Insulin-Like Growth Factor-I (IGF-I) and IGF Binding Protein-5 in Schwann Cell Differentiation

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Schwann cells (SCs) are the myelin producing cells of the peripheral nervous system. During development, SCs cease proliferation and differentiate into either a myelin-forming or non-myelin forming mature phenotype. We are interested in the role of insulin-like growth factor-I (IGF-I) in SC development. We have shown previously SCs proliferate in response to IGF-I in vitro. In the current study, we investigated the role of IGF-I in SC differentiation. SC differentiation was determined by morphological criteria and expression of myelin proteins. Addition of 1 mM 8-bromo cyclic AMP (cAMP) or growth on Matrigel matrix decreased proliferation and induced differentiation of SCs. IGF-I enhanced both cAMP and Matrigel matrix-induced SC differentiation, as assessed by both morphological criteria and myelin gene expression. Cultured SCs also express IGF binding protein-5 (IGFBP-5), which can modulate the actions of IGF-I. We examined the expression of IGFBP-5 during SC differentiation. Both cAMP and Matrigel matrix treatment enhanced IGFBP-5 protein expression and cAMP increased IGFBP-5 gene expression five fold. These findings suggest IGF-I potentiates SC differentiation. The concomitant up-regulation of IGFBP-5 may play a role in targeting IGF-I to SCs and thus increase local IGF-I bioavailability. **J. Cell. Physiol. 171:161–167, 1997.** © 1997 Wiley-Liss, Inc.

Insulin-like growth factor I (IGF-I) can serve as a differentiating factor for a variety of cell types including osteoblasts, myoblasts and neuroblasts (Pahlman et al., 1991; James et al., 1993; Ewton and Florini, 1995; Thrailkill et al., 1995). The biological effects of IGF-I are mediated via the type I IGF receptor (IGF-IR) (Jones and Clemmons, 1995; Schmid, 1995) and modulated by six IGF binding proteins (IGFBP-1 to IGFBP-6) (Holly and Martin, 1994; Jones and Clemmons, 1995). Expression of IGF-I, IGF-IR and IGFBP is tissue and cell-specific and regulated during development (Bondy et al., 1992; Cerro et al., 1993; Green et al., 1994). IGFBPs transport IGF-I to target tissues and modulate IGF-I interactions with IGF-IR in either a stimulatory or inhibitory manner (Holly and Martin, 1994; Jones and Clemmons, 1995).

IGFBP-5 is the most conserved IGFBP with 97% identity between human and rodent homologues (Kou et al., 1994). IGFBP-5 can associate with cell surface proteins (Andress, 1995; Jones and Clemmons, 1995) and bind to components of the extracellular matrix (Jones et al., 1993; Clemmons et al., 1994). While IGFBP-5 may have IGF independent effects (Andress and Birnbaum, 1992; Jones et al., 1993; Jones and Clemmons, 1995), it also can potentiate IGF-I action. For example, in fibroblasts, IGFBP-5 binds to the extracellular matrix and augments IGF-I action (Jones et al., 1993).

We are interested in the role of IGF-I in peripheral nervous system myelination. Schwann cells (SCs) are the myelin-forming cells in the peripheral nervous system. Differentiating, myelin-forming SCs express myelin specific proteins, including protein 0 (P0), myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and P_2 (Trapp and Quarles, 1984; Trapp et al., 1984) and form a relationship with axons. In vivo, axonal contact is required for the induction of SC differentiation (Knight et al., 1993), yet, the cellular signals underlying axonal regulation of SC differentiation and myelination are not understood. In vitro, agents which elevate intracellular cAMP level, such as forskolin and cAMP analogues, mimic axonal effects in cultured SCs to alter SC morphology and activate the expression of myelin proteins including P0 and MBP (Jessen and Mirsky, 1991; Morgan et al., 1991). Similarly, SC differentiation requires the presence of a SC basement membrane (Bunge et al., 1989) and, in vitro, SCs undergo phenotypic differentiation when grown on substrata of basement membrane components such as Matrigel matrix (Yoshino et al., 1990).

We recently reported that IGFBP-5 is expressed in

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developing rat SCs (Cheng et al., 1996b). Additionally, after peripheral nerve injury in adult rodents, we found that invading macrophages produce IGF-I and activated SCs express both IGF-IR and IGFBP-5 (Cheng et al., 1996a). In vitro, we discovered that undifferentiated SCs express IGF-IR and IGFBP-5 (Cheng et al., 1996a) and that IGF-I protects SC secreted IGFBP-5 from proteolysis (Cheng et al., 1996a). These data have led us to speculate that during development or after injury, Schwann cells secrete IGFBP-5, which may increase local IGF-I bioavailability (Cheng et al., 1996a). We believe IGF-I, in turn, may enhance Schwann cell differentiation and myelination. In the current study, we address these ideas and propose a role for IGF-I and IGFBP-5 in Schwann cell differentiation.

MATERIALS AND METHODS

Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco BRL (Gaithersburg, MD) and fetal bovine serum (FBS) from Hyclone (Logan, UT). Other tissue culture supplies were obtained from Corning Glass Works (Corning, NY) and Costar (Cambridge, MA). Restriction enzymes were from Gibco BRL and New England Biolabs (Beverly, MA). ^{32}P-dCTP was obtained from Amersham (Arlington Heights, IL). Recombinant human IGF-I was a generous gift of Cephalon (West Chester, PA) and was stored in 100 mM acetic acid in −80°C until use. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Antibodies

The anti-IGFBP-5 antibody (Upstate Biotechnology, Lake Placid, NY) used in this study is a polyclonal antisera raised against immunoaffinity purified IGFBP-5 from the conditioned media of transfected CHO cells. This antisera recognizes IGFBP-5 from human and rat. The cross-reactivities with other binding proteins are as follows: IGFBP-1, <0.5%; IGFBP-2, <0.1%; IGFBP-3, <0.1%; and IGFBP-4, less than 0.5%. The monoclonal anti-MBP antiserum (Boehringer Mannheim, Indianapolis, IN) recognizes residues 130–137 of MBP from human, chimpanzee, monkey, rabbit, bovine, and rat. The anti-P0 antiserum (P07) is a gift from Dr. Michael Shy, Wayne State University, Detroit, MI and was originally developed by Dr. J. J. Archelos, Julius-Maximilians-Universität Wurzbürg, Wurzbürg, Germany.

cDNA probes

The rat IGFBP-5 cDNA was received from Dr. Shunichi Shimasaki, The Whittier Institute, La Jolla, CA. A 300-bp rat IGFBP-5 cDNA, which encodes portions of the mature peptide, in pBluescript SK+ plasmid was generated by *Sac*II and *Hin*dIII digestion. The cDNA probes were purified with a Magic PCR Prep DNA Purification Kit from Promega (Madison, WI).

Cell culture

MT4H1 rat SC (SC) were provided kindly by Dr. Gihan Tennekoon, Children's Hospital of Philadelphia, Philadelphia, PA. These cells have been transfected with simian virus 40 (SV 40) large T antigen under the control of the metallothionine promoter (Tennekoon et al., 1987). Expression of SV 40 large T antigen is suppressed in the absence of zinc. Under these conditions, the cells behave like primary SCs expressing myelin genes and undergoing myelination when cocultured with sensory neurons (Tennekoon et al., 1987; Peden et al., 1990). In the presence of forskolin, MT4H1 SCs express a similar phenotype to primary SCs. We previously reported IGF-I is a mitogen for MT4H1 SCs and demonstrated these cells express both the type I IGF receptor and IGFBP-5 (Cheng et al., 1996a). MT4H1 cells were maintained in DMEM containing 10% FBS, 100 μM $ZnSO_4$, and 100 μg/ml G418 at 37°C in a humidified atmosphere with 10% CO_2. When reaching 80% confluency, cells were subcultured with trypsin-ethylenediamine tetraacetic acid (EDTA) into T75 flasks. In some experiments, cells were grown in defined glial cell medium, designated G3, containing 50 μg/ml transferrin, 10 ng/ml biotin, and 30 nM selenium in DMEM (Cheng et al., 1996a).

Morphological studies of SC differentiation

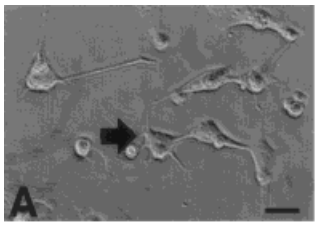
Cells were washed twice and plated into Lab-Tek chamber slides (Nunc, Naperville, IL) with DMEM containing 0.3% FBS at a density of 3×10^4 cells/cm^2 in the presence of increasing concentrations of (0, 1, 3, 10 nM) IGF-I in the presence or absence of 1 mM 8-bromo cAMP. In some studies, cells were plated on Growth Factor Reduced-Matrigel matrix from Collaborative (Bedford, MA). After 24 hr, the morphology of cells was examined under a phase-contrast microscope.

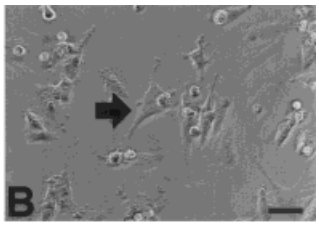
Northern analysis

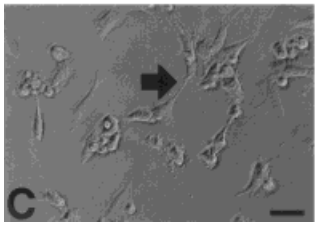
SC were plated (3×10^6 cells/T75 flask) in DMEM and 10% FBS. After 2 days, the medium was removed, cells were rinsed twice in DMEM, and placed in G3 media with 10 nM IGF-I in the presence or absence of 1 mM 8-bromo cAMP. After 24 hr, the culture media was collected and the cells harvested for RNA using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). Total RNA (10 μg) was separated by size in formaldehyde-agarose gels and transferred to nylon nytran membranes (Schleicher and Schuell, Keene, NH). Membranes were hybridized with ^{32}P-dCTP labeled ($0.1–10 \times 10^8$ cpm/μg) cDNA probes for rat IGFBP-5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Individual nytran membranes were hybridized by stripping and reprobing, as described in the manufacturer's instructions (Schleicher and Schuell, Keene, NH). Relative optical density for each hybridization was determined densitometrically by averaging several exposures in the linear range of the film. These values were expressed as a percentage of the G3 control and were divided by the percentage of control values similarly obtained from GAPDH hybridization (Cheng et al., 1996a).

Immunoblotting

Protein (50 μg) of concentrated conditioned media (for IGFBP-5 immunoblots) or whole cell lysates (for P0 immunoblots) were mixed at a 5:1 ratio with 10× SDS-sample buffer [100 mM Tris (pH 8), 10 mM EDTA, 10% SDS, 100 mM dithiothreitol, 0.1% bromphenol blue, and 20% glycerol] and boiled for 3–5 min. Samples were separated by SDS-PAGE (12.5%) and electrophoretically transferred to nitrocellulose. Nitrocellulose membranes were blocked with 5% nonfat dried milk in TBST [20 mM Tris, 0.16 M NaCl, and 0.10% Tween-20 (pH 7.4)]. The membranes were then incu-







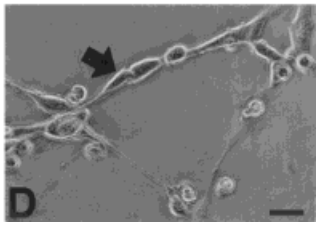
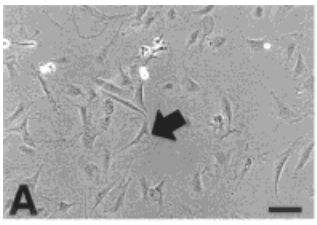
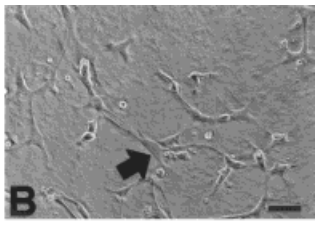
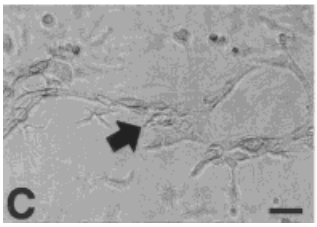


Fig. 1. The effects of 8-bromo cAMP and IGF-I on SC morphology. **A:** SCs extended processes and became smaller polygonal cells after 24 hr of treatment with 1 mM 8-bromo cAMP (arrow). **B:** Differentiating SCs clumped together after treatment with 1 nM IGF-I + 1 mM 8-bromo cAMP (arrow). **C:** IGF-I (3 nM) with 1 mM 8-bromo cAMP further promoted SC clumping and process extension (arrow). **D:** SCs became very slender bipolar cells that bundled together to form fascicles (arrow) in the presence of 10 nM IGF-I + 1 mM 8-bromo cAMP. Data are from one of three representative experiments. A and D, bar = 25 μM. B and C, bar = 50 μM.







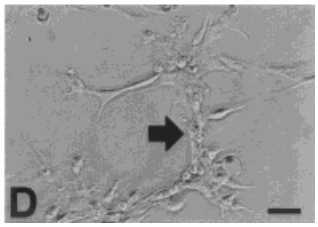


Fig. 2. The effects of Matrigel matrix and IGF-I on SC morphology. **A:** When plated on Matrigel-coated plates, SCs became polygonal and extended processes (arrow). **B:** In the presence of 1 nM IGF-I, SCs cultured on Matrigel-coated plates became slender with long processes. The cells tended to adhere to each other (arrow). **C:** IGF-I (3 nM) further promoted Matrigel-coupled SC aggregation and process elongation (arrow). **D:** SCs on Matrigel formed fascicles (arrow) with 10 nM IGF-I. Data are from one of three representative experiments. Bar = 50 μM.

bated for 2 hr with IGFBP-5 antiserum (1:1000) or anti-P0 (1:1000), in TBST + 5% milk, washed extensively during a 30-min period with TBST, and then incubated for 1 hr with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:7500, Santa Cruz Biotechnology) for IGFBP-5 or horse anti-mouse (1:1000, Santa Cruz Biotechnology) for P0 in TBST + 5% milk. After 40 min of extensive washing, nitrocellulose membranes were incubated with enhanced chemiluminescence reagents (Amersham). Bound protein-antibody complexes were visualized by autoluminography on reflection-NEF film (Du Pont, Boston, MA).

RESULTS

8-bromo cAMP and matrigel matrix induce SC differentiation

SCs express different phenotypes while undergoing differentiation (Yoshino et al., 1990; De Deyne et al., 1994). We and others have observed that undifferentiated MT4H1 SCs have a flattened, round morphology when cultured on plastic tissue culture dishes (Yoshino et al., 1990; De Deyne et al., 1994). Upon differentiation, SCs assume a distinct morphology, form polygonal and/or tubular aggregates and extend cellular processes (Yoshino et al., 1990; De Deyne et al., 1994). We initially induced SC differentiation using two known SC differentiating factors: the cAMP analogue, 8-bromo cAMP and Matrigel matrix (Knight et al., 1993). After treatment with 1 mM 8-bromo cAMP for 24 hr, SCs became smaller, polygonal cells and extended processes (Fig. 1A). To test the effect of IGF-I on cAMP induced morphological changes, SCs were treated with increasing concentrations of IGF-I in the presence of 1 mM 8-bromo cAMP. With the addition of 1 nM IGF-I, SCs began to group together and form aggregates, with long cellular processes (Fig. 1B). Higher concentrations of IGF-I enhanced the morphological changes. IGF-I (3 nM) treatment induced more cell aggregation (Fig. 1C), whereas in the presence of 10 nM IGF-I, SCs assumed a slender bipolar configuration (tubular aggregates) with long processes. Cells also formed colonies with fascicles consisting of bundles of long cell processes radiating out of each colony connecting to neighboring colonies (Fig. 1D).

We also used the artificial basement membrane matrix, Matrigel matrix, to induce SC differentiation. SCs plated on Matrigel changed from a round, flattened morphology to a more bipolar or triangular configuration (Fig. 2A). SCs became elongated after the addition of 1 nM IGF-I (Fig. 2B) whereas 3 nM IGF-I treatment promoted the formation of cell fascicles and the extension of processes (Fig. 2C). In the presence of 10 nM IGF-I, SCs formed similar structures to the cells treated with IGF-I and 8-bromo cAMP (Fig. 2D). Thus, IGF-I affected SC morphology in at least two ways: by promoting cell clustering and by facilitating the extension of processes. Essentially, all SCs underwent the morphological changes depicted in Figs. 1 and 2.

Differentiation not only induces changes in SC morphology but also activates the expression of myelin proteins including P0 and myelin basic protein (MBP). To quantitate enhanced SC differentiation, we examined P0 protein expression. After incubation in the presence or absence of 8-bromo cAMP and/or 10 nM IGF-I, SC whole cell lysates were collected and the extracted proteins were separated by SDS-PAGE followed by P0 immunoblotting procedures. P0 immunoblots demonstrated the presence of the 30 kD P0 protein (Fig. 3). IGF-I (10 nM) enhanced P0 expression in comparison with untreated cells or cells treated with 1 mM 8-bromo cAMP alone (Fig. 3). Maximal P0 protein expression was detected in cells treated for 2 days with both 8-bromo cAMP and 10 nM IGF-I (Fig. 3). In parallel,

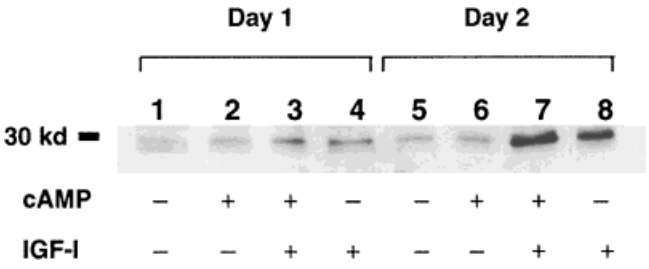


Fig. 3. IGF-I enhances P0 expression. P0 immunoblots were prepared from whole-cell lysates of SCs cultured in 1 mM 8-bromo cAMP ± 10 nM IGF-I. IGF-I (10 nM) alone promoted P0 expression (lanes 4 and 8) with maximal enhancement by day 2 (lane 8). 8-bromo cAMP enhanced P0 protein expression only in the presence of 10 nM IGF-I (compare lanes 2 and 6 with lanes 3 and 7, respectively). Data are from one of three representative experiments.

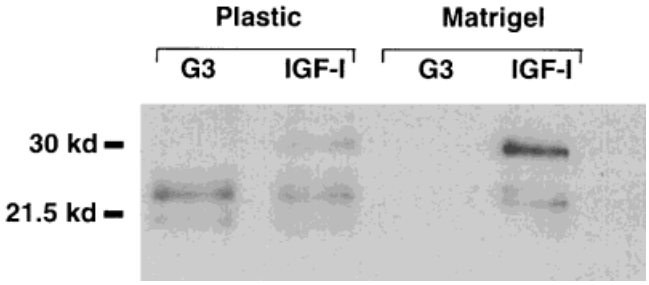


Fig. 4. Matrigel matrix promotes SC IGFBP-5 expression. IGFBP-5 immunoblots were prepared using concentrated conditioned media from SCs grown in G3 media on Matrigel-coated or plastic plates ± 10 nM IGF-I. There was no 30-kD IGFBP-5 signal detected under either plating conditions in the absence of IGF-I. In the presence of 10 nM IGF-I, the level of IGFBP-5 in the media of SCs cultured on Matrigel-coated plates was higher than that from SCs grown on plastic uncoated plates. Data from one of three representative experiments.

we observed low amounts of MBP immunoreactivity in untreated SCs. Treatment with 1 mM 8-bromo cAMP modestly enhanced the expression of MBP immunoreactive protein while the most intense immunofluorescence was observed in cells treated with both cAMP and IGF-I (data not shown).

IGFBP-5 expression is increased with SC differentiation

SCs express IGFBP-5 (Cheng et al., 1996a); interestingly, IGFBP-5 is enhanced during differentiation in multiple cell types and may play a role in the maintenance of a differentiated phenotype (Birnbaum and Wiren, 1994; Ewton and Florini, 1995; Rotwein et al., 1995). In the current study, we examined the levels of IGFBP-5 produced by SCs upon differentiation. Conditioned media of SCs cultured on Matrigel-coated plates were collected and concentrated, and equal amounts of protein were separated by SDS-PAGE followed by IGFBP-5 immunoblotting. As we have previously observed (Cheng et al., 1996a), secreted IGFBP-5 was degraded in the absence but protected in the presence of IGF-I. Matrigel coating significantly enhanced the expression of IGFBP-5 in the presence of 10 nM IGF-I (Fig. 4).

Similar results were observed with cAMP treatment. SCs were incubated in defined media in the presence or absence of 1 mM 8-bromo cAMP and/or 10 nM IGF-I. Conditioned media were collected and concentrated, and proteins were separated by SDS-PAGE followed by IGFBP-5 immunoblotting. As in our previous experiments, we again observed that secreted IGFBP-5 was degraded in the absence but protected in the presence of IGF-I. 8-bromo cAMP (1 mM) significantly enhanced the expression of IGFBP-5 in the presence of 10 nM IGF-I (Fig. 5A). We then examined the effects of two additional agents that elevate intracellular cAMP, forskolin and dibutyryl cAMP, on IGFBP-5 expression. These agents had the same effects as 8-bromo cAMP and enhanced IGFBP-5 expression in SC conditioned media in the presence of IGF-I (Fig. 5B).

We then asked if the effects of cAMP on IGFBP-5 protein expression was secondary to enhanced IGFBP-5 gene expression. Northern analysis was performed using RNA isolated from SCs cultured under the same conditions as the immunoblot experiments. As we have previously reported, IGF-I had no effect on IGFBP-5 gene expression (Cheng et al., 1996a). However, addition of cAMP increased IGFBP-5 gene expression fivefold (Fig. 6).

DISCUSSION

The IGF family of ligands, receptors and binding proteins are essential for normal nervous system development (Carson et al., 1993; Liu et al., 1993) and instrumental in nervous system regeneration (Hansson, 1993). We are interested in how IGF family members affect the development and repair of the peripheral nervous system (Sullivan and Feldman, 1994; Cheng et al., 1996a,b). Although these are separate processes, each is dependent on successful myelination of axons by differentiated SCs (Trapp et al., 1981, 1984; Trapp and Quarles, 1984; Owens and Bunge, 1990; Jessen and Mirsky, 1991). In the current study, we examined the role of IGF-I and IGFBP-5 in SC differentiation, as a first step in understanding the role of IGF family members in peripheral nervous system biology.

We have shown previously that IGF-I is a mitogen for newly cultured SCs (Cheng et al., 1996a). However, once SCs have begun to mature, we found that IGF-I assumed a new role, that of a differentiating factor. After treatment with cAMP, IGF-I enhanced SC differentiation. Markers of SC differentiation included morphological changes and enhanced P0 and MBP expression. In parallel, De Deyne and colleagues have reported (1994) that SCs respond to cAMP by assuming a differentiated morphology characterized by reorganization of microtubules. In our studies, IGF-I not only enhanced this process but also promoted cell clustering and enhanced P0 and MBP expression. Interestingly, cell clustering is important for P0 expression (Knight et al., 1993). Consequently, we do not know whether the increase in P0 expression was due to a direct effect of IGF-I signaling or secondary to the IGF-I coupled morphological changes.

Similar phenomena were observed when SCs were grown on Matrigel. Matrigel contains many of the components of the SC basement membrane including laminin, type I and type IV collagen (McGarvey et al., 1984). Laminin, the major component of Matrigel, promotes

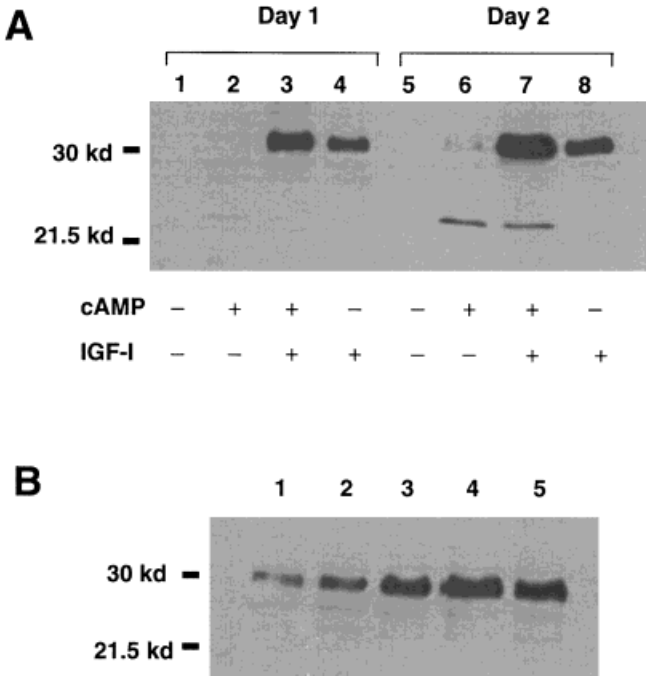


Fig. 5. cAMP analogues promote SC IGFBP-5 expression. IGFBP-5 immunoblots were prepared using concentrated conditioned media from SCs grown on plastic plates ± 10 nM IGF-I for 1 or 2 days. **A:** In the absence of IGF-I, IGFBP-5 was degraded in SC conditioned media (lanes 1, 2, 5, and 6). A 23-kD proteolytic fragment was visualized after 2 days. In the presence of IGF-I, 1 mM 8-bromo cAMP enhanced the levels of IGFBP-5 in SC conditioned media (compare lanes 3 and 7 with lanes 4 and 8). **B:** In the presence of 10 nM IGF-I, 50 μM forskolin (lane 3), 1 mM 8-bromo cAMP (lane 4), and 1 mM dibutyryl cAMP (lane 5) each increased IGFBP-5 expression in SC conditioned media in comparison with G3 control (lane 1) and 10 nM IGF-I alone (lane 2). Data from one of three representative experiments.

the adhesion and differentiation of cultured SCs and Schwannoma cells by interacting with integrin receptors (McGarvey et al., 1984; Baur et al., 1995). In the current study, IGF-I enhanced Matrigel-coupled morphological differentiation in SCs. Similar enhancement has been reported when SCs are cultured on Matrigel in 5% fetal bovine serum (Yoshino et al., 1990). Our results suggest that IGF-I is one of the serum factors that contributes to these previous reports of serum enhancement of Matrigel-mediated SC differentiation.

Like our observations with cAMP, we do not yet know the underlying mechanism of IGF-I enhancement of Matrigel coupled SC differentiation. However, both our own work in neurons (Leventhal and Feldman, 1996; Leventhal et al., 1996) and a recent report in breast cancer cells (Doerr and Jones, 1996) suggest an interaction between IGF-I receptor downstream signaling cascades and integrin-mediated intracellular signaling pathways, known to induce SC differentiation. We have reported recently that IGF-I, as well as integrin binding, stimulates the activation of two proteins involved in neuronal adhesion, focal adhesion kinase and paxillin (Leventhal and Feldman, 1996; Leventhal et al., 1996). In parallel, Doerr and Jones (1996) have reported that interaction of vitronectin with the $\alpha v\beta 5$ integrin or collagen with the $\alpha 2\beta 1$ integrin is required for IGF-I stimulation of cellular migration. Collectively, our results with SCs and neurons (Leventhal and Feldman, 1996; Leventhal et al., 1996) as well as the recent data of Doerr and Jones with breast cancer cells (Doerr and Jones, 1996) imply important cross-talk between IGF-I and integrin signaling cascades. We speculate that this link may be instrumental in IGF-I enhancement of SC differentiation.

We recently reported that cultured SCs primarily express IGFBP-5 (Cheng et al., 1996a). In the current study, we observed that treatment with either Matrigel or cAMP increased IGFBP-5 expression while mediating SC differentiation. Several other examples of IGFBP-5 enhancement with cellular differentiation imply this process may be an important component of normal tissue maturation. For example, enhanced expression of IGFBP-5 occurs during terminal muscle differentiation (James et al., 1993; Ewton and Florini, 1995; Rotwein et al., 1995; Duan et al., 1996). Although IGFBP-5 is not detectable in dividing myoblasts (Ewton and Florini, 1995), there is a marked increase in both IGFBP-5 mRNA and protein during myoblast differentiation (James et al., 1993; Ewton and Florini, 1995), coincident with onset of myogenin gene expression (Rotwein et al., 1995). Rotwein and colleagues recently found that the rapid upregulation of IGFBP-5 during muscle differentiation is mediated, in part, by a 156 nucleotide region of the IGFBP-5 promoter containing several potential binding sites for muscle cell-related transcription factors (Rotwein et al., 1995).

We also found that secreted IGFBP-5 was degraded in the absence but protected in the presence of IGF-I. These results parallel our previous reports that IGF-I protects SC secreted IGFBP-5 from proteolysis (Cheng et al., 1996a). In both our past (Cheng et al., 1996a) and current studies, proteolysis of IGFBP-5 resulted in a 23-kD degraded fragment. Several IGFBP-5 proteases have been isolated and characterized (Conover et al., 1993; Clemmons et al., 1994; Nam et al., 1994; Thrailkill et al., 1995). A set of 52- to 72-kD IGFBP-5 proteases isolated from osteoblast conditioned media are cation-dependent matrix metalloproteinases (Thrailkill et al., 1995). Interestingly, these IGFBP-5 proteases are upregulated during osteoblast differentiation (Thrailkill et al., 1995), suggesting IGFBP-5 proteases also are involved in IGF-I regulation of cellular differentiation.

The marked increase in IGFBP-5 expression during SC differentiation led us to examine the effects of cAMP analogues on IGFBP-5 gene expression. As has been observed previously in human fibroblasts (Duan and Clemmons, 1995), we found that treatment with cAMP analogues increased IGFBP-5 gene expression. Recently, an AP-2 binding site was identified on the IGFBP-5 promoter, suggesting a direct interaction between the cAMP-response element and the IGFBP-5 promoter (Duan and Clemmons, 1995). Our results corroborate this concept and imply that previous reports of increased IGFBP-5 gene expression by hormones (Camacho-Hubner et al., 1992) may actually be mediated via cAMP. Similarly, our results of enhanced IGFBP-5 gene expression during SC differentiation also parallel the upregulation of IGFBP-5 observed during myoblast (James et al., 1993; Ewton and Florini, 1995; Rotwein et al., 1995; Duan et al., 1996) and osteo-

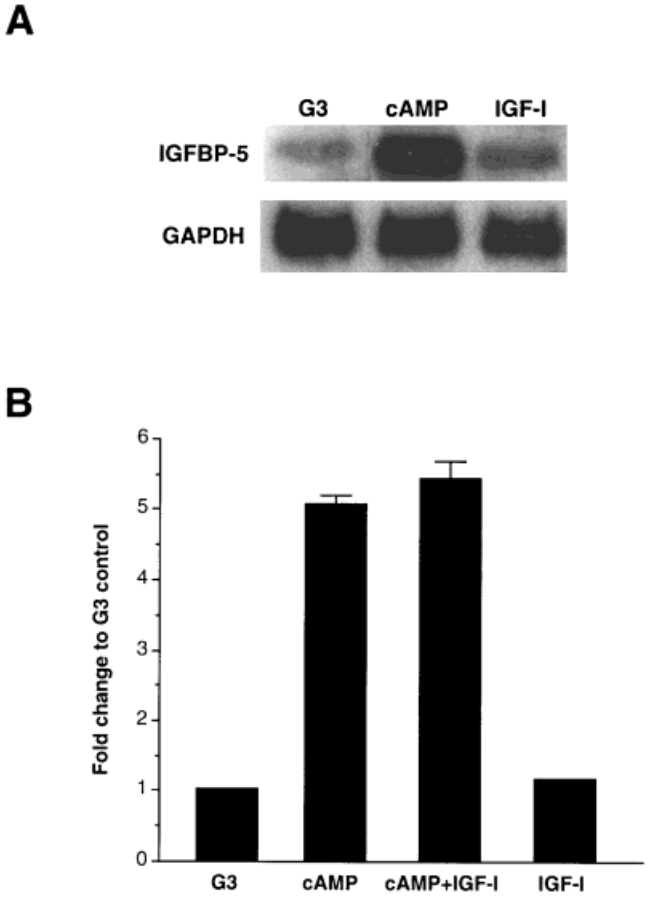


Fig. 6. cAMP enhances SC IGFBP-5 gene expression. SCs were cultured in G3 media as described in MATERIALS AND METHODS. After 48 hr of incubation, ±1 mM 8-bromo cAMP ± 10 nM IGF-I, total RNA was isolated, and Northern analysis on 20 μg RNA was performed using ^{32}P-labeled cDNA probes for IGFBP-5 and GAPDH as indicated in Materials and Methods. **A:** Autoradiograph from a Northern blot of IGFBP-5 and GAPDH mRNA, exposed for 2 days. 8-bromo cAMP (1 mM) promoted IGFBP-5 gene expression. Data are from one of three representative experiments. **B:** Densitometric analysis of IGFBP-5 mRNA. Autoradiographs from multiple exposures of the experiment shown in (A) and two additional experiments were quantitated using relative optical densities, as described in Materials and Methods. Values are means ± SEM of the ratio of IGFBP-5 mRNA relative optical density to GAPDH relative optical densities, expressed as percentage of G3 untreated control.

blast (Thrailkill et al., 1995) differentiation. However, we have not observed IGF-I regulation of SC IGFBP-5 gene expression in either the current study or our past work (Cheng et al., 1996a). This is in contrast to reports in aortic smooth muscle cells (Duan et al., 1996), rat FRTL-5 thyroid cells (Backeljauw et al., 1993), and osteoblasts (Dong and Canalis, 1995) but in agreement with work in human fibroblasts (Camacho-Hubner et al., 1992; Conover et al., 1995), U2 osteosarcoma cells (Conover et al., 1993), and breast carcinoma cells (Shemer et al., 1993). These differences emphasize that stimulation of IGFBP-5 gene expression by IGF-I is a cell-type specific event and discrepancies in regulation may reflect both cell-specific as well as species-specific regulation.

In summary, we believe terminal SC differentiation is augmented by the ability of SCs to secrete IGFBP-5, which, in turn, may bind SC basement membrane and increase local IGF-I bioavailability. IGF-I then serves to maintain the differentiated SC phenotype. In support of this concept, we have demonstrated an upregulation of SC IGFBP-5 gene and protein expression during SC differentiation as well as the ability of IGF-I to enhance this process. Further investigation into the function of IGF-I/IGFBP-5 in the peripheral nervous system may provide additional insights into the role of the IGF family in nervous system development and differentiation.

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