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A Gene Expression Study of the TGF-β Signaling Pathway Components in Differentiating PC12 Cells

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Abstract: The neuronal differentiation process has been studied in great detail from many aspects. In the PC12 system, NGF was found to induce neuronal differentiation as measured by neurite extensions and neuronal marker expression. This effect was seen to be mainly conducted through the Ras/MAPK pathway; however, additional pathways are suspected to play a significant role in this cellular response. One such pathway is Smad signaling – NGF was previously reported to induce the expression of the TGF-β signal, although the physiological relevance of this was not completely understood. In this study, we analyzed the expression profiles of several TGF-β pathway components, in an attempt to understand the potential role of this pathway in NGF-induced differentiation of PC12 cells.

Key Words: PC12, TGF-β, Smad, NGF, gene expression profiling, neuronal differentiation

Farklılaşan PC12 Hücrelerindeki TGF-β Sinyalleme Yolağı Elemanlarına Ait Bir Gen Anlatımı Çalışması

Özet: Sinir hücresi farklılaşması işlemi pek çok açıdan oldukça detaylı olarak çalışılmıştır. PC12 sisteminde, NGF'in sinir hücresi farklılaşmasını indüklediği hem nörit uzantıları hem de nöronal işaret anlatımı ölçülmek suretiyle gösterilmiştir. Bu etkinin ana olarak Ras/MAPK yolağı tarafından ortaya çıkarıldığı bilinmekle birlikte, başka yolakların da bu hücresel tepkide büyük rol oynadığından şüphelenilmektedir. Bu yolaklardan bir tanesi Smad sinyallemesidir – NGF'in daha önce TGF-β sinyalinin anlatımına sebep olduğu gösterilmiştir ancak bunun fizyolojik önemi tam olarak anlaşılamamıştır. Bu çalışmada biz çeşitli TGF-β yolak elemanlarının anlatım profillerini inceleyerek bu yolağın NGF-indüklü PC12 farklılaşmasındaki olası işbirliğini anlamaya çalışmaktayız.

Anahtar Sözcükler: PC12, TGF-β, Smad, NGF, gen anlatımı profillemesi, sinir hücresi farklılaşması

Introduction

Rat pheochromocytoma (PC12) cells derived from tumors of chromaffin cells are an excellent model system to study neuronal differentiation: when these cells are treated with nerve growth factor (NGF) or basic fibroblast growth factor (bFGF), neurite extensions are observed along with electrical conductivity and expression of neuronal markers, indicative of neuronal differentiation (1,2). On the other hand, when cells are exposed to epidermal growth factor (EGF), they simply proliferate and no differentiation response occurs (1,3). Although all these growth factors act through their respective receptor tyrosine kinases (RTKs) and activate mitogen-activated protein kinase (MAPK) pathway, they generate different responses in the cell (1-4).

NGF-induced differentiation of PC12 cells is also known to involve other signaling pathways such as Jun N-terminal kinase (JNK) or phosphoinositide 3-kinase (PI3K) pathways parallel to MAPK (2), and involves the transcription and secretion of a TGF-β (5,6). PC12 cells have been reported to show differentiation in response to a bone morphogenic protein-2 (BMP-2) signal, a member of the TGF-β superfamily, through activation of the p38 pathway (7,8). In addition, BMP proteins appear to potentiate NGF-induced differentiation in PC12 cells, and cotransfection of Smad proteins and upstream activators induce reporter gene activity in NGF-stimulated cells (9).

The TGF-β superfamily of signals act as potent inhibitors of growth in many tissues (10). TGF-β signals include TGF-β, activin, and BMPs – BMPs were originally

identified as cytokines required for bone and cartilage formation (10,11). However, it is now known that BMPs play an important role in neural development, and act as instructive signals for neuronal lineages (12). TGF-β itself has been shown to be a neurotrophic factor for the dorsal root ganglia (13).

