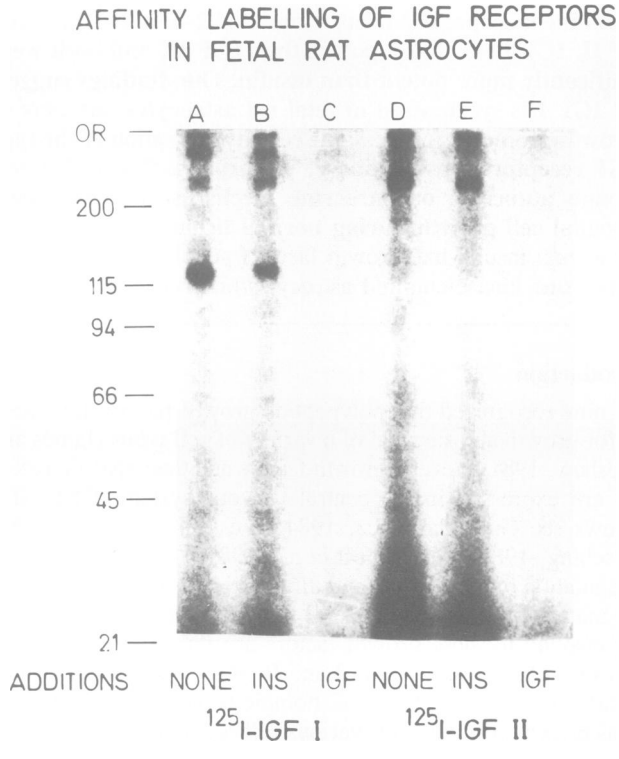

Fig. 3. Affinity labelling of cellular IGF receptors. Confluent rat astroglial cells in 25-cm^2 flasks were incubated for 2 h at 20°C with [^{125}I]IGF I (**A–C**) or [^{125}I]IGF II (**D–F**) in concentrations of 1 nM in the absence or presence of insulin (0.1 μM) or IGF I + IGF II (0.1 μM) as indicated. Bound [^{125}I]IGF I or [^{125}I]IGF II was cross-linked with disuccinimidyl suberate, followed by solubilization of the cells in SDS (3% w/v) and reduction with mercaptoethanol (1.4 M). The ^{125}I-labelled proteins were analysed by SDS–polyacrylamide (7.5% w/v) gel electrophoresis and autoradiography.

