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Regulation of neuronal differentiation and apoptosis by Brn-3 POU family transcription factors: - potential use in gene therapy (review)

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Summary
The Brn-3 sub group of POU family transcription factors has three members:- Brn-3a, Brn-3b and Brn-3c, all of which are expressed in distinct but overlapping groups of neuronal cells in the developing and adult nervous systems. Although these factors are closely related to one another, they have distinct functions. Thus, Brn-3a activates the expression of a number of different genes expressed in neuronal cells whereas Brn-3b represses their expression and inhibits activation by Brn-3a. These stimulatory effects of Brn-3a are paralleled by its ability to stimulate neurite outgrowth by neuronal cells and to protect them from apoptosis/programmed cell death. Thus Brn-3a may be of potential use in a gene therapy approach to human neurological diseases which involve either a failure of neurite outgrowth, such as spinal injury, or excessive neuronal cell death such as Alzheimer's or Parkinson's diseases.

I. Introduction

The POU (Pit-Oct-Unc) family of transcription factors was originally identified on the basis of a 150-160 amino acid domain which was common to the mammalian transcription factors Pit-1, Oct-1 and Oct-2 and the nematode regulatory protein Unc-86 (for reviews see Verrijzer and van der Vliet, 1993; Ryan and Rosenfeld, 1997). The POU domain constitutes the DNA binding domain of these factors and is divided into a POU-specific domain and a POU homeodomain which is homologous to that found in the homeobox family of transcription factors. These two sub domains are separated by a short linker region (**Figure 1**).

The four original POU factors all play critical roles in the regulation of gene expression particularly in neuronal cells. Thus, for example, Pit-1 was originally defined on the basis of its being essential for normal development of the pituitary gland and its absence results in congenital dwarfism (Anderson and Rosenfeld 1994) whilst the nematode *unc-86* mutation results in the absence of specific neuronal cell types in this organism (Desai et al., 1988).

The essential role of these original POU factors led a number of groups to attempt to identify novel members of the POU family which might play similarly critical roles in the regulation of gene transcription in specific cell types. This was done using the approach developed by He et al., (1989) who used degenerate primers derived from conserved regions at each end of the POU domain in a reverse transcriptase/polymerase chain reaction (RT/PCR) using RNA prepared from rat brain. Evidently, RNAs capable of encoding novel POU proteins will contain these conserved regions and will thus be amplified in the RT/PCR reaction.

II. Identification of the Brn-3 POU family transcription factors

We utilized this approach to identify the nature of the POU proteins which were expressed in the ND7 cell line which was obtained by the immortalization of dorsal root ganglion (DRG) sensory neurons (Wood et al., 1990). This was of particular interest since the ND7 cells can be induced to cease dividing and differentiate to a non-dividing neuronal phenotype bearing numerous neurite processes by

Figure 1. POU domain sequences of the four founder POU factors. Conserved amino acids are shown in black boxes and the final line shows a consensus sequence.

removal of serum from the growth medium or treatment with cyclic AMP (Wood et al., 1990; Suburo et al., 1992). Hence these cells can serve as a model for studies of neuronal differentiation.

In this study (Lillycrop et al., 1992) we isolated cDNA clones derived from both Oct-1 and Oct-2 which were known to be expressed in ND7 cells. In addition however, we also isolated cDNA clones derived from Brn-3, a factor initially identified by He et al., (He et al., 1989) as a novel POU protein expressed in the brain. Moreover, we also isolated cDNA clones derived from another POU factor which was much more closely related to Brn-3 than to any of the other POU factors. We therefore renamed the original Brn-3 factor Brn-3a and named our novel factor Brn-3b (Lillycrop et al., 1992). In subsequent studies, others have referred to Brn-3a as Brn-3.0 (Gerrero et al., 1993) and to Brn-3b as Brn-3.2 (Turner et al., 1994). In addition a third member of this family Brn-3c (Ninkina et al., 1993) has been isolated and is also known as Brn-3.1 (Gerrero et al., 1993).

The three Brn-3 factors constitute a closely related sub-family of POU factors which are encoded by distinct genes (Theil et al., 1993). In intial studies, all three Brn-3 POU factors were shown to be expressed predominantly in neuronal cells with distinct but overlapping patterns of expression being observed in the developing and adult brain (Lillycrop et al., 1992; Gerrero et al., 1993; Turner et al., 1994; Ninkina et al., 1993; Theil et al., 1993; Fedtsova and Turner, 1996). More recently however, expression of Brn-3a and Brn-3b has also been observed in the cervix (Ndisdang et al., 1998) and the breast and ovary where both Brn-3a and Brn-3b are able to regulate the activity of the oestrogen receptor (Budhram-Mahadeo et al., 1998).