The TGF-β family of signals act through serine/threonine kinase receptors Type I and Type II (10,11,14,15). Type-II receptors are constitutively active; upon binding of ligand, both receptors engage in heteromer formation, whereby the Type-II receptor activates the Type-I receptor, which in turn generates the signal-specific response further downstream (10). The Type-I receptor therefore can be considered to be downstream of the Type-II receptor, and confers specificity to the signal, by phosphorylating receptor-activated Smads, or R-Smads. Activated R-Smads then form complexes with common-mediator Smads, or co-Smads, inducing nuclear translocation, and target gene expression (10,11,14) (Table 2).

In this study, we attempted to understand which TGF-β pathway downstream components were regulated in uninduced or growth factor-induced cells, in order to gain insight into the possible involvement of this signaling pathway in the differentiation process.

Materials and Methods

Cell culture

PC12 pheochromacytoma cells (a generous gift from Dr. Reuven Stein, Tel Aviv University, Israel) were maintained as described previously (7). NGF 2.5 S, (Sigma N6009), EGF (Sigma E4127) and bFGF (Sigma, F0291) were used commonly as per the manufacturer's instructions.

Complementary DNA (cDNA) and Reverse Transcriptase Polymerase Chain Reactions (RT-PCRs)

Total cytoplasmic RNA was prepared using an RNAeasy kit (Qiagen, cat no 74104) using the manufacturer's instructions. Mainly, 1 mg RNA was used for each first strand cDNA synthesis reaction (M-Mu-LV-Rtase, Roche) as per the manufacturer's instructions, using random primers (Boehringer Mannheim).

The amount of cDNA used was normalized using GAPDH and the linear range of the polymerization reaction was determined. Typically the RT-PCR reactions were performed using 10-50 ng cDNA template in a 25 ml reaction with BioTaq polymerase at 50 ºC for 30 cycles. The RT-PCR products were resolved in 1.5%-2.5% Nu-Sieve agarose gels and were analyzed using QuantiOne imaging software (BioRad). The RT-PCR primers can be found in Table 1.

Multiple Alignment of Sequences

Multiple alignment of rat Smad sequences (Figure 1), as well as various TGF-β receptors, was carried out using the CLUSTAL W software available in the toolbox of the Biology Workbench (San Diego Supercomputing Center, http://workbench.sdsc.edu). The aligned sequences were then used to determine unique regions for each Smad family member protein for designing specific primers (see Table 1 for primer sequences).

Results

In order to carry out a semi-quantitative analysis of the expression of TGF-β pathway components, we first normalized the cDNAs using GAPDH expression as the standard as described in the Materials and Methods, and cycle 25 was determined to be within the linear range, making it possible to carry out semi-quantitative analysis of gene expression. For the assays, PC12 cells were either treated with differentiation signals, NGF or bFGF, or the proliferation signal EGF, for 5 days. Untreated cells were grown in parallel as controls. A typical GAPDH RT-PCR result for 5 days of growth factor treatment is shown (Figure 2A). H2O was included as a control against any external DNA contamination.

We then conducted a similar RT-PCR analysis with TGF-β1 primers, and observed a strong increase in TGF-β1 mRNA levels after 5 days of NGF exposure (Figure 2B). It should be noted that there was no TGF-β1 message detected when cells were stimulated with either EGF or bFGF (data not shown). We next analyzed BMP-4 expression. Although some mRNA message was present in untreated cells on day 5, the level of expression was increased at least 2- to 3-fold in the presence of the NGF signal (Figure 2C, lower band, 340 bp expected product; compare to Figure 2A). The level was unaltered in the presence of EGF, but there was a similar increase in the mRNA when cells were induced with another differentiation signal, bFGF (Figure 2C). Although the gene sequence of rat BMP-4 suggests a 340 bp PCR

Table 1. Sequences of primers used in the RT-PCR studies.

Primer	Sequence	Fragment size
GAPDH	F 5'AGACAAGCTTCAGAGCCACCCGGGACC	
	R 5'AGACTCTAGATCGGAGTCAACGGATTTGG	400 bp
TGF-β1	F 5'-AGACGAATTCATGGCCCTGGATACC	
	R 5'-AGACGGATCCTCAGCTGCACTTGCAGG	341 bp
BMP-4	F 5'-GTGACGTGGGCTGGAATG	
	R 5'-GTGGGGACACAGCAGGC	340 bp
TGF-β R I	F 5'-CCCTCACTAGATCGCC	
	R 5'-CCAACAATCTCCATGCG	475 bp
Activin RI	F 5'-CCAAGGTCAACCCGAAGC	
	R 5'-CGTCGGGAAGACCATCTG	133 bp
BMP-4 RI	F 5'-CTGGGAGCCTGTCTGTTC	
	R 5'-CTATTGTCCTGCGTAGC	330 bp
Smad 1	F 5'-CTGGCAGCAGCAGCAGC	
	R 5'-GGTGCCGTCATGTTCGTC	160 bp
Smad 2	F 5'-CAGAACTGCCGCCTCTGG	
	R 5'-CTAGTTTCTCCATCTTCACTG	145 bp
Smad 4	F 5'-CAGTGTCACCGGCAGATG	
	R 5'-CACCAATGCCAGCAGCAG	150 bp
Smad 5	F 5'-ATGACGTCAATGGCCAGCC	
	R 5'-ACTTGCTTGGCTGTCCTGG	200 bp
Smad 7	F 5'-CCATCTTCATCAAGTCCGC	
	R 5'-GGCTGCTGCATGAACTCG	175 bp

Table 2. Pathway-specific components in the TGF-β superfamily.

	Activin	TGF-β	BMP
Receptors	Activin-RI	TGFβR-I	BMPR-I
	TGFβR-II Activin-RII	TGFβR-II Activin-RII	BMPR-II
R-Smads	Smad 2, Smad 3	Smad 2, Smad 3	Smad 1, Smad 5, Smad 8
Co-Smads		Smad 4	
I-Smads	Smad 7	Smad 7	Smad 7, Smad 6

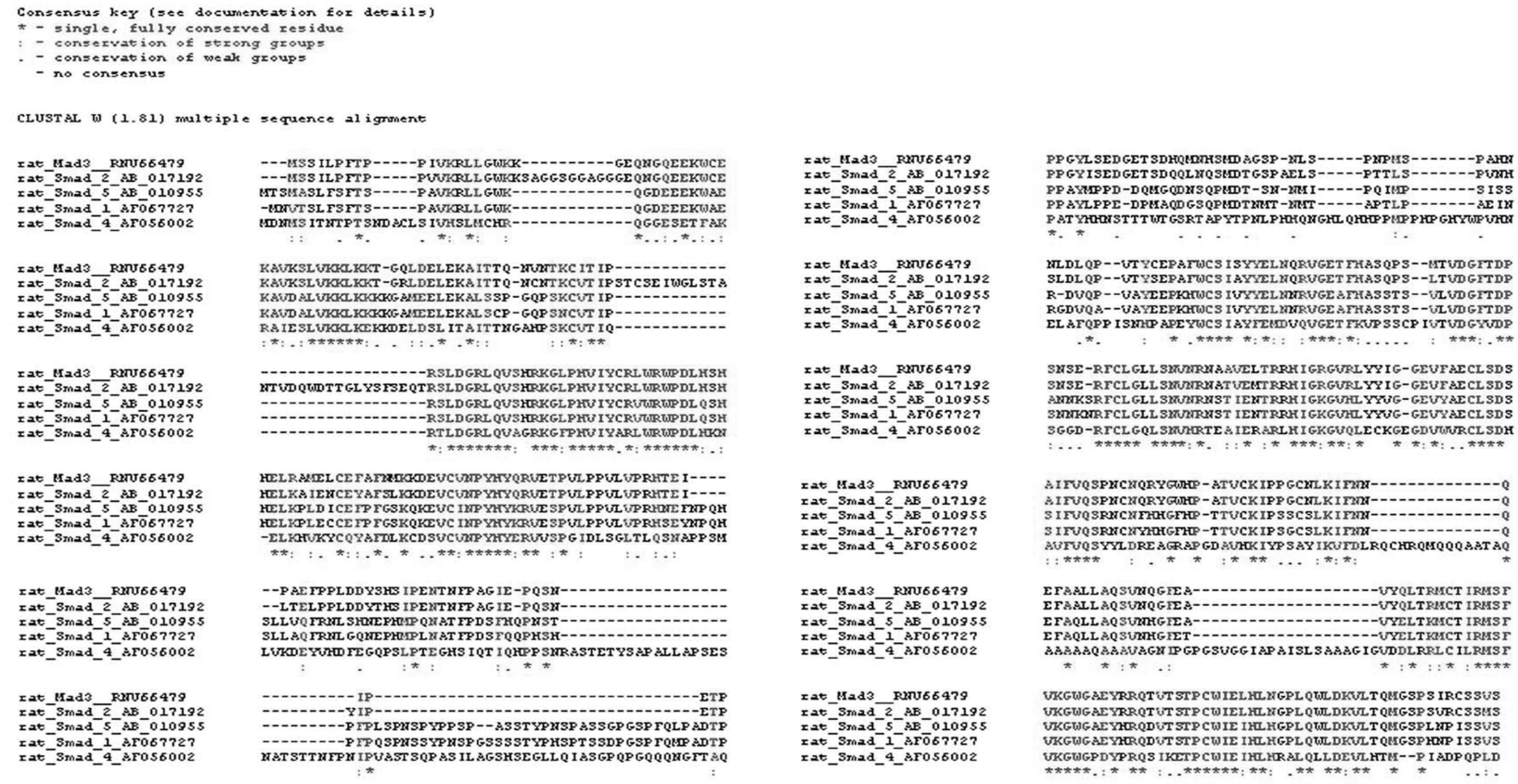

Figure 1. Alignment of rat Smad protein sequences. Protein sequences were obtained from GenBank (see Materials and Methods for GenBank accession numbers), and multiple aligned using the CLUSTAL W program.

product, we have observed a higher band of around 400 bp (Figure 2A, higher band). We suspect this could be an alternative splicing product, although such a splicing has not been reported for BMP-4 in the literature to date.

The expression of TGF-β1 and BMP-4 messages in NGF-induced differentiation leads to a conclusion of a possible autocrine loop whereby these signals augment the cell cycle withdrawal and differentiation. Therefore, we next analyzed the presence of signal-specific Type-I receptors. There appeared to be no change in the basal mRNA levels of TGFb-RI after NGF stimulation of 5 days (Figure 3A), nor at EGF or bFGF inductions (data not shown). The expression of an activin Type-I receptor was practically nonexistent in the absence of the NGF signal; however, when the cells were exposed to 5 days of NGF stimulation, a strong signal specific for activin-RI was observed (Figure 3B), while the induction was far less when the cells were stimulated with EGF, and there was no activin receptor expression in response to bFGF treatment (Figure 3B). In contrast, BMPR-I was downregulated under differentiation signals NGF and bFGF, while basal levels were maintained when cells were treated with EGF (Figure 3C, lower band). Similar to BMP-4 messages, we observed a doublet with the BMPR-I message, the upper band being around 50 bp longer than the expected PCR product. This band is likely to correspond to a possible alternative splicing product, although no such alternative splicing has been reported for rat BMPR-I previously.

We next investigated the expression levels of various Smad proteins that are specific for different TGF-β subfamilies. Smad 1 was previously shown to be

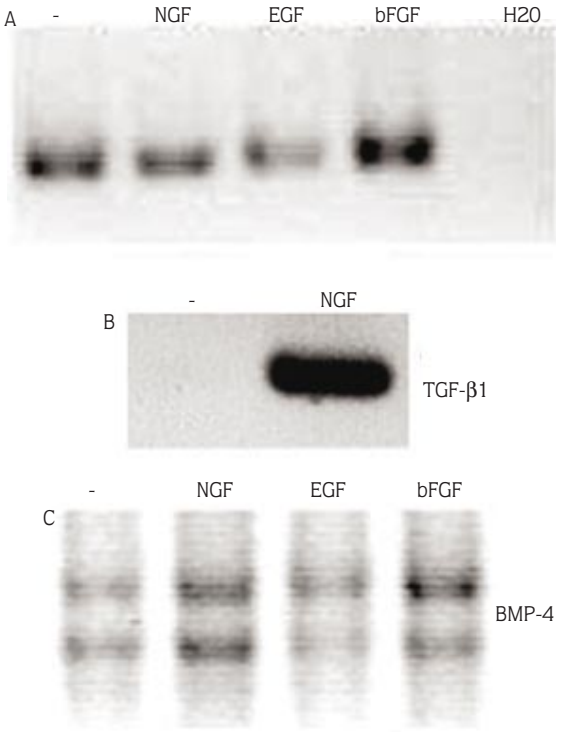

Figure 2. Upregulation of mature TGF-β and BMP-4 messages in NGF, bFGF and EGF-stimulated PC12 cells. (A) GAPDH normalization of RT-PCR for semi-quantitative analysis. cDNA was isolated from PC12 cells either untreated (-), or treated with NGF, EGF or bFGF for 5 days, and subjected to RT-PCR analysis. (B) RT-PCR analysis of TGF-β1 expression from cells either untreated (-) or NGF-treated PC12 cells. (C) RT-PCR analysis of BMP-4 expression from cells either untreated (-) or NGF-, EGF- or bFGF-treated PC12 cells. The images are representative of at least 2 independent experiments.

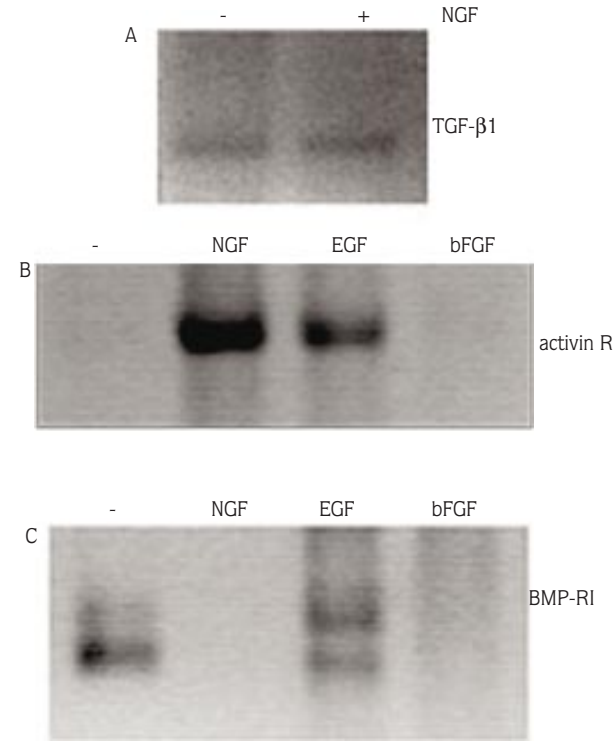

Figure 3. Expression profiling of TGF-β-, BMP-, and activin-receptor messages upon various growth factor treatments. (A) RT-PCR analysis of activin R-I expression from cells either untreated (-) or NGF-treated PC12 cells. (B) RT-PCR analysis of TGFbR-I expression from cells either untreated (-) or NGF-treated PC12 cells. (C) RT-PCR analysis of BMPR-I expression from cells either untreated (-) or NGF-, EGF- or bFGF -treated PC12 cells. The images are representative of at least 2 independent experiments.

phosphorylated in response to BMP stimulation of PC12 cells (16-18). However, we could not detect any change in the transcript levels with or without growth factor stimulation (Figure 4A), suggesting that Smad 1 is regulated mainly at the translational or post-translational level. Activin- and TGF-β-specific receptor-mediated Smad 2 and the common mediator Smad 4, on the other hand, were both found to be induced in response to NGF treatment (Figures 4B and 4C). Similar to activin receptor, Smad 4 and Smad 2 messages were also present in EGF-induced cells, while there is very little, if any, of either message in response to bFGF (Figures 4B and 4C).

In contrast, the inhibitor Smad 7 was inhibited by NGF after 5 days of treatment (Figure 4D). These results show that NGF appears to directly or indirectly affect the expression of Smad pathway components. Although the levels of Smad 7 message were decreased in bFGF- and EGF-induced cells, the decrease was not as pronounced as in NGF treatment (Figure 4D).

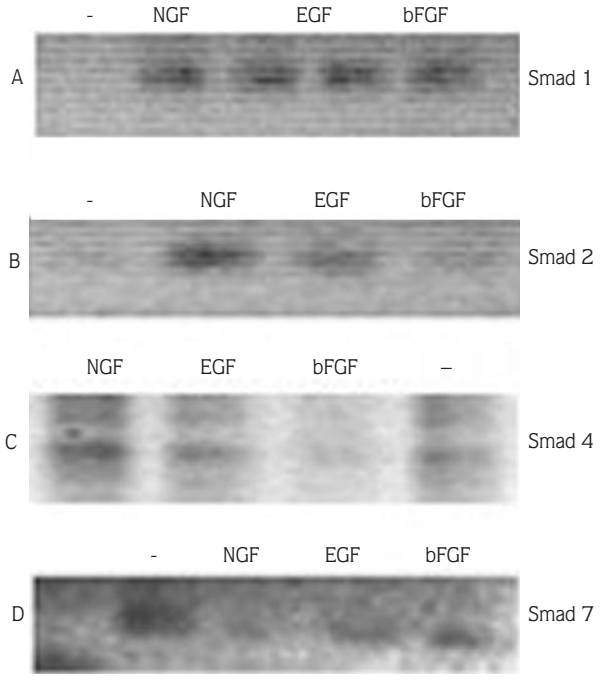

Figure 4. Expression profiling of rat Smad mRNA levels in NGF, bFGF and EGF-treated PC12 cells. (A) RT-PCR analysis of Smad 1 expression from cells either untreated (-) or NGF, EGF- or bFGF-treated PC12 cells. (B) RT-PCR analysis of Smad 2 expression from cells either untreated (-) or NGF-treated PC12 cells. (C) RT-PCR analysis of Smad 4 expression from cells either untreated (-) or NGF-treated PC12 cells. (D) RT-PCR analysis of Smad 7 expression from cells either untreated (-) or NGF-treated PC12 cells. The images are representative of at least 2 independent experiments.

Discussion and Conclusion

The aim of this study was to analyze whether the components of the TGF-β signaling pathway were induced at the transcriptional level in response to growth factors, mainly NGF. We confirmed that a mature TGF-β message was upregulated, and, similarly, a BMP-4 message was expressed in response to differentiation signals such as NGF or bFGF upon 5 days of treatment, while the basal levels did not change in response to EGF.

The expression of TGF-β1 and BMP-4 messages in NGF-induced differentiation leads to a conclusion of a possible autocrine loop; however, TGFbR-I expression was unaltered in the presence of NGF stimulus (Figure 4A), and BMP-4 receptor Type-I expression is greatly diminished by differentiation signals NGF and bFGF, suggesting that the secreted product acts on other cell types in a paracrine manner, rather than on the PC12 cells themselves. Interestingly, however, there was great induction of activin-receptor Type-I message in response to NGF (Figure 4B).

We also investigated the downstream signal transaduction proteins. The lack of increased Smad 1 transcription in the presence of growth factor stimulation (Figure 4A) indicates that the immediate activation response takes place with the existing Smad 1. Parallel to an increase in activin RI, we observed a transcriptional upregulation of Smad 2, which is specific to the activin and TGF-β signals, with a simultaneous increase in the common mediator Smad 4. On the other hand, the downregulation of the inhibitory Smad 7 by NGF indicates that a maintenance signal could be essential for the differentiation process initiated by NGF.

Another point that needs to be addressed in future experiments is the possible alternative splicing products for activin receptor and BMPR-I messages in rat cells, since doublets were observed in our gene expression studies, which show a similar pattern in response to different growth factors.

In order to fully understand and appreciate the mechanism of differentiation, these expression profiles need to be supported by detailed studies of all parallel-running pathways activated by NGF, and how these relate

to the activation of Smads in the presence or absence of the TGF-β superfamily of signals. We are currently investigating the potential activation of Smads by the Ras/MAPK pathway as an alternative to the typical Smad activation scheme.

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