The CES-2-related transcription factor E4BP4 is an intrinsic regulator of motoneuron growth and survival

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Summary

The regulation of neuronal growth and survival during development requires interplay between extrinsic and intrinsic factors. Among the latter, transcription factors play a key role. In the nematode, the transcription factor CES-2 predisposes neurosecretory motoneurons to death, whereas E4BP4 (NFIL3), one of its vertebrate homologs, regulates survival of pro-B lymphocytes. We show that E4BP4 is expressed by embryonic rat and chicken motoneurons in vivo, with levels being highest in neurons that survive the period of naturally occurring cell death. Overexpression of E4BP4 by electroporation of purified motoneurons in culture protected them almost completely against cell death triggered by removal of neurotrophic factors or activation of death receptors. Moreover, E4BP4 strongly enhanced neuronal cell size and axonal growth. Axons of motoneurons transfected with E4BP4 were 3.5-fold longer than control neurons grown on laminin; this effect required the activity of PI3 kinase. In vivo, overexpression of E4BP4 in chicken embryos reduced the number of dying motoneurons by 45%. Our results define E4BP4 as a novel intrinsic regulator of motoneuron growth and survival. Pathways regulated by E4BP4 are of potential interest both for understanding neuromuscular development and for promoting neuronal survival and regeneration in pathological situations.

Key words: Motoneuron, Survival pathways, Axon growth, In ovo electroporation

Introduction

During neuronal development, successful innervation of the target is in many cases rapidly followed by a period of naturally occurring cell death that typically leads to the loss of more than half of the neurons initially generated (Oppenheim, 1991; Pettmann and Henderson, 1998). Although the functional significance of this cell death is not fully understood, it is a feature shared by invertebrate and vertebrate nervous systems. The basic underlying mechanisms of programmed cell death (PCD) are highly conserved, and the principal elements of the apoptotic pathway – CED-9/Bcl2 family, CED-4/Apaf1 and CED-3/caspases – are involved in neuronal death in all species studied.

One apparent difference between vertebrates and invertebrates lies in the degree to which cell death is predetermined on a cell-by-cell basis. Within large populations of vertebrate neurons, the neurotrophic hypothesis suggests that those neurons that survive do so as a result of successful competition for access to target-derived neurotrophic factors, in an essentially stochastic manner (Davies, 1996; Lewin and Barde, 1996). However, in the nematode *C. elegans*, the developmental pathway is more hard-wired, in that the same specific neurons die in each animal (Sulston, 1983). However, even in vertebrates, the action of extrinsic regulators (e.g. neurotrophic factors) may be modulated by intrinsic factors that predispose a given neuron to die or to survive. This would be of interest for studies of both normal development and neurodegenerative pathologies.

Cell-death specification (CES) genes in *C. elegans* provide a particularly interesting model (Ellis and Horvitz, 1991). During development of the NSM neurons (neurosecretory motoneurons) that innervate the pharynx, a precursor divides to form two daughter cells, one of which normally undergoes programmed cell death and the other of which becomes an NSM. In the absence of *CES-2* activity, both sister cells survive and become NSM neurons. Thus, the principal role of CES-2, which is a bZIP transcription factor related to the PAR (proline acidic rich) family, is to predispose 50% of the NSM population to cell death (Metzstein et al., 1996).

Vertebrate homologs of CES-2 have been identified. Although their sequence similarity is distant, they share a consensus DNA-binding site (Cowell et al., 1992; Drolet et al., 1991; Falvey et al., 1995; Fonjallaz et al., 1996; Haas et al., 1995; Hunger et al., 1992). One of these, E4BP4 (also known as NFIL3), can act in different contexts as a transcriptional repressor or activator (Cowell et al., 1992; Lai and Ting, 1999; Zhang et al., 1995). In pro-B lymphocytes, which depend on the cytokine IL3 for their survival, E4BP4 is induced by IL3, and is itself sufficient for survival in the absence of cytokine (Ikushima et al., 1997; Kuribara et al., 1999). Thus, E4BP4 in pro-B cells, in contrast to CES-2 in the nematode, is an anti-apoptotic factor.

We therefore asked whether E4BP4 might play a role in the survival of vertebrate motoneurons. We show that *E4BP4* is expressed by motoneurons at the time at which their survival is being determined. Moreover, overexpression of E4BP4 in cultured motoneurons protects them against cell death. Importantly, overexpression in vivo reduces the number of dying motoneurons during development. Together with our observations that E4BP4 potently enhances motoneuron growth, these results define E4BP4 as a novel potential player in vertebrate motoneuron development and pathology.

Materials and methods

Molecular cloning of chicken *E4BP4* and RT-PCR amplification

We used an *Eco*RI/*Pst*I fragment of mouse *E4BP4* (generous gift from T. Look, Boston) to screen a chicken bursa filter cDNA library (RZPD, Berlin library no. 426). One positive clone (DKFZp426K217Q2) contained the cE4BP4 cDNA from bases 591-1856, including the poly-A tail. In parallel, the cDNA corresponding to bases 216-1164 was cloned from E2 chicken whole embryo cDNA by using a degenerated primer touch down PCR approach. Bases –170 to +437 were cloned by performing 5′-RACE (Life Technology). The assembled sequence was verified by sequencing of RT-PCR products from E2 whole embryo and E9 chicken retina cDNA (three independent clones each). The DNA corresponding to bases –70 to +1856 was cloned into the β-actin promoter-driven expression vector pCAGGS or into pBSK.

For RT-PCR analysis, total RNA was isolated either from purified mouse motoneurons or from chicken spinal cords at various stages. cDNAs were synthesised with random oligonucleotide primers. The primers used for PCR amplification of *E4BP4* sequences were: chicken, 5′-aaggatgctatgtattgggaga-3′ and 5′-acagttgttgagctactgag–3′; and mouse, 5′-taccagacatccaaggctgc-3′ and 5′-ccaatcttgaatgttcgtcac-3′. All fragments were sequenced to verify the amplified sequences. The *GAPDH* control was amplified with the following PCR primers: 5′-gtcaacggatttggccgtat-3′ and 5′- aatgccaaagttgtcatggatg-3′.

In situ hybridisation

Plasmids containing full-length chicken *E4BP4* or islet 1 (*ISL1*) (chicken or rat) were used to synthesise digoxigenin-labelled antisense riboprobes according to the supplier's protocol (Roche) and purified on spin columns (Qiagen). In situ hybridisation on tissue sections was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993; Yamamoto and Henderson, 1999). Briefly, chicken embryos of various stages were fixed overnight (4% PFA in PBS), cryopreserved (20% sucrose in PBS) and embedded in OCT (Miles). Cryosections (12-14 μm) were acetylated [0.1 M triethanolamine/HCl (pH 8.0), 0.25% acetic anhydride, 10 minutes at room temperature] and hybridised with riboprobe (150 ng/slide) overnight at 65°C. The sections were washed (1×SSC, 50% formamide, 0.1% Tween20, 2×45 minutes, 65°C) and blocked in the presence of 20% inactivated goat serum prior to incubation overnight with AP-conjugated anti-DIG-Fab-Fragments (Roche, 1:3000). After extensive washing, hybridised riboprobes were revealed by performing a NBT/BCIP reaction. Negative controls were performed using sense probes.

Whole-mount in situ hybridisation on E14 rat spinal cord was performed as previously described (Garcès et al., 2001). Riboprobes were hybridised at 68°C overnight. Hybridisation was detected using AP-conjugated anti-DIG Fab-fragments and NBT/BCIP. The spinal cords were flat-mounted as an 'open book' preparation.

Electroporation, culture and morphometrical analysis of primary rat motoneurons

Rat motoneurons were purified from E14 spinal cords using a $p75^{NTR}$ antibody and magnetic beads as previously described (Arce et al., 1999). Purified motoneurons were resuspended in electroporation buffer [125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES (pH 7.4)] at a density of 50,000 cells in 50 μl of buffer and transferred to an electroporation cuvette (4 mm). E4BP4-pCAGGS expression vector (3 μg) and/or GFP-pCAGGS (at least 1 μg/μl in PBS) were added, gently mixed and incubated for 15 minutes at room temperature. Electroporation was performed with the following protocol: 200 V, three pulses, 5 ms pulse length, 1 second interval (BTX, ECM 830). Immediately after electroporation, 500 μl of complete Neurobasal culture medium (Life Technologies) with supplements (2% B27 supplement, 2% horse serum, 0.5 mM L-glutamine, 25 μM β-mercaptoethanol) were added. The transduction rate judged by GFP expression after 2 days varied between 30 and 70% of surviving motoneurons. Previous experiments using GFP and tagged proteins had shown that the degree of co-transfection obtained by this method is high (>90%) (Raoul et al., 2002). The cells were distributed into four 16-mm wells and growth factors or enzyme inhibitors were added either alone or in combination (GDNF 1 ng/ml, BDNF 10 ng/ml, CNTF 10 ng/ml, LY294002 10 μM). Given that transfection is monitored by GFP expression, it was not possible to determine directly the number of transduced motoneurons at the time of seeding. We therefore quantified the survival rate by counting all GFP-positive motoneurons in each culture dish after 24 hours or 48 hours, and expressing them as a percentage of the number surviving in the presence of neurotrophic factors at the same time. To trigger death, cells were treated with BDNF at seeding, treated with soluble Fas ligand (0.1 μg/ml, Alexis) and Fas enhancer (1 μg/ml, Alexis) after 24 hours, and counted 24 hours later. For morphological analysis, pictures of motoneurons (80-200 of each condition) were taken on an inverted fluorescence microscope after first counting the cells for *E4BP4* survival effects. Neurite outgrowth was analysed by measuring the longest and the total neurite length of all GFP-positive motoneurons in each experiment. Pictures were taken without prior fixation of the cells. All processes were marked by hand and the length finally determined by software (Visiolab, Biovision). Cell body growth was addressed by measuring the pixel area of each GFP-positive motoneuron cell body. All pictures were analysed and normalised using Lucia G software (Nikon).

Semi-quantitative RT-PCR for *E4BP4*

E4BP4 mRNA was quantified in cultures of dissociated cells from E13 rat ventral spinal cord. For each data point, 300,000 cells were cultured for 6, 16, 18 or 24 hours in the absence or presence of neurotrophic factors (GDNF, 2 ng/ml; BDNF, 10 ng/ml; CNTF, 10 ng/ml). Total RNA was isolated by using the Qiagen RNeasy protocol. The RNA was treated with DNAse (Invitrogen) for 30 minutes, cleaned (RNeasy, Qiagen) and finally dried using a speed vac. First strand cDNA was synthesised using random oligonucleotide primers (Superscript II protocol, Invitrogen). The following primers were used for PCR amplifications: rat *E4BP4*, 5′-gctctcggatgtgtctgagc-3′ and 5′-tggggacctgctgctcgtct-3′; rat actin, 5′-ttgtaaccaactgggacgatatgg-3′ and 5′-gatcttgatcttcatggtgctagg-3′. