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Cloning and Characterization of the 5'UTR of the Rat Anti-Apoptotic *Bcl-w* Gene

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Abstract

The anti-apoptotic Bcl-w regulator, which is expressed in the developing and mature brain, not only promotes neuronal survival, but also neuronal differentiation. However, its transcriptional regulation remains to be elucidated due to a lack of knowledge of the Bcl-w promoter. Here, we report the mapping and characterization of the rat Bcl-w promoter, which is highly conserved between the human, mouse, and rat species. Using a series of 5' and 3' deletions, we mapped the TATA-less minimal Bcl-w promoter and showed that it is under a combinatorial regulation with the neurogenic bHLH transcription factor NeuroD6 mediating its activation, validating our previous finding of increased expression of the Bcl-w protein in stably transfected PC12-NeuroD6 cells. Upon stress, NeuroD6 promotes colocalization of Bcl-w with mitochondria and endoplasmic reticulum. Finally, we provide the first evidence of Bcl-w localization in the growth cones of differentiating neuronal cells, suggestive of a potential synaptic neuroprotective role.

Keywords

Neuronal differentiation; neuronal survival; basic Helix-Loop-Helix transcription factors; transcriptional regulation; anti-apoptotic Bcl2 family

Introduction

During neural development, neurotrophic factors induce a variety of signaling cascades, which ultimately results in the modulation of gene expression promoting differentiation and survival. A wealth of studies has demonstrated that developing neurons are programmed to die by an apoptotic pathway unless rescued by extrinsic growth factors to promote the survival pathway, which encompasses the active repression of the apoptotic pathway and the activation of the anti-apoptotic pathway [1,2]. In contrast, adult neurons no longer require growth factors to prevent cell death, as the default apoptotic pathway is shut down and the intrinsic anti-apoptotic pathway is programmed to respond to stress-induced signals [3].

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Members of the basic Helix-Loop-Helix (bHLH) NeuroD family, which includes NeuroD, NeuroD2, NeuroD4 (NeuroM/MATH-3), and NeuroD6 (also known as Nex1/MATH-2), are excellent candidates to link the neurotrophin-induced differentiation pathway to the survival pathway at the transcriptional level in developing and/or mature brain. Knockout studies on NeuroD members have revealed substantial cell death of differentiating neurons belonging to distinct lineages [4–7]. Using our in vitro PC12-ND6 cellular paradigm, which constitutively expresses NeuroD6, we provided the first direct evidence of NeuroD6 intrinsic neuroprotective properties and the molecular mechanism by which NeuroD6 promotes neuronal survival, independently of any extrinsic differentiation signaling. While NeuroD6 initiates neuronal differentiation by regulating the expression of a wide spectrum of genes involved in neuronal cytoskeleton, synaptic activity, cell cycle regulation, and mitochondrial biogenesis, many of them being downstream regulators of the NGF pathway, it stimulates the expression of heat shock proteins and anti-apoptotic regulators, such as Bcl-x_L, Bcl-w, XIAP, survivin [8–10]. Interestingly, Bcl-w is the only member of the pro-survival group of the bcl2 family to be expressed upon constitutive expression of NeuroD6 in the absence of stress, suggestive of a potential NeuroD6-mediated transcriptional regulation of the *Bcl-w* gene. Such hypothesis is strengthened by their co-expression in developing and mature brain [11–12].

Bcl-w has emerged as a critical anti-apoptotic regulator to delay cell death during early neurofibrillary lesions associated with Alzheimer's disease [13,14]. Bcl-w anti-apoptotic functions have recently been extended to dorsal root ganglia neuron survival [15]. Despite its increasing role in brain development [16] and neuronal survival, little is known about the transcriptional regulation of the *Bcl-w* gene, as the Bcl-w promoter has not been cloned and characterized. Controlling Bcl-w expression levels is critical, as the ratio of anti-apoptotic versus pro-apoptotic regulators dictates the survival capacity of neurons in response to stress stimuli [17] and susceptibility for specific neurodegenerative diseases.

Thus, the main objectives of this study are: 1) to clone the 5'UTR of the rat *Bcl-w* gene; 2) to elucidate the Bcl-w promoter region in the human, mouse, and rat species using a phylogenetic approach; 3) to identify key regulatory elements of the Bcl-w promoter during neuronal differentiation; and 4) to investigate the functional link between NeuroD6 and Bcl-w in the context of neuronal differentiation.

Materials and methods

RNA Isolation

DNA-free total RNA was isolated from untreated and 6-day NGF-treated PC12 cells using the RNAqueous kit (Applied Biosystems, Austin, TX). Poly(A) RNA was prepared using the Poly (A) Purist MAG kit (Applied Biosystems) and poly(A) RNA from rat adult whole brain was obtained from Applied Biosystems.

Primer Extension Analysis

Transcription start sites were mapped by primer extension assay using a primer (Table S1) complementary to the genomic rat sequence (accession number **NW_047454**). Primer and poly (A) mRNA (1 μg) were denatured and annealed at 55 °C for 30 min. First strand cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) for 50 min at 50 °C as described in [18].

5' Rapid Amplification of cDNA Ends

Transcription start sites were mapped by 5'- rapid amplification of cDNA ends (5'RACE) using the GeneRacer kit (Invitrogen) according to the manufacturer's recommendations and as described in [18]. First strand cDNA synthesis was carried out at 50 C for 1 hour using

Superscript III Reverse Transcriptase and gene-specific primers GSP-Bclw (Table S1). Reactions were analyzed on agarose gel and purified fragments were cloned into the pCR4-TOPO vector (Invitrogen) and sequenced (Veritas, Inc., Rockville, MD).

