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Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade

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ABSTRACT

The Bcl-2 protein has an anti-apoptotic effect in neuronal and other cell types. We show for the first time that the Bcl-2 promoter is activated by the neuronal survival factor nerve growth factor (NGF) and that this effect is dependent on a region of the promoter from –1472 to –1414. This activation requires the Rap-1 G protein and the MEK-1 and p42/p44 MAPK enzymes but is independent of other NGF-activated signalling pathways involving protein kinase A or protein kinase C.

INTRODUCTION

The process of programmed cell death (apoptosis) plays a key role in the development and functioning of a number of different organ systems such as the immune system and the nervous system and alterations in the amount of such cell death have been implicated in a number of different human diseases including autoimmune diseases and neurodegenerative diseases (reviewed in 1). The Bcl-2 protein has been shown to play a key role in the regulation of programmed cell death, being able to protect a wide variety of cell types, including B cells and neuronal cells, from apoptosis (reviewed in 2,3). In neuronal cells, for example, overexpression of Bcl-2 has been shown to enhance the survival of both central and peripheral nervous system neurons in culture as well as counteract the effects of a variety of apoptotic stimuli such as the withdrawal of neurotrophic factors (4,5). Similarly, overexpression of Bcl-2 in transgenic animals protects neurons from cell death during development, resulting in animals with increased numbers of neurons and also protects neurons in the adult from axotomy-induced cell death (6,7).

As well as being protected by overexpression of Bcl-2, it is clear that normal levels of Bcl-2 expression in neuronal cells play a key role in their survival during normal development when a high proportion of the neuronal cells which initially form, normally die by apoptosis (8). Thus, the inactivation of the Bcl-2 gene in knock-out mice results in losses of motor neurons, sympathetic and sensory neurons (9) and also impaired survival in cultured neurons derived from such mice (10). Similarly, reduction of Bcl-2 levels in cultured neurons using an anti-sense approach inhibits the enhanced survival normally observed on adding neurotrophic factors (11).

Therefore, overexpression of Bcl-2 results in enhanced neuronal survival even in the absence of neurotrophic factors whilst in its absence, neurons die even in the presence of such survival factors. It is, therefore, of great interest that treatment with the neurotrophic factor, nerve growth factor (NGF), has been shown to greatly enhance the level of Bcl-2 expression in mast cells (12), melanocytes (13) and the PC12 phaeochromocytoma cell line (14) which resembles sympathetic neurons and in cerebellar granule neurons (15). Moreover, the artificial overexpression of Bcl-2 in PC12 cells results in enhanced survival following NGF withdrawal (16) and, conversely, inhibiting the rise in Bcl-2 levels induced by NGF in melanocytes or PC12 cells prevents the normal anti-apoptotic effect of NGF treatment in these cells (13,14). Hence the protective effect of NGF treatment is dependent upon NGF-induced up regulation of Bcl-2.

These experiments suggest, therefore, a model in which NGF produces its effect on the survival of neuronal cells in the developing and adult nervous system, at least in part, via the stimulation of Bcl-2 expression. It is thus of critical importance to elucidate the mechanisms/signalling pathways by which such effects on Bcl-2 expression are achieved. Despite this and the intense attention devoted to the function of the Bcl-2 protein, relatively little work has been done on the mechanisms regulating *bcl-2* gene expression. Thus, some reports have appeared describing regulatory elements in the Bcl-2 promoter which regulate its expression in specific non-neuronal cell types (e.g. 17–20) and we have recently shown that the POU family transcription factor Brn-3a can activate the Bcl-2 promoter in neuronal cells resulting in a protective effect against apoptosis (21,22). However, no study has investigated the manner in which NGF activates Bcl-2 expression. Therefore, we have carried out a study aimed at directly investigating whether NGF can activate the Bcl-2 promoter and determining the mechanisms/signalling pathways by which it mediates this effect.

MATERIALS AND METHODS

Materials

2.5S NGF was obtained from Sigma. The specific MAPK inhibitors PD98059 (p42/p44) and SB203580 (p38) were from NEB and Calbiochem, respectively, and were used at concentrations of 50 and 10 μM, respectively. Cell culture media and serum were from Gibco BRL.