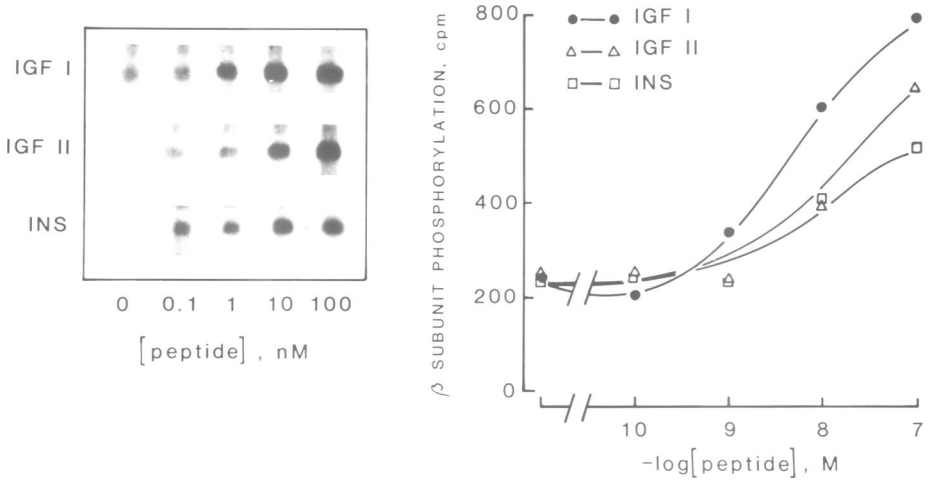

Fig. 4. Phosphorylation of solubilized IGF I receptors. Aliquots (50 μl) of purified rat astroglial cell glycoproteins (5 μg of protein) were incubated for 2 h at 20°C with 10 μl buffer or IGF I, IGF II or insulin in final concentrations of 0.1–100 nM. Protein phosphorylation was initiated by addition of 15 μl [γ-^{32}P]ATP (15 μM) for 15 min at 20°C and stopped by boiling in SDS (3% w/v) and mercaptoethanol (1.4 M). The phosphoproteins were analysed by SDS–polyacrylamide gel electrophoresis followed by autoradiography. **Left panel:** autoradiograms of ^{32}P-labelled proteins with M_r 94 000. **Right panel:** incorporation of radioactivity in the protein with M_r 94 000 measured by Cerenkov counting of the corresponding polyacrylamide gel fragments.

II binding to solubilized receptor showed an apparent K_d for IGF II of ~10 nM, IGF I being ~3 times less potent (Figure 2, right panel). On both types of IGF receptor, insulin showed a partial inhibition with ~1000 times lower potency (Figure 2). In solubilized astroglial cells the amounts of type I and type II IGF receptors were almost equal: 260 and 320 pmol/g of purified glycoprotein respectively. The amount of insulin receptors in the solubilized cell preparation was only 3 pmol/g glycoprotein or ~1% of that of the two types of IGF receptors (data not shown). In intact astroglial cells the binding specificities of the two types of IGF receptor were similar to those of the solubilized receptor preparations. The amount of type II IGF receptor in intact cells is, however, significantly higher than the amount of type I receptors: 500 versus 100 pmol/g cell protein, respectively (data not shown). The difference between receptor numbers in intact and solubilized astrocytes may be due to a lower recovery of type II IGF receptors than of type I receptors during solubilization and lectin-affinity chromatography, in agreement with recent observations in isolated fat cells (Corvera *et al.*, 1986).

Affinity labelling of IGF receptors

IGF receptors on intact astroglial cell monolayers in secondary culture were affinity labelled by chemical cross-linking the ^{125}I-labelled peptides with disuccinimidyl suberate followed by analysis on SDS–polyacrylamide gels under reducing conditions. As shown in Figure 3, labelling with [^{125}I]IGF revealed three bands of M_r 130 000, 250 000 and 300 000, which were completely inhibited by addition of IGF I + IGF II 0.1 μM, but only slightly affected by insulin 0.1 μM. Only one band of M_r 250 000 was labelled by [^{125}I]IGF II, the labelling of which was completely inhibited by 0.1 μM of IGF I + IGF II and partly by 0.1 μM insulin. These data suggest that two molecular types of IGF receptor exist in astroglial cells. The M_r 130 000 protein corresponds to the type I IGF receptor α-subunit and the M_r 250 000 protein represents the type II IGF receptor. The M_r 300 000 band specifically labelled by [^{125}I]IGF I may represent an oligomer of type I IGF receptor, which has been covalently cross-linked by disuccinimidyl suberate before reduction of disulfide bridges. Additional bands of M_r 66 000 and 44 000 specifically labelled by IGF I and IGF II were also observed and may represent IGF binding proteins (data not shown). Affinity

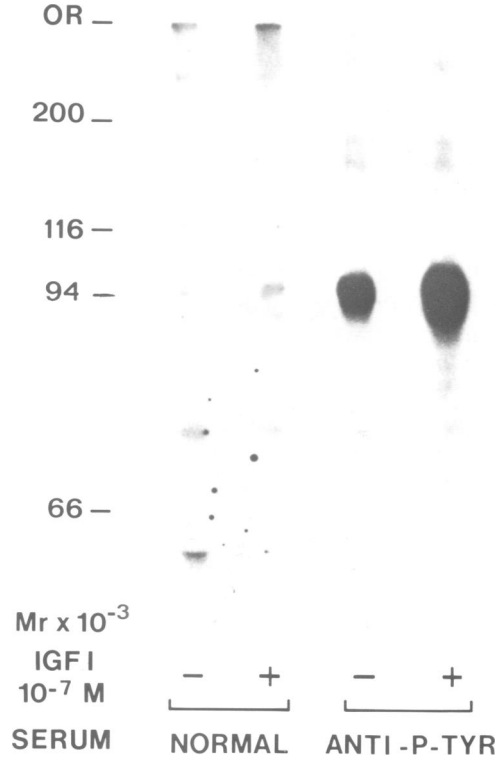

Fig. 5. Immunoprecipitation of phosphorylated IGF receptors by antibody to phosphotyrosine. Purified rat astroglial cell glycoproteins were incubated in the presence or absence of IGF I (100 nM) for 2 h at 20°C, and phosphorylated with [γ-^{32}P]ATP. Phosphoproteins were immunoprecipitated by incubation for 16 h at 4°C with rabbit antiserum to phosphotyrosine (**right**) or normal rabbit serum (**left**) followed by precipitation with protein A. The immunoprecipitates were solubilized in SDS (3%) and analysed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

labelling of secondary cultures of astroglial cells with [^{125}I]-insulin showed only one band of M_r 130 000 which was inhibited more efficiently by IGF I than insulin in concentrations from 0.1 nM to 0.1 μM, suggesting that it represents the type I IGF receptor and not the insulin receptor (data not shown).

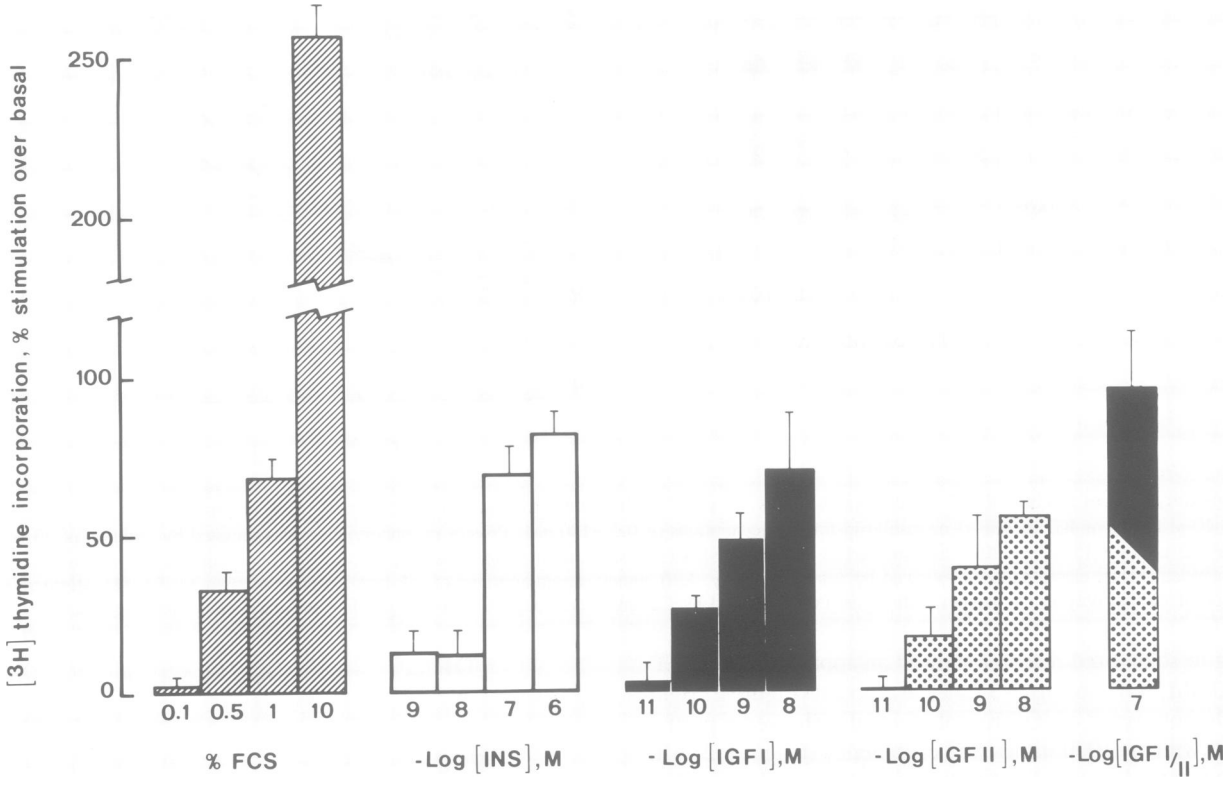

Fig. 6. Stimulation of DNA synthesis in fetal rat astroglial cells. After 24 h culture in RPMI 1640 medium without FCS, cells were incubated for 20 h at 37°C with [^{3}H]thymidine (3 μM) in serum-free medium (basal), or in medium with added FCS, insulin, IGF I or IGF II. Incorporation of radioactivity was determined and expressed in per cent stimulation over basal. The columns represent the mean of three experiments.

Kinase activity of type I IGF receptor

Phosphorylation of the type I IGF receptor β-subunit was studied in a preparation of WGA-purified glycoproteins from Triton X-100 solubilized astroglial cells. When added to such preparations, IGF I markedly enhanced the incorporation of ^{32}P into one protein with M_r 94 000 whereas no other ^{32}P-labelled proteins were observed on the SDS–polyacrylamide gel. As shown on Figure 4, phosphorylation was stimulated 4-fold with 0.1 μM IGF I, and half-maximal effect occurred at ~3 nM. IGF II and insulin were less potent in stimulating phosphorylation of the 94 000 protein with half-maximal effects at 10 nM IGF II and 0.1 μM insulin respectively. The specificity of the peptide-stimulated protein phosphorylation is similar to that of inhibition of [^{125}I]IGF I binding to the type I IGF receptor in solubilized astroglial cells (cf. Figure 2). Furthermore, the molecular size of the phosphoprotein 94 000 is similar to that of the type I IGF receptor β-subunit previously described in human brain cortex (Gammeltoft *et al.*, 1985).

The IGF-sensitive protein kinase activity of solubilized astroglial cells was also demonstrated by phosphorylation of added substrate, histone H2B (data not shown). IGF I, IGF II and insulin stimulated histone phosphorylation with potencies similar to those observed for [^{125}I]IGF I receptor binding and receptor β-subunit phosphorylation, suggesting that the kinase activity is associated with the type I IGF receptor.

In order to investigate whether the IGF-sensitive protein phosphorylations occur on tyrosine residues, solubilized astroglial glycoproteins were phosphorylated followed by immunoprecipitation with an antibody to phosphotyrosine. As seen in Figure 5, the phosphorylated protein (M_r 94 000) was precipitated by antiserum but not by control serum. Furthermore, IGF I enhanced the amount of phosphotyrosine on the 94 000 protein (Figure 5). IGF II was significantly less potent (data not shown). These results may be interpreted as the type I IGF receptor being a tyrosine-specific protein kinase which autophosphorylates its own β-subunit and other substrates, such as histones.

Stimulation of thymidine incorporation in astroglial cells

Secondary astroglial cells cultured under serum-free conditions were tested for stimulation of DNA synthesis by IGF I, IGF II, insulin and fetal calf serum (FCS) (Figure 6). After starvation of the cells for 24 h in the absence of FCS, incorporation of [^{3}H]thymidine during 24 h was augmented approximately 2-fold by IGF I (10 nM–0.1 μM). Half-maximal stimulation was obtained at an IGF I concentration of ~1 nM. IGF II was slightly less potent, whereas insulin was significantly less active in concentrations up to 1 μM. FCS (10%) was ~2.5 times more efficient in stimulating thymidine incorporation than IGF I and IGF II, suggesting that FCS contains growth factors other than IGFs which promote DNA synthesis in astroglial cells. The relatively weak response of DNA synthesis to FCS in astroglial cells corresponds to a slow rate of cell division, i.e. the time required for a 2-fold increase in cell number was ~40 h with 10% FCS (data not shown). The dose–response relationships of IGF I, IGF II and insulin for DNA synthesis are similar to those of inhibition of [^{125}I]IGF I receptor binding and stimulation of type I IGF receptor tyrosine kinase activity, which may mean that the growth response of astroglial cells to IGF I is mediated by the type I receptor.

Discussion

In this study, IGF I mRNA has been demonstrated in cultured astroglial cells from newborn rat brain, by hybridization of human IGF I cDNA with poly(A)$^+$ RNAs. The size of the transcript

(~1.9 kb) in astroglial cells is similar to the major mRNA species from whole fetal rat brain. In contrast, IGF II mRNA was only found in whole fetal rat brain and not in cultured astroglial cells. The patterns of hybridizing IGF I and IGF II mRNAs are in agreement with a recent study by Lund *et al.* (1986). Our observations suggest that IGF I is synthesized by astroglial cells in developing rat brain. The cellular sites of IGF II production in fetal and adult rat brain remain to be established. It is not yet known whether IGF I is released from rat astroglial cells into the culture medium, but studies to address this question are in progress.

Two types of IGF receptor have been identified in cultures of astroglial cells from fetal rat brain by their binding specificity, subunit structure and protein phosphorylation. They are similar to those described in other fetal and adult tissues (Nissley and Rechler, 1984), but the apparent mol. wt of the type I IGF receptor α-subunit in fetal rat astroglial cells (M_r 130 000) is significantly larger than that described previously on adult rat brain cortical plasma membranes (M_r 115 000) (Gammeltoft *et al.*, 1985; Heidenreich *et al.*, 1986). The reason for this difference is not known, but one possible explanation is that the type I IGF receptor in brain undergoes developmental changes in its biosynthesis as has been reported for rat brain insulin receptors (Grizzard *et al.*, 1984; Brennan, 1986). Another possibility is that the IGF I receptor α-subunit examined in adult rat brain plasma membrane preparations may originate from neuronal rather than astroglial cells. This is based on recent findings of a faster electrophoretic mobility (corresponding to M_r 115 000) of type I IGF receptors in primary cultures of post-mitotic neuronal cells (Burgess *et al.*, 1987). Interestingly, recent studies in our laboratory have revealed that type I IGF receptors on glioma cell lines derived from adult brain tumours are similar to those on fetal astroglial cells (Gammeltoft *et al.*, 1987b).

Our studies suggest that the type I IGF receptor in rat astroglial cells has tyrosine kinase activity, which autophosphorylates its own β-subunit, and phosphorylates an exogenous substrate, histone H2B. This is based on the following observations. The kinase is stimulated in a dose-dependent manner by IGF I, IGF II and insulin with potencies approximately similar to those estimated from receptor binding competition experiments. In a preparation containing partially purified receptors the detected phosphoprotein has an electrophoretic mobility (M_r 94 000) which is similar to that of type I IGF receptor β-subunits in human brain and other tissues (Jacobs *et al.*, 1983; Gammeltoft *et al.*, 1985). Finally, antibody to phosphotyrosine precipitated quantitatively the phosphorylated β-subunit of the type I IGF receptor.

Incorporation of thymidine into DNA of astroglial cells is stimulated ~2-fold by IGF's in the absence of FCS. In our study IGF I was ~5 times more potent than IGF II. These results are in agreement with previous work by Lenoir and Honegger (1983), who observed that IGF I was significantly more potent than IGF II and insulin for stimulating thymidine incorporation 2-fold in fetal rat brain cells cultured in a chemically defined medium. Recently, McMorris *et al.* (1986) reported that IGF I produced an up to 60-fold increase of oligodendrocyte numbers, and a 1.8-fold increase in the number of non-oligodendroglial cells in serum-free cultures established from neonatal rat brain. The increase in oligodendrocyte numbers is produced mainly by inducing oligodendrocyte precursor cells to differentiate in culture (M.Dubois-Dalcq and F.A.McMorris, personal communication). In these studies IGF I was also much more potent that insulin. Finally, Han *et al.* (1987) have demonstrated that IGF I and IGF II increase DNA synthesis 1.6-fold in primary cultures of neonatal rat astrocytes after 48 h in the absence of FCS. All of these results agree that IGF I produces a weak stimulation of DNA synthesis in rat astrocytes. It is suggested that IGF I acts as a growth and differentiation factor on astroglia and oligodendroglia in developing brain.

Our data indicate that the growth response of astroglial cells to IGF I is mediated by the type I rather than the type II IGF receptor. This conclusion is based on a comparison of the dose–response curves of type I receptor binding, kinase activation and stimulation of thymidine incorporatoin by IGF I, IGF II and insulin. For all three responses IGF I is more potent than IGF II or insulin. In contrast, IGF II is more effective than IGF I in inhibiting [^{125} I]IGF II binding to the type II receptor. Insulin receptors show low affinity for IGFs and are present in very small amounts in secondary cultures of fetal rat astrocytes. Thus, it seems possible that IGF I induces DNA synthesis in astroglial cells by binding to the type I IGF receptor and activating its tyrosine kinase. Our conclusion is in agreement with recent studies of the mitogenic mechanism of IGF I in human skin fibroblasts and L6 myoblasts where the action of IGF I is apparently mediated by the type I receptor (Flier *et al.*, 1986; Ewton *et al.*, 1987). At present the role of the type II IGF receptor in brain and other tissues is not understood, but a role in endocytosis of IGF II has been suggested (Nissley and Rechler, 1984; Czech, 1985; Gammeltoft *et al.*, 1987a). Recently it was reported that the cDNA sequence of the IGF II receptor is similar to that of the mannose-6-phosphate receptor (Morgan *et al.*, 1987).

We have shown that the IGF I gene is transcribed in cultured rat astroglial cells, that IGF I binds to two types of IGF receptor and that IGF I activates type I IGF receptor tyrosine kinase activity and DNA synthesis in the same cell cultures. Thus, IGF I may act as an autocrine or paracrine growth promoter in the regulation of astroglial cell divisions *in vitro*. A formal proof for an involvement of the endogenously produced IGF I in stimulation of astroglial cell growth in culture would require that cell replication be arrested by addition of specific antibodies to IGF I or its receptor. Evidence for autocrine or paracrine mechanisms in growth regulation by IGF I has been obtained with other cell types, such as cultured human fibroblasts and porcine smooth muscle cells (Clemmons and Van Wyk, 1985), and rat chondrocytes *in vivo* (Nilsson *et al.*, 1986). In these two studies, production of IGF I was found to be stimulated by platelet-derived growth factor and growth hormone respectively. How IGF I gene expression is regulated in fetal rat astroglial cells is not known, but it has recently been shown that growth hormone administered into the cerebral ventricles of hypophysectomized rats increased the production of IGF I mRNA in brain (Hynes *et al.*, 1987). In conclusion, our results suggest that IGF I may play an important role in autocrine or paracrine stimulation of astroglial cell proliferation during normal brain development.

Materials and methods

Preparation of cultured astroglial cells

Primary cultures of mixed brain cells were prepared from brains of 8–10 fetal rats at 16–18 days of gestation (Schousboe, 1980). Cerebral cortices were stripped of meninges and washed in phosphate-buffered saline (PBS, pH 7.4) at room temperature. The tissue was dissociated by pressing through a nylon filter (80 μm) followed by washing, and resuspension in 200 ml of RPMI 1640 medium containing glucose (10 g/l) and FCS (20%). The cell suspension was divided in five 175-cm^2 culture flasks (NUNC). After 2–4 days of culture the medium was changed to remove dead and unattached cells, and thereafter medium was changed every 3 days. After 2–4 weeks the cells were trypsinized, transferred to culture flasks (NUNC) or multidishes (Falcon) and cultured for an additional

1–2 weeks. These secondary brain cell cultures were enriched for glial cells which were characterized as type 1 astrocytes by morphological and immunocytochemical criteria (Raff *et al.*, 1983; Burgess *et al.*, 1987). About 90% of the cells were flattened, fibroblast-like cells with cytoplasmic filaments which were positively stained with antibody to glial fibrillary acidic protein (GFAP) and peroxidase-coupled second antibody (data not shown). Of the residual 10% of cells, some were tentatively identified as oligodendrocytes by their morphology, whereas neuronal cells were not present.

Preparation and analysis of astrocyte RNA

Astrocyte cultures were prepared essentially as described above, except that the cultures were vigorously agitated to remove cells adhering to the astrocyte monolayer, and treated with cytosine arabinoside for 48 h to preferentially kill rapidly dividing cells such as fibroblasts (McCarthy and de Vellis, 1980). The final cultures contained >98% astrocytes, judged by GFAP immunoreactivity. Total cell RNA was prepared by the lithium chloride–urea method of Auffray and Rougeon (1980), and poly(A)$^+$ RNA selected by oligo(dT)–cellulose chromatography. Poly(A)$^+$ RNA (10 μg; estimated by absorbance at 260 nm) was denatured and electrophoresed on a 1% agarose gel containing formaldehyde (Maniatis *et al.*, 1982). After transfer to Gene Screen Plus nylon membrane (New England Nuclear) the RNA was hybridized with ^{32}P-labelled DNA probes specific for IGF I or IGF II. cDNA clones of human prepro IGF I (650 bp) and human prepro IGF II (800 bp) (Bell *et al.*, 1984) were generous gifts from J.Scott, Molecular Medicine Group, MRC Clinical Research Centre, Harrow, Middlesex, UK. Blots were hybridized and washed under conditions of high stringency, according to the manufacturer's recommendations, then dried and exposed to pre-sensitized Kodak XAR film at −70°C with an intensifying screen.

Partial purification of IGF receptors

Cells from 6–8 NUNC flasks (175 cm^2) were solubilized for 1 h at 4°C in 50 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, Triton X-100 (1.0% v/v), aprotinin (100 KIE/ml), bacitracin (1.8 mg/ml) and PMSF (0.17 mg/ml). After ultracentrifugation (100 000 *g* for 1 h at 4°C) the supernatant was applied to a WGA-agarose column. The glycoproteins were eluted with *N*-acetylglucosamine (0.3 M), and used for binding and protein kinase assays (Van Obberghen *et al.*, 1983; Gammeltoft *et al.*, 1984).

Receptor binding assays on solubilized receptors

Purified glycoproteins (5–10 μg of protein) were incubated with ^{125}I-labelled IGF I, IGF II or insulin (150 pM), and increasing concentrations of unlabelled peptides, for 15 h at 4°C in 100 μl of Hepes buffer (30 mM, pH 7.6) plus NaCl (30 mM), Triton X-100 (0.1% v/v) and BSA (4 mg/ml). The receptor-bound ^{125}I-activity was determined by precipitating with polyethylene glycol (0.25 g/ml) and human gamma globulin (3 mg/ml), and subtracting non-specific binding determined in the presence of unlabelled IGF I + IGF II or insulin (0.1 μM) (Van Obberghen *et al.*, 1983; Gammeltoft *et al.*, 1984, 1985).

Affinity labelling

Confluent cells in 25-cm^2 NUNC flasks were washed and incubated for 2 h at 20°C in Krebs–Ringer–Hepes buffer containing BSA and bacitracin with ^{125}I-labelled IGF I, IGF II or insulin (1 nM) in the presence or absence of unlabelled IGF I + IGF II or insulin (0.1 μM). After washing with cold PBS three times, the cells were incubated with KRH buffer (without BSA and bacitracin) at 4°C. Cross-linking was achieved by addition of disuccinimidyl suberate (0.1 mM) dissolved in dimethyl sulfoxide (2% v/v) for 15 min followed by addition of Tris buffer (10 mM, pH 7.4) and EDTA (1 mM). Labelled proteins were analysed by SDS–polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography (Gammeltoft *et al.*, 1985, 1987b).

Protein kinase assay

Purified glycoproteins (5 μg of protein) were incubated in 50 μl of 2 h at 15°C in the presence or absence of peptide at the indicated concentrations followed by addition of histone H2B (50 μg), [γ-^{32}P]ATP (sp. act. 370 MBq/mol), $MgCl_2$ (8 mM), and $MnCl_2$ (4 mM). After 15 min the reaction was stopped by adding SDS to 3% w/v, and boiling for 3 min. The labelled proteins were analysed by SDS–polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography. The radioactivity in phosphorylated IGF receptor β-subunits or histones was measured by Cerenkov counting of the corresponding gel fragments (Van Obberghen *et al.*, 1983; Gammeltoft *et al.*, 1985, 1987b).

Immunoprecipitation with antibody to phosphotyrosine

Antibody to phosphotyrosine (Ek and Heldin, 1984) was a generous gift of C.-H.Heldin, Ludwig Institute for Cancer Research, Biomedicum, Uppsala, Sweden. For immunoprecipitation the phosphorylation reaction was stopped by addition of EDTA (20 mM) and NaF (100 mM) followed by incubation with rabbit antibody to phosphotyrosine (80 μg IgG/ml) for 16 h at 4°C. IgG was precipitated by addition of protein A for 2 h at 4°C and centrifugation (10 000 *g*, 5 min). The pellets were washed twice with Hepes buffer (50 mM, pH 7.6) with NaCl (150 mM), Triton X-100 (0.1% v/v) and solubilized in SDS (3%). Labelled proteins were analysed by SDS–polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography.

Thymidine incorporation

DNA synthesis was determined by thymidine incorporation in subconfluent cells on Falcon 24-well multidishes. The cells were cultured for 24 h in medium without FCS, but with 0.2% defatted BSA, followed by culture for 24 h in the same medium containing [^{3}H]thymidine (3 μM) in the presence of different concentrations of FCS or peptides. After washing three times with PBS, proteins and DNA were precipitated by trichloroacetic acid (0.1 g/ml) followed by thorough washing with PBS and solubilization in NaOH (0.5 N), neutralization with HCl (0.5 N) and counting in a β-counter (Van Obberghen-Schilling *et al.*, 1983). The assay was optimized regarding the duration of culture in serum-free medium and labelling with [^{3}H]thymidine. Cultured astrocytes are viable for 1–2 weeks under serum-free conditions and the response to FCS or peptides is maximal 24–48 h after removal of serum. The incorporation of [^{3}H]thymidine increases with increasing periods of labelling up to about 24 h.

Peptides

Purified human serum IGF I and IGF II (Rinderknecht and Humbel, 1976) were generous gifts from R.E.Humbel, Biochemisches Institut, Universität Zürich, Switzerland. A partially purified preparation of IGF I and IGF II (30% purity) was used in incubations at concentrations >10 nM. This mixture contains approximately equal amounts of IGF I and II. Insulin was purchased from NOVO Research Institute, Copenhagen, Denmark.

Isotopes

[^{125}I]IGF I and [^{125}I]IGF II were prepared by the chloramine T method. [Methyl 1^1, $2^{1,3}$-^{3}H]thymidine, [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from the Radiochemical Centre, Amersham, UK.

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