Recombinant Plasmids

The rat Bclw promoter region was amplified by PCR using the BAC clone CHO230-118C1 (BAC Resources PAC; Children's Hospital Oakland Research Institute, Oakland, CA) as template and cloned into the MluI and XhoI sites pGL3-basic vector (Promega Inc., Madison, WI). Primers to generate nested Bcl-w deletions are listed in Table S1.

Cell Culture and Luciferase Assay

PC12 cells and PC12-ND6 cells were grown as described in [8]. For luciferase assays, cells were transfected by electroporation with specific Bcl-w promoter-reporter firefly luciferase constructs (5 μg) and eukaryotic expression vectors (8 μg), as described in [18]. As an internal control for transfection normalization, cells were transfected with 500 ng of the thymidine kinase (TK) promoter driven Renilla luciferase construct pRL-TK (Promega). Transfected cells were either untreated or treated with NGF (50 ng/ml) for 3 days, before performing a dual Firefly-Renilla luciferase assay. Triplicate samples were measured for each construct, and each transfection experiment was independently repeated at least three times. Luminescence was measured with an LMax luminometer (Molecular Devices, Sunnyvale, CA). The relative luciferase activity was defined as the ratio of the light units of Firefly luciferase vs. Renilla luciferase.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay

Nuclear extracts from untreated PC12-ND6 cells, untreated and NGF-treated PC12 cells were prepared as described in [18]. DNA binding reactions and EMSA were done as described in [18]. The sequences of the top strand of oligonucleotides used as probes, specific and mutated competitors are listed in Table S1. For supershift assay, PC12-ND6 nuclear extracts were incubated for 30 min with 2 μg of pre-immune serum or anti-NeuroD6 polyclonal antibody as described in [8].

Immunoblot analysis

PC12 and PC12-ND6 cells were lysed in M-per mammalian protein extraction buffer (Pierce, Rockford, IL, USA) in the presence of protease inhibitors (Roche Molecular Biochemicals) as described in [8]. Mitochondrial-enriched factions from serum-grown or serum-deprived PC12-ND6 cells were prepared as described in [19]. Total proteins (40 μg) or mitochondrial proteins (12 μg) were resolved on a 10% NuPAGE Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membrane, which was stained with Ponceau-S (Sigma, St Louis, MO) to confirm uniform transfer, blocked using Superblock™ blocking buffer (Pierce Biotechnology, Rockford, IL, USA), and probed with Bcl-w polyclonal antibody (Cat#13525; Abcam, Cambridge, MA). Blots were then stripped using Restore™ western blot stripping buffer (Pierce) and re-probed with an anti-GAPDH antibody to confirm equal protein loading.

Immunocytochemistry and confocal microscopy

Cells were seeded on Poly-D-Lysine–coated coverslips, fixed and permeabilized as described in [10]. Mitochondria were visualized using an antibody against the mitochondrial marker COXVα (cat# MS502; Mitosciences, Eugene, OR), while the endoplasmic reticulum was visualized using an antibody against poly-D-isomerase (cat# ab2792; Abcam). Bcl-w protein was detected using a polyclonal antibody. Cells were incubated with the nuclear counterstain DAPI and mounted with Fluoromount G. Images were acquired using a Zeiss LSM 510 confocal system (Zeiss, Thornwood, NY, USA). Colocalization between Bcl-w and organelles

was analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) by drawing a line scan extending throughout the soma or growth cone of serum-grown or serum deprived PC12-ND6 cells. The intensity of the pixels along the line was recorded to generate a histogram as described in [20].

Results and Discussion

Identification of the Rat Bcl-w Transcription Initiation Sites by Primer Extension and 5' RACE

Besides its chromosomal localization on 15p13, little is known about the genomic structure of the rat *Bcl-w* gene and its regulatory sequences. Similarly, not much is known about the mouse *Bcl-w* gene, except for its exon composition, with exons 1 and 2 being non-coding regions, and exons 3 and 4 containing the entire coding sequence [21]. To initiate the transcriptional studies of the rat Bcl-w promoter, we first evaluated the timing of Bcl-w expression upon NGF exposure in PC12 cells for direct comparison with NeuroD6-induced Bcl-w expression in PC12-ND6 cells, as NeuroD6 is a critical effector of the NGF pathway [8,9]. We found that Bcl-w expression was triggered after four days NGF exposure, when PC12 cells withdraw from cell cycle to undergo terminal neuronal differentiation (Fig. 1). Thus, both NeuroD6 and Bcl-w proteins are co-expressed in NGF treated-PC12 cells [9], concurring with their overlapping expression patterns in developing brain [11,12].

To map the transcription start sites of the rat *Bcl-w* gene by primer extension, we designed a primer complementary to the sense strand located +13 to +32 nt downstream of the ATG start site (Table S1). We detected multiple transcription start sites using mRNA isolated from adult rat brain and NGF-treated PC12 cells with major transcription start sites located 430, 315 and 240 nucleotides upstream of the 3' end of the primer (Fig. 2A).

We next performed 5' RACE using the GeneRacer method, as it allowed capturing full-length transcripts by targeting 5' capped mRNAs. During the course of this analysis, we mapped a novel alternative splice form exon 1b (Fig. 2B). Figure 2B shows the location of the major and minor multiple transcription start sites mapping in both non-coding exons 1a and 1b (Fig. 2B). We identified the shortest transcripts starting at −888 and −889 with respect to the ATG translational initiation site and extending through the exon 1a splice site located at −844. The longest transcripts initiated at sites −888, −847 and −838, while extending through the novel exon 1b splice site located at −652. The intermediate sized transcripts were found to contain a mixture of exons 1a and 1b transcripts, with exon 1a transcripts starting from −941 to −953 and exon 1b transcripts starting at −801 and −775. The presence of alternative Bcl-w splice variants in neuronal cells is in keeping with previously reported Bcl-w splice forms during oligodendroglial differentiation [22].

Conserved Functional Features of the Rat Bcl-w Promoter

ClustalW alignment of mouse, rat and human bcl-w sequences shows that the multiple transcription start sites and the alternative non-coding exons are located in a 0.68 kb region displaying very high homology between the three species (Fig. 3A; Fig. S1). Homology between the three species remained significant in the 5' and 3' flanking regions, with homology dropping to 35% beyond 1.8 kb (Fig. 3A). No TATA box was identified within the putative promoter region, in keeping with the typical multiple transcription sites of TATA-less promoters.

Potential regulatory elements were identified within the first1.8 kb region using Transfac (v. 4.0) weight matrices and TESS binding site analysis and included Sp1 binding sites interspersed throughout the region of the clustered transcription start sites (Fig. 3A). Our phylogenetic analysis also revealed the presence of seven E-boxes, three of which (E5, E4 and E3) are

conserved in terms of sequence and position between the mouse, rat and human sequences (Fig. 3A). Their proximity to the multiple transcription start sites is in accordance with the well-documented interactions between bHLH transcription factors and the basal transcriptional machinery.

Our analysis highlighted conserved putative binding sites for MEF2, Ets-1, Ets-2, NF-κB, and C/EBP (Fig. 3A). The presence of Ets binding sites near E-boxes is particularly relevant, as they are critical to the bHLH-mediated transcriptional activity [23]. Interestingly, the MEF2D transcription factor promotes sensory neuron survival via Bcl-w expression upon activation of Trk-dependent ERK5/MEF2 pathway [15]. Thus, this molecular architecture suggests a combinatorial regulation involving distinct transcriptional factors depending on the cellular and developmental context.

Transcriptional regulation of the rat Bcl-w gene

To determine whether the cloned 5' UTR of the rat *Bcl-w* gene contains intrinsic promoter activity, we cloned the first 1800 bp of the Bcl-w 5'UTR upstream of the firefly luciferase gene to generate the construct Bcl-w (−1800/+1) (Fig. 3A). We generated a 5' and 3' Bcl-w deletion series, referred to as Bcl-w (−1453/+1) and Bcl-w (−1800/−750). Luciferase expression from the Bcl-w (−1800/+1) and Bcl-w (−1453/+1) constructs was about 40-fold greater than that produced by the promoterless pGL3-basic vector, confirming that the identified 5'UTR region of the rat *Bcl-w* gene contains an active promoter (Fig. 3B). No essential regulatory elements, located between −1800 and −1453 bp, appeared to contribute to the overall Bcl-w promoter activity upon NGF-induced differentiation (Fig. 3B). In contrast, deletion of the first −750 bp led to increased Bcl-w promoter activity, suggestive of the presence of a negative regulatory element(s) between the initiation translation start site (+1) and the transcription start sites (−750) (Fig. 3A,B).

We next examined whether NeuroD6 could stimulate the Bcl-w promoter activity, as suggested by increased expression of the Bcl-w protein in untreated PC12-ND6 cells (Fig. 1), a notion further supported by the presence of multiple conserved E-boxes in the Bcl-w promoter region (Fig. 3A). We used two complementary cellular paradigms: 1) PC12 cells, which were transiently transfected with the pcDNA6/NeuroD6 expression construct and the Bcl-w (−1800/+1) reporter construct in the presence or absence of NGF; and 2) the stable PC12-ND6 cell line, which was transfected with the Bcl-w (−1800/+1) reporter construct. NeuroD6 stimulates Bcl-w promoter activity by three-fold, when either transiently in untreated PC12 cells or constitutively expressed in untreated PC12-ND6 cells (Fig. 3C). While NGF-treated PC12 cells transfected with the Bcl-w (−1800/+1) reporter construct only displayed a four-fold induction of Bcl-w promoter activity, transfected NGF-treated PC12-ND6 cells displayed an eight-fold increase in Bcl-w promoter activity, suggesting a synergistic effect between NGF and NeuroD6 (Fig. 3C). As expected, NGF-treated PC12 cells transfected with a dominant negative-like form of NeuroD6 (NeuroD6-mut1), which lacks one of the transaction domains [8], failed to display increased Bcl-w promoter activity (Fig. 3C).

The notion that NeuroD6 modulates the Bcl-w promoter activity is further strengthened by the observed Id2-mediated repression, as Id2 behaves as a dominant-negative HLH regulator lacking a basic domain [24]. Overexpression of Id2 abolished Bcl-w promoter activity in untreated PC12-ND6 cells, while it partially repressed it in both NGF-treated PC12 and PC12-ND6 cells, suggesting that the Bcl-w promoter activity is most likely controlled by a combinatorial regulation involving NeuroD6 and other transcriptional regulators (Fig. 3C). This is in accordance with our phylogenetic analyses, highlighting the presence of conserved binding sites for Sp1 and the MEF2 transcription factor, which has recently been shown to stimulate Bclw expression in sensory neurons [15].

To determine which E-box is recognized by NeuroD6, we examined the occupancy of the E3 and E5 (CAAGTG) E-boxes, as they are conserved in the three species (Fig. 3A). We purposely did not include the conserved E4 E-box in our initial analysis, as it is located in the alternative exon1b, while the E5 and E3 E-boxes are located either in exon 1a or exon 2, respectively. We performed EMSA using nuclear extracts from naïve PC12 cells, NGF-treated PC12 cells, and untreated PC12-NeuroD6 cells. A single retarded protein-DNA complex was observed with a radiolabeled oligonucleotide containing the E3 E-box (CAGCTG), which was successfully competed by the addition of a 100-fold excess of unlabeled E3 specific competitor (Fig. 3D). The specificity of this complex was confirmed by supershift using our anti-NeuroD6 polyclonal antibody (Fig. 3D). Finally, a mutant oligonucleotide containing a 2-base substitution at positions 1 and 6 of the E3 E-box failed to compete (Fig. 3D). We did not observe a similar DNA-protein complex with the E5 E-box (data not shown), an expected finding since NeuroD6 does not recognize this unusual E-box [25]. Collectively, our results from the reporter-promoter luciferase assay combined with EMSA and phylogenetic analyses show that NeuroD6 regulates the Bcl-w promoter activity, which is in agreement with the correlated increase of NeuroD6 and Bcl-w expression in the absence of NGF or stress [9].

Subcellular localization of the Bclw protein in PC12-ND6 cells prior to and upon stress stimulus

We supplemented our transcriptional studies with immunocytochemistry analyses to investigate whether NeuroD6 could promote proper subcellular localization of the Bcl-w protein compatible with its anti-apoptotic functions. The pro-survival Bcl2 members promote cell survival by binding to the outer membrane of mitochondria through their C-terminal hydrophobic domain to prevent the release of cytochrome c and SMAC/DIABLO from mitochondria into the cytoplasm [17]. Bcl-w protein is mainly cytosolic in the soma and growth cones of untreated PC12-ND6 cells (Fig. 4A). Since Bcl-w remains expressed in serum-deprived PC12-ND6 cells [9], we examined whether such stress stimulus would impact Bcl-w subcellular localization. By confocal microscopy, we detected partial colocalization with mitochondria located in the soma and growth cones of serum deprived PC12-ND6 cells, and ER located in the soma of serum-deprived PC12-ND6 cells (Fig. 4A). Line scans drawn through the soma and growth cones confirmed such colocalization upon serum deprivation (Fig. S2). Finally, increased Bcl-w expression levels in mitochondria upon serum deprivation were further confirmed by immunoblot analysis using mitochondria-enriched fractions isolated from serum-grown or serum-deprived PC12-ND6 cells (Fig. 4B).

Conclusions

In conclusion, we have characterized the rat Bcl-w promoter, which displays significant homology with the human and mouse Bcl-w genomic sequences. The minimal promoter lies within the non-coding exon 1a, mapping 750 bp upstream of the translation start site and is TATA-less with typical clustered SP1 binding sites in the vicinity of the multiple transcriptional start sites. Using a phylogenetic approach, we identified several conserved enhancers elements, such as E-boxes, Ets and MEF2 bindings sites. By luciferase assays, we showed that the rat Bclw promoter is under a combinatorial transcriptional regulation involving NeuroD6 and other transcription factors that remain to be identified. NeuroD6 not only stimulates the Bcl-w promoter activity, resulting in increased expression of the Bcl-w protein, but also promotes proper subcellular localization of the Bcl-w protein upon stress. Finally, our studies provide the first demonstration of Bcl-w protein being localized in the growth cones of neuronal PC12-ND6 cells with partial colocalization with mitochondria upon stress stimulus, suggesting a potential neuroprotective role at the synapse level. These studies set the stage for future studies to unravel the transcriptional regulation of the *Bcl-w* gene in distinct

neuronal lineages and developmental stages, which may help in understanding the molecular mechanisms responsible for specific neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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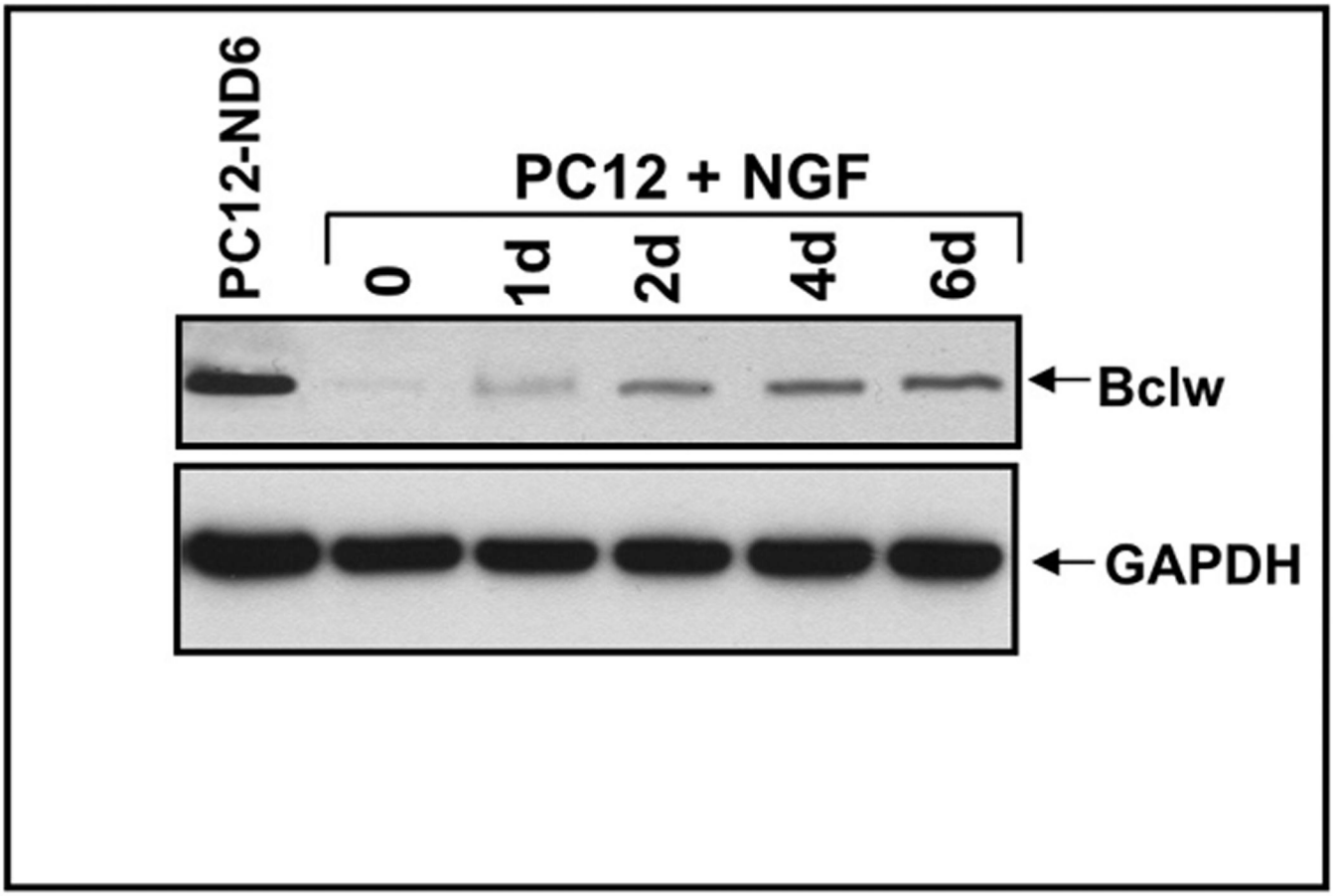

Fig. 1.

Constitutive expression of NeuroD6 induces expression of the Bcl-w protein at level similar to that of NGF-induced neuronal differentiation of PC12 cells. Cell lysates from untreated PC12-ND6 cells and NGF-treated PC12 cells were analyzed by Western blot using an anti-Bcl-w polyclonal antibody. Equal loading was verified using an anti-GAPDH antibody.

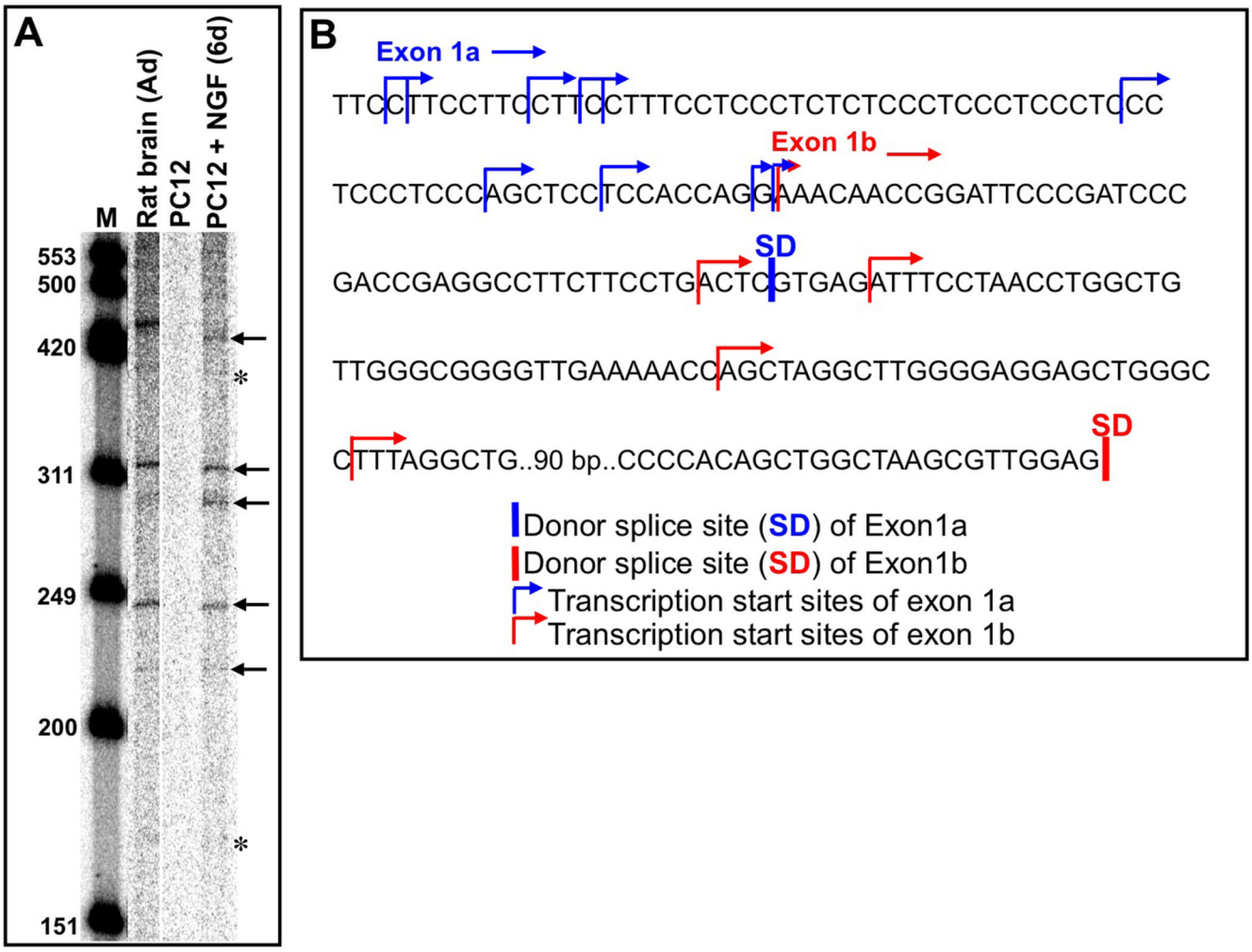

Fig. 2.

The rat Bcl-w promoter is TATA-less and contains multiple transcription start sites. (A). Primer extension of the rat Bcl-w promoter during neuronal differentiation. Poly (A) RNA isolated from adult rat brain, untreated and NGF-treated PC12 cells was annealed to an end-labeled primer. The arrows indicate major transcription start sites found in common with adult rat brain and NGF-PC12 cells, while the star symbol indicates minor transcription start sites specific to adult rat brain. (B). Mapping of the multiple Bcl-w transcription start sites and exon boundaries by 5'RACE. We identified a novel alternative non-coding exon 1b in the 5'UTR of the rat *Bcl-w* gene. Transcription start sites mapping in the non-coding exons 1a and 1b are indicated with blue and red arrows, respectively. Donor splice sites (SD) of non-coding exons 1a and 1b are indicated by a blue and red vertical line, respectively.

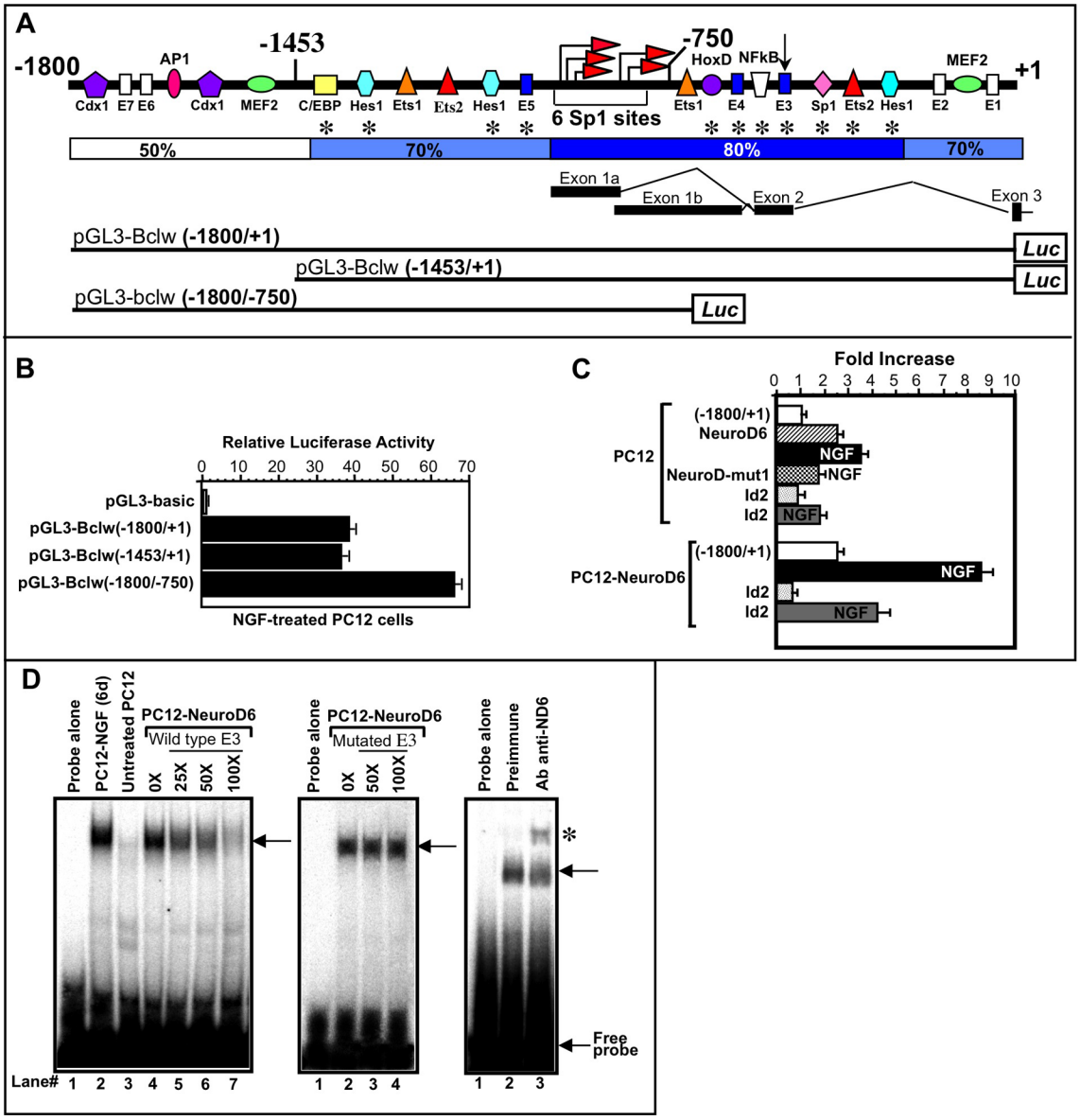

Fig. 3.

Regulation of the rat Bcl-w promoter. (A). Map of the rat Bcl-w promoter and Bcl-w luciferase reporter constructs. The schematic representation illustrates the degree of homology between human, mouse, and rat genomic sequences. The numbering is relative to the translation start site (+1). Multiple transcription start sites span from −750 to −870, with clustered Sp1 binding sites indicated by red arrows. Relevant conserved enhancer elements are schematically represented by different symbols with their names indicated below. Asterisks indicate enhancer elements conserved in the three species, while the arrow indicates the protected E3 E-box as shown by EMSA. (B). Bcl-w promoter activity in PC12 cells treated with NGF (50 ng/ml) for three days. Results are expressed as means ± SD from 4 independent experiments done in triplicate. (C). NeuroD6 upregulates the Bcl-w promoter activity. PC12 and PC12-ND6 cells were transfected with the Bcl-w (−1800/+1) reporter construct, with or without eukaryotic expression vector containing NeuroD6, NeuroD6-mut1, or Id2. Cells were either untreated or treated with NGF for 3 days. Results are expressed as means ± SD of fold of induction relative

to the Bcl-w (−1800/+1) reporter construct from 4 independent experiments done in triplicate. (D). The E3 E-box is occupied by nuclear proteins from both untreated PC12-ND6 cells and NGF-PC12 cells. Nuclear extracts (8 μg) were incubated in the presence of increasing amounts specific (left panel), mutated (middle panel) competitors or pre-immune IgG or purified anti-NeuroD6 IgG (right panel).

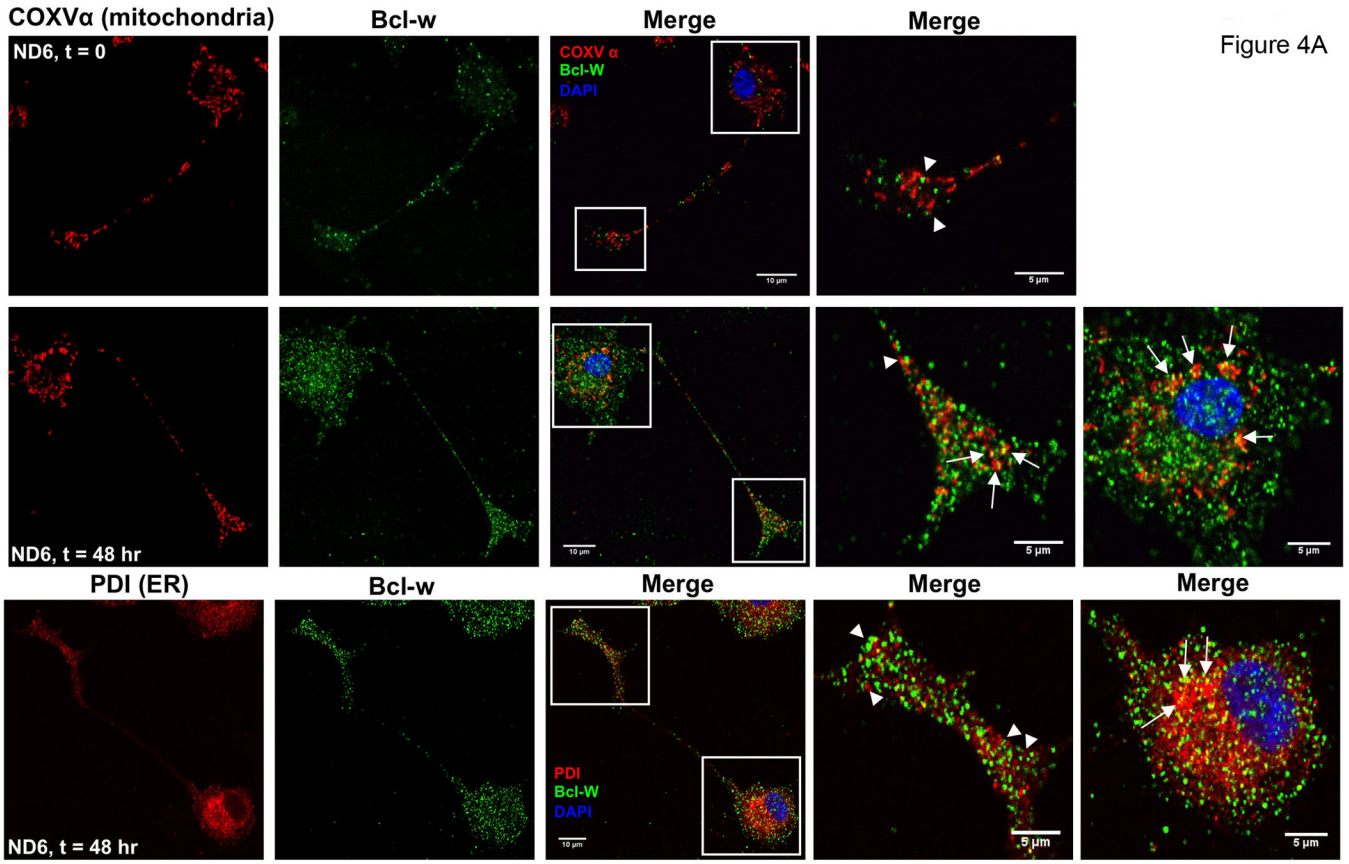

Figure 4A

Figure 4B

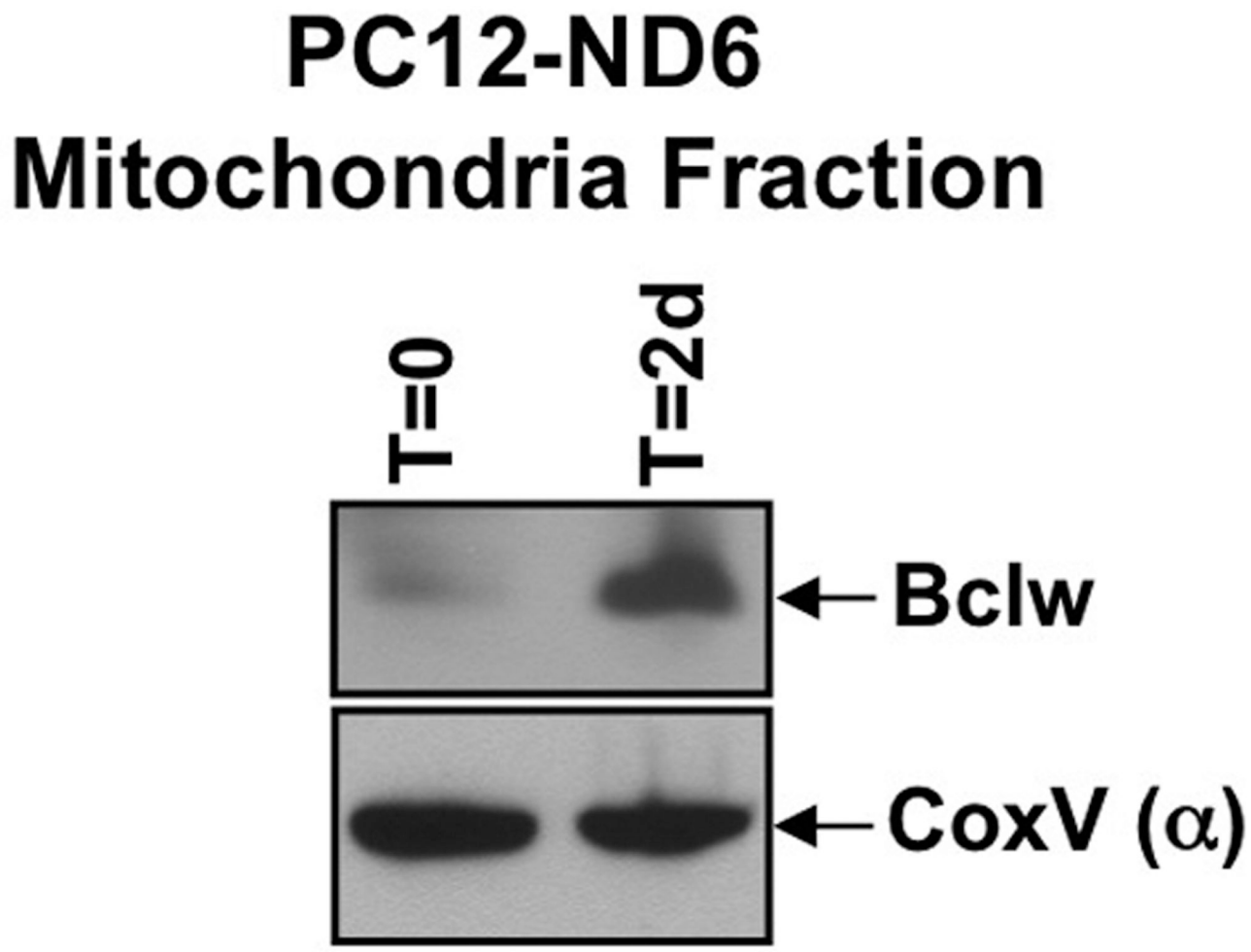

Fig. 4.

Subcellular localization of Bcl-w protein in the absence or presence of stress stimulus. (A). Immunocytochemistry was performed on serum-grown (t=0) and serum-deprived (t=48 hr) PC12-ND6 cells to examine Bcl-w subcellular localization (green) and its overlapping expression with mitochondria and endoplasmic reticulum (ER), using COXV α subunit as a mitochondrial marker (red) and poly-D-isomerase (PDI) as an ER marker (red). Cells were incubated with the nuclear counterstain DAPI (blue). Scale bars are indicated at the bottom right corner of merged images. Magnification of the soma and growth cone is shown on right panels. Colocalization between Bcl-w and mitochondria and ER is indicated by arrows, while Bcl-w expression in the vicinity of either organelle is indicated by arrowheads. (B). Expression levels of Bcl-w protein were analyzed by immunoblot analysis using mitochondria-enriched fractions isolated from serum grown (t= 0) or serum-deprived (t=2d) PC12-ND6 cells. Equal loading was verified using an antibody against subunit α of COX V.