Although the importance of the Brn-3 factors in the non neuronal cells where they are expressed is not yet clear, they have been shown to play a critical role in neuronal cell function and differentiation. Thus, Brn-3a expression defines the earliest post-mitotic neurons to form in the central nervous system (Fedtsova and Turner, 1996) and its inactivation in knock out mice results in widespread losses of sensory and motor neurons leading to death of the mice shortly after birth (McEvilly et al., 1996). Similar losses of neurons are also observed in mice lacking functional Brn-3b and Brn-3c, although these are more restricted in nature affecting specifically the visual and auditory systems respectively (Erkmann et al., 1996). This critical role for Brn-3c in the auditory system has recently been shown to apply in humans also, with cases of progressive deafness having been shown to result from mutation in the gene encoding Brn-3c (Vahua et al., 1998).

III. Expression of Brn-3a and Brn-3b in ND7 cell differentiation

To further investigate the role of Brn-3 factors in neuronal differentiation we used the ND7 cell system and investigated whether the expression of the Brn-3 factors changed during their differentiation. Most interestingly, we observed that the expression of Brn-3a increased from a very low level in the undifferentiated cells to a much higher level in the differentiated cells which were produced following exposure to cyclic AMP or removal of serum. Conversely, Brn-3b was expressed at a high level in the undifferentiated cells and at a much lower level in the differentiated cells whereas the level of Brn-3c remained unchanged upon differentiation (Lillycrop et al., 1992; Budhram-Mahadeo et al., 1994). This observation was of particular interest in view of the results of experiments in which expression vectors containing full length cDNAs encoding Brn-3a or Brn-3b were

transfected with a test promoter containing their binding site upstream of a reporter gene. Thus, in these experiments (Budhram-Mahadeo et al., 1994; Morris et al., 1994) Brn-3a activated the promoter whereas Brn-3b repressed it and interfered with activation by Brn-3a. Similar activation by Brn-3a and repression by Brn-3b has also been observed for a number of promoters derived from genes expressed in neuronal cells such as those encoding the synaptic vesicle protein SNAP-25 (Lakin et al., 1995) the intermediate filament protein -internexin (Budhram-Mahadeo et al., 1995) and the neurofilaments (Smith et al., 1997c). Hence, Brn-3a activates the expression of a number of neuronally expressed genes whilst, Brn-3b represses them. Moreover, during the process of ND7 cell differentiation the levels of the activator Brn-3a rise whilst the level of the Brn-3b repressor decreases.

IV. Role of Brn-3a and Brn-3b in ND7 cell differentiation.

Both Brn-3a and Brn-3b are transcription factors which evidently act by regulating the expression of other genes encoding specific proteins. Hence, the opposite activities of these factors and their opposite changes in expression pattern during ND7 cell differentiation raises the possibility that the changes in expression of these factors might actually be involved in the differentiation event. Thus, the rise in Brn-3a and fall in Brn-3b levels could result in the activation of a number of different target genes whose protein products were required for either the growth arrest or neurite outgrowth associated with differentiation. Thus, several of the genes which are activated by Brn-3a encode intermedeate filament proteins such as the neurofilaments and -internexin whilst SNAP-25 has been shown to be essential for the process of neurite outgrowth (Osen-Sand et al., 1993).

To test directly the possibility that the changes in Brn-3a/Brn-3b levels were involved in the differentiation event, we prepared ND7 cell lines which over expressed either Brn-3a, Brn-3b or Brn-3c under the control of a steroid-inducible promoter. In these experiments, the induction of Brn-3a expression by steroid treatment was able to induce the ND7 cells to put out neurite processes even in the absence of the normal stimuli such as serum removal or cyclic AMP treatment which are required to induce this effect (Smith et al., 1997a). This effect was not observed in the cells containing the steriod inducible Brn-3b or Brn-3c constructs or in ND7 cells which had been transfected with plasmid expression vector lacking any insert, all of which showed no response to steroid treatment, paralleling the lack of steroid effect on parental ND7 cells (Smith et al., 1997a).

Hence, the over expression of Brn-3a can indeed induce differentiation, as assayed by the outgrowth of neurite processes, in ND7 cells even in the absence of stimuli which normally induce it. This enhancement of neurite outgrowth in the cells expresssing Brn-3a was also accompanied by the enhanced expression of a number of different genes whose promoters had previously been shown to be activated by Brn-3a such as those encoding SNAP-25 (Smith et al., 1997a) and the neurofilaments (Smith et al., 1997c). Hence Brn-3a is able to activate the promoters of these genes both in transfection experiments and when the endogenous gene is in its natural chromatin structure in the cell lines.

Hence, the rise in Brn-3a which is produced by serum removal or cyclic AMP treatment of ND7 cells plays a direct role in the differentiation process by activating the expression of several different target genes allowing their protein products to then produce neurite

outgrowth. Interestingly, the cell lines over-expressing Brn-3b show a failure of neurite outgrowth even when stimulated by serum removal or treatment with cyclic AMP (Smith et al., 1997b). Hence, the effect of serum removal or cyclic AMP treatment on the outgrowth of neurite processes by ND7 cells is produced both by a rise in the activating transcription factor Brn-3a and by a corresponding fall in the level of the inhibitory transcription factor Brn-3b. This results in the activation of the appropriate target genes and produces neurite outgrowth.

V. Role of the Brn-3a POU domain

Interestingly, it has been shown that Brn-3a exists in two different forms which are generated by alternative splicing of its RNA (Theil et al., 1993) (**Figure 2**). Although both these forms contain the C-terminal POU domain they differ in that the long form of Brn-3a contains an additional 84 amino acids at the N-terminus of the protein which are absent in the short form. The relative proportions of these two forms are regulated during neuronal development and in response to specific stimuli (Liu et al., 1996). We therefore wished to determine whether both these forms would promote neurite outgrowth in ND7 cells. In fact, ND7 cell lines engineered to express either the long or the short form of Brn-3a were induced to put out neurite processes when Brn-3a expression was induced. Such induction of neurite processes was also observed in ND cell lines expressing only the isolated POU domain of Brn-3a indicating that this region of the protein is sufficient for this effect (Smith et al., 1997a). This parallels the ability of the isolated

Figure 2. Schematic diagram of the long and short forms of Brn-3a showing the genes and processes requiring only the C-terminal POU domain for induction and those which also require the N-terminal domain unique to the long form of the molecule.

POU domain of Brn-3a but not that of Brn-3b to activate the promoters of the SNAP-25 and neurofilament genes (Smith et al., 1997c; Morris et al., 1997b). Hence activation of the genes involved in neurite outgrowth and of neurite outgrowth itself requires only the C-terminal POU domain (**Figure 2**).

These effects of the POU domain of Brn-3a are not observed with the POU domain of Brn-3b which can neither induce neurite outgrowth or activate target genes. These differences in activity must be dependent upon one or more of the seven amino acid differences between the closely related POU domains of Brn-3a and Brn-3b (Lillycrop et al., 1992; Morris et al., 1994). The POU-specific domain is identical in Brn-3a and Brn-3b, the POU homeodomains differ by only a single amino acid and the remaining six differences are contained in the linker region between the two sub-domains which is poorly conserved between different POU proteins.

In fact, it is the single difference at position 22 in the POU homeodomain which controls the different activities of the two factors. Thus, alteration of the isoleucine at this position in Brn-3b to the valine found at the equivalent position in Brn-3a, converts Brn-3b into an activator of an artifical test promoter (Dawson et al., 1996) and of the SNAP-25 promoter (Morris et al., 1997a) and allows it to stimulate neurite outgrowth (Smith et al., 1997a). Conversely, mutant Brn-3a containing an isoleucine at this position represses the SNAP-25 promoter (Morris et al., 1997a) and inhibits neurite outgrowth (Smith et al., 1997b). Although the POU domain is the DNA binding domain of these factors, these effects are not dependent on any differences in DNA binding ability with wild type Brn-3a, Brn-3b and the mutant forms binding to DNA with equal affinity. Rather, position 22 has been shown to play a critical role in protein-protein interactions of other POU proteins (Lai et al., 1992) and it is likely therefore that the valine/isoleucine difference may control the ability of Brn-3a/Brn-3b to recruit co-activator or co-repressor molecules which are necessary for their effect on transcription.