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Plasmid DNA

Bcl-2 promoter–reporter constructs containing various regions of the Bcl-2 promoter linked to the luciferase reporter gene have previously been described (17,20–22). The expression vectors for the constitutively active p42/p44 MEK-1 mutant (SS/DD) and the dominant negative p42/p44 MAPK mutants (MAPKTA and MAPKYF) were a kind gift of Dr J. Pouyssegur (23). The expression plasmid containing the constitutively active subunit of PKC delta was a kind gift of Dr M. Marber and Dr P. Parker. The PKI and constitutively active PKA vectors were a kind gift of Dr R. Maurer whilst the Rap-1 N17 construct was a kind gift of Dr P. J. Stork.

Cell culture and transfections

PC12 cells (24) were maintained in L15 medium supplemented with 10% (v/v) FCS and 1 mM glutamine. Transient transfections of PC12 cells were performed using the calcium phosphate procedure as described by Gorman (25). Transfections were carried out with 4×10^5 cells/well on 6-well tissue culture plates. For each transfection, a β-galactosidase expression plasmid was used as an internal control. Following calcium phosphate transfections, PC12 cells were glycerol-shocked and incubated for 16 h in media containing 1% horse serum. Cells were then treated with 2.5S NGF for 30 h. In some experiments, cells were pre-treated with appropriate kinase inhibitor for 1 h before addition of NGF. Cells were then harvested and assayed for luciferase activity according to the manufacturer's instructions (Promega). Results were normalised to β-galactosidase activity and by using relative protein content as determined by the Bradford protein assay (26).

RESULTS

Although treatment of PC12 cells with NGF has been shown to result in enhanced levels of Bcl-2 protein (14), no previous study had demonstrated a direct effect of NGF on the Bcl-2 promoter. We therefore transfected PC12 cells with a construct containing the 3934 bases upstream of the translational start site of the *bcl-2* gene linked to a luciferase reporter gene (17,20; Fig. 1). As shown in Figure 2, this construct (LB322) was ~6-fold inducible by NGF. To map the site of NGF responsiveness, we carried out similar experiments with several deletion constructs containing progressively less extensive upstream regions of the Bcl-2 promoter. In these experiments (Fig. 2) the construct containing 1642 bases of upstream sequence (LB329) was inducible by NGF to a similar extent to the full-length promoter whereas one containing 1281 bases (LB335) was virtually uninducible. This indicates that promoter elements between bases –1642 and –1281 are essential for inducibility of the Bcl-2 promoter by NGF.

Interestingly, this region contains a cyclic AMP response element (CRE) located between bases 1546 and 1537 which is involved in the induction of Bcl-2 expression during B-cell activation. It has previously been shown the CRE motifs in the c-*fos* (27) and calcitonin gene related peptide (28) promoters are involved in their inducibility by NGF. We therefore tested whether a construct containing a mutated CRE within the Bcl-2 promoter was impaired in its response to NGF. However, this construct (MUCRE) was as inducible by NGF as the corresponding promoter containing an intact CRE (LB334; Fig. 1). Moreover, none of the Bcl-2 promoter constructs showed a response to

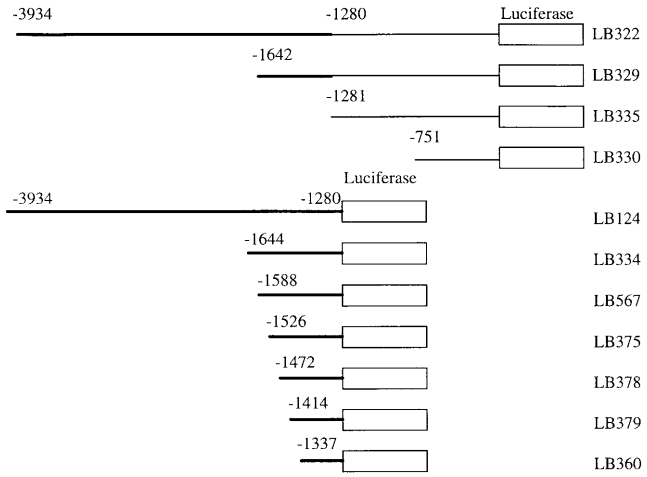

Figure 1. Structure of the Bcl-2 promoter constructs used in this study.

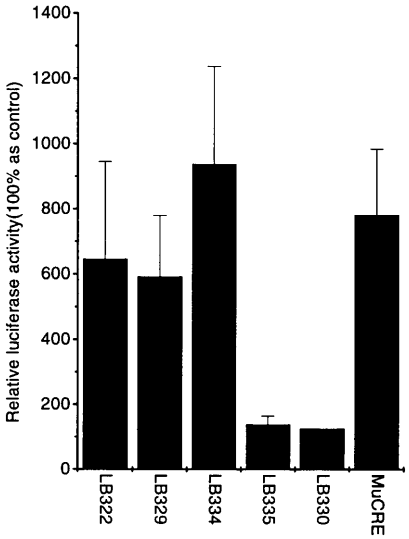

Figure 2. Activation of various Bcl-2 promoter constructs (see Fig. 1) by NGF. In all cases, promoter activity in the presence of NGF is compared to that of the same construct in the absence of NGF (set at 100%). Values are the mean of five experiments whose standard error is shown by the bars. MuCRE indicates the result with the LB334 construct further modified by mutation of the AMP CRE.

treatment of the PC12 cells with cyclic AMP (data not shown) indicating that the CRE in the Bcl-2 promoter does not function to produce a response to NGF or cyclic AMP in PC12 cells.

Having established that the region from –1642 to –1281 bases was involved in NGF inducibility but that this effect did not involve the CRE, we wished to map this NGF response element further. It has previously been demonstrated that Bcl-2 has two promoters, an upstream P1 promoter and a downstream P2 promoter which is located entirely downstream of base –1280. Therefore, to more precisely map the NGF element, we used a series of constructs (Fig. 1) containing only the P1 promoter upstream of base –1280 linked to progressively shorter regions of upstream sequence. In these experiments (Fig. 3) a construct containing the region from base –3934 to base –1281 was readily

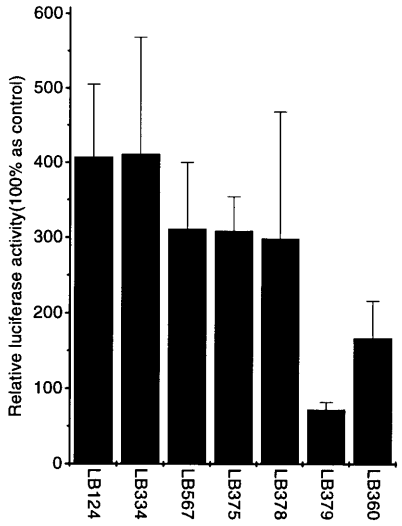

Figure 3. Activation of various Bcl-2 promoter constructs (see Fig. 1) by NGF. In all cases, promoter activity in the presence of NGF is compared to that of the same construct in the absence of NGF (set at 100%). Values are the mean of five experiments whose standard error is shown by the bars.

inducible by NGF in PC12 cells confirming that this effect could be demonstrated for the isolated P1 promoter. In a deletion analysis of this promoter (Fig. 3) NGF inducibility was observed for all constructs down to that which contained the region from –1472 to –1281 (LB378) but was not observed for the next shortest construct containing the region from –1414 to –1281 (LB379). This indicates, therefore, that the region from –1472 to –1414 is critical for the inducibility of the Bcl-2 promoter by NGF. This region is distinct from, for example, the region which mediates induction of Bcl-2 during B-cell activation (20) and from the region of the promoter which we have previously shown renders it responsive to the POU family transcription factor Brn-3a (21,22).

We next wished to study the signalling pathways by which NGF activates the Bcl-2 promoter. We therefore investigated the effect of specifically inhibiting or activating various kinase-mediated signalling pathways upon the activation of the Bcl-2 promoter by NGF. Thus, it is known that binding of NGF to its receptor activates the Ras/Raf/MEK/ERK pathway which results in the activation of the mitogen-activated protein kinases (MAPK) p42 MAPK (ERK2) and p44 MAPK (ERK1) (32,33). To inhibit the activity of the p42/p44 MAPK enzymes, we used the synthetic compound PD098059 which has been shown to specifically inhibit the activation of these kinases by growth factors *in vivo* (34). In these experiments (Fig. 4a) the PD compound completely blocked the activation of the full-length Bcl-2 promoter by NGF suggesting that the p42/p44 MAPK signalling pathway was involved in this effect. In contrast, addition of the compound SB203580 which specifically inhibits the p38 MAPK pathway has no effect on the activation of the promoter by NGF indicating that the p38 MAPK pathway was unlikely to be involved and demonstrating the specific effect of the PD098059 compound. In neither case did the compounds affect the basal activity of the Bcl-2 promoter in the absence of NGF (Fig. 4a), indicating that the effect of PD098059 was specific to NGF induction.

To further demonstrate the involvement of the p42/p44 MAPK pathway, we made use of dominant negative mutants of the p44 MAPK enzyme which can interfere with the activity of the endogenous enzyme (23). In these experiments, two different dominant negative forms of p44 MAPK specifically inhibited the activation of the Bcl-2 promoter by NGF (Fig. 4b). A similar effect was also observed with a dominant negative form of the MEK-1 enzyme which is the upstream activator of the p42/p44 MAPK enzyme but was not observed with the protein kinase A inhibitor (PKI) which binds with high affinity to the active subunit of PKA and inhibits its activity (35) (Fig. 4b). It is known that the small G protein Rap-1 is required for NGF-induced sustained activation of MAP kinase (36). To test whether such sustained activation was required for Bcl-2 promoter activation by NGF, we overexpressed a dominant negative mutant of Rap-1 (N-17 Rap-1; 36) in PC12 cells. As shown in Figure 4b, this mutant blocked the NGF-mediated activation of the Bcl-2 promoter indicating the involvement of Rap-1 in this effect.

Hence, inhibition of the p42/p44 MAPK pathway either with a chemical inhibitor or with dominant negative forms of the enzymes or a G-protein in this pathway greatly reduces inducibility of the Bcl-2 promoter by NGF indicating that this pathway is likely to be critical for such activation. To confirm that constitutive activation of the p42/p44 pathway could mediate the activation of the Bcl-2 promoter, we transfected our Bcl-2 promoter construct with a constitutively active mutant (SS/DD) of the MEK-1 enzyme (37) which is the upstream activator of p42/p44 MAPK enzymes. In this experiment (Fig. 4c) this constitutively active MEK-1 was able to strongly activate the Bcl-2 promoter. In contrast, constitutively active forms of protein kinase A and protein kinase C (38) were unable to activate the Bcl-2 promoter. Hence protein kinase A cannot activate the Bcl-2 promoter in PC12 cells in accordance with its lack of activation by cyclic AMP (see above) whilst a similar lack of inducibility occurs for protein kinase C, although this enzyme is responsible for the phosphorylation of CREB protein in B cells allowing it to activate the Bcl-2 promoter (20). Taken together, therefore, these findings indicate that the PKA, PKC and p38MAPK pathways are not involved in the activation of the Bcl-2 promoter by NGF which is, however, dependent upon the p42/p44 MAPK pathway.

DISCUSSION

Several previous studies (17–21) have defined different regions of the predominant P1 Bcl-2 promoter which can mediate its responses to various stimuli and differentiation events in non-neuronal cells such as B lymphocytes. In previous studies in neuronal cells (21,22) we showed that the neuronally-expressed POU family transcription factor Brn-3a is able to activate the Bcl-2 P2 promoter via a sequence located between bases –584 and –594 upstream of the translational start site. Here we show, however, that a distinct region located between bases –1472 and –1414 upstream of the translational start site is critical for the NGF responsiveness of the Bcl-2 P1 promoter. Hence, at least two distinct regions of the promoter are involved in its regulation in neuronal cells mediating the response to NGF and to the POU family transcription factor, Brn-3a, respectively.

Although the region mediating the response to NGF which we have defined is distinct from these other regulatory regions, it does contain a sequence which has previously been shown to bind the WT1 transcription factor in a B cell lymphoma (29). The

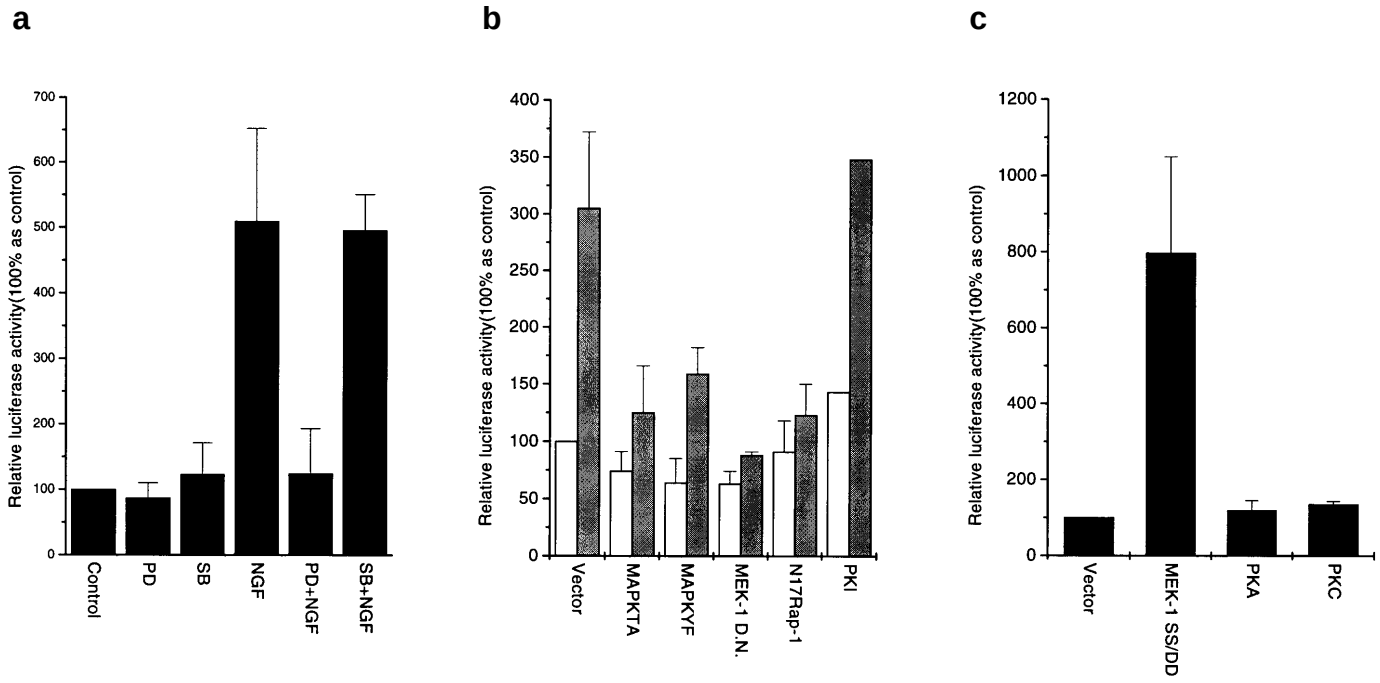

Figure 4. Role of different signalling pathways in the activation of the Bcl-2 promoter by NGF. (**a**) Effect of the p42/p44 MAPK inhibitor PD098059 (at a final concentration of 50 μM) and the p38 MAPK inhibitor SB203580 (at a final concentration of 10 μM) on the response of the full-length Bcl-2 promoter to NGF. The effect of the two inhibitors on the activity of the promoter is shown both in the presence and absence of NGF. (**b**) Effect on the NGF response of co-transfecting the full-length Bcl-2 promoter with dominant negative mutants of p42/p44 MAPK (TA and YF), MEK-1 (D-N) or Rap-1 (N17) or with a PKA inhibitor (PKI). In each case, promoter activity is shown in the absence (open bar) or the presence (solid bar) of NGF. (**c**) Response of the Bcl-2 promoter in the absence of NGF to co-transfection with constitutively active mutants of MEK-1 (SS/DD), PKA or PKC. All values are the mean of five experiments whose standard error is shown by the bars.

binding site for WT1 within the Bcl-2 promoter was shown to be occupied on the inactive silent allele of Bcl-2 and to be unoccupied on the active translocated allele. The implication of this finding (that WT1 binding to this site represses Bcl-2 gene expression) was supported by transfection studies in which a WT1 expression vector was able to repress the Bcl-2 promoter when its binding site was intact but not when it was mutated (29). Although the NGF-induced transcription factors NGFI-A and NGFI-C are members of the same transcription factor family as WT1 and bind to similar sites (30,31), we have been unable to detect an NGF-inducible protein which binds to this site in the Bcl-2 promoter (data not shown). In agreement with this, overexpression of NGFI-A has previously been shown not to activate the Bcl-2 promoter (29).

It is possible, therefore, that the NGF-mediated activation of the Bcl-2 promoter is mediated via a post-translational modification of a transcription factor which we have detected bound to this site both prior to and after NGF treatment. Evidently, this post-translational modification could neutralise the inhibitory activity of a repressor protein or could result in the enhanced activating ability of a previously inactive protein. More detailed analysis and mutagenesis of this region will be necessary to determine the role of the WT1 binding site and/or other putative binding sites in this region, in mediating the effect of NGF on the Bcl-2 promoter.

These considerations draw attention to the signalling pathways by which NGF mediates its effect upon the Bcl-2 promoter. It has previously been shown that NGF treatment of PC12 cells activates several different signal transduction pathways including protein kinase C (39), protein kinase A (40) and the Ras-dependent mitogen-activated protein kinases (41). Here we have shown by a combination of chemical inhibitors and mutant forms of various kinase enzymes that neither PKC, PKA or p38MAPK enzymes are apparently involved in the stimulation of the Bcl-2 promoter by NGF. In contrast, such stimulation can be inhibited by both a chemical inhibitor of the p42/p44 MAPK enzymes as well as by dominant negative forms of these enzymes and Bcl-2 gene expression can be stimulated by a constitutively active form of the MEK-1 enzyme which is the upstream activator of the p42/p44MAPK enzymes. These experiments thus establish the requirement for this p42/p44 MAPK pathway in the activation of the Bcl-2 promoter by NGF.

Using a similar approach, it has previously been shown that this pathway is also required for the NGF-induced differentiation of PC12 cells (41). Similarly, we have previously shown that the transcriptional activating ability of the CBP co-activator is directly stimulated by NGF in PC12 cells and this effect depends upon the p42/p44MAPK pathway (42). Thus, if CBP is recruited to DNA by linkage to the DNA binding domain of a heterologous transcription factor, its ability to stimulate transcription from promoters containing binding sites for this factor is enhanced by NGF in a p42/p44 MAPK-dependent manner (42). Moreover, we have recently shown that CBP and the p42/p44MAPK enzymes specifically associate with one another and can be co-immuno-precipitated (43).

It is possible, therefore, that the transcription factor which activates Bcl-2 expression following NGF treatment uses CBP as a co-activator which itself is stimulated by NGF treatment.

Although CBP was originally defined as a co-activator binding to the phosphorylated form of the CREB factor, it is unlikely that CREB is involved in the responses we have observed, since the CRE binding site for CREB is not involved in the response to NGF and the promoter itself does not respond to cyclic AMP treatment of PC12 cells.

Further analysis of the transcription factor(s) involved in the activation of the Bcl-2 promoter by NGF will be required in order to determine the manner in which the promoter is activated via the p42/p44 MAPK pathway. It is already clear, however, that NGF can directly activate the Bcl-2 promoter paralleling its previously described ability to stimulate Bcl-2 protein synthesis in PC12 cells (14) and that this effect is dependent on the p42/p44 MAPK pathway rather than upon other NGF activated pathways such as those involving PKC, PKA or the p38MAPK pathway.

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