VI. Role of Brn-3a in the regulation of apoptosis

The finding that neurite outgrowth and the expression of a number of Brn-3a target genes can be induced by the isolated POU domain of Brn-3a leads to the question of the function of the remaining regions of the protein and the reason why two different forms of the protein differing at the N-terminus are generated during neuronal development. To investigate this question we attempted to identify genes which were over expressed in the cell lines over expressing the long form of Brn-3a but not in those over expressing the short form of Brn-3a. In these experiments, we were able to show that the Bcl-2 gene was strongly over expressed in the cells overexpressing the long form of Brn-3a but not in those overexpressing the short form. Moreover, in co-transfection experiments the Bcl-2 promoter could be activated by the long form of Brn-3a but not by the short form (Smith et al., 1998). This finding parallels our previous observation that the activation of the -internexin promoter also requires the N-terminus of Brn-3a (Budhram-Mahadeo et a., 1995) and suggests that two classes of Brn-3a regulated genes exist with some genes being

activated by the POU domain alone and others requiring the region at the N-terminus unique to the long form of Brn-3a (**Figure 2**).

The regulation of Bcl-2 by Brn-3a is of considerable importance because of the known ability of Bcl-2 to protect both neuronal and other cell types from programmed cell death or apoptosis (White, 1996). We therefore investigated whether the ND7 cells overexpressing the long form of Brn-3a and Bcl-2 would be protected from stimuli which induce apoptosis using a model system in which apoptosis is induced in ND7 cells by removal of serum together with addition of retinoic acid (Howard et al., 1993). In these experiments a clear protective effect was observed in the cells overexpressing the long form of Brn-3a and Bcl-2 compared to the cell death observed in cells overexpressing the short form of Brn-3a, or Brn-3b or Brn-3c neither of which induces enhanced Bcl-2 levels. Similar protection by over-expression of Brn-3a was also observed when we introduced the Brn-3a expression vector into primary cultures of dorsal root ganglion neurons or trigeminal ganglion neurons indicating that these effects are not unique to ND7 cells (Smith et al., 1998) (**Figure 3**). Hence, the long form of Brn-3a but not the short form is able to up regulate Bcl-2 levels and protect cells from apoptosis (**Figure 2**).

VII. Potential use of Brn-3a in gene therapy procedures

Many human neurological diseases involve excessive cell death which can occur acutely as in stroke or chronically as in Alzheimer's or Parkinson's diseases. The ability of the long form of Brn-3a to prevent apoptotic death suggests that its over-expression may be of use in such diseases. Similarly, the ability of both the long and short forms of Brn-3a to induce neurite outgrowth could be of use in situations such as human spinal injury which involve a failure of neurite outgrowth following injury.

Such alterations of Brn-3a expression for therapeutic benefit could involve the use of pharmacological procedures to enhance endogenous Brn-3a expression or the use of gene therapy procedures to deliver an exogenous Brn-3a gene. Although many potential viral or non-viral means are available for such *in vivo* gene delivery we have concentrated on herpes simplex virus (HSV)-based vectors in view of the ability of this virus to establish latent infections specifically in neuronal cells (for review see Latchman, 1990). We have now constructed disabled HSV-based vectors which can safely and efficiently deliver genes to the central and peripheral nervous systems *in vivo* (Coffin et al., 1996; Howard et al., 1998).

The long form of Brn-3a has recently been introduced into this vector and we have shown that infection with this Brn-3a expressing virus can protect trigeminal ganglion neurons from apoptosis induced by withdrawal of nerve growth factor (Smith et al., 1998) (**Figure 3**). Hence this virus may respresent an effective means of elevating Brn-3a levels *in vivo* for therapeutic benefit.

Figure 3. Survival of trigeminal ganglion neurons infected with a recombinant herpes simplex virus vector expressing Brn-3a or control vector (c) either in the presence of nerve growth factor (+) or following its removal (-). Note the protective effect of infection with the Brn-3a expressing virus.

VIII. Conclusions

The experiments described here have shown that the Brn-3a transcription factor is a bi-functional factor which contains two domains capable of stimulating the expression of specific genes and thereby modifying neuronal phenotype (**Figure 2**). Thus, the C-terminal POU domain can alone activate the transcription of specific genes and promote neurite outgrowth with this effect being opposed by the Brn-3b transcription factor. In addition however, the N-terminal region of the protein is also able to activate the expression of specific genes such as the Bcl-2 gene and thereby protect neuronal cells against apoptosis whereas Brn-3b has no effect. These findings suggest therefore that Brn-3a is likely to have a critical role in the correct development of the nervous system by regulating both neuronal differentiation and the rate of apoptosis which plays a critical role in the proper development of the nervous system (Oppenheim, 1991). Moreover, the manipulation of its expression by gene therapy procedures may be of importance in the treatment of neurological disorders involving excessive neuronal death or a failure of neurite outgrowth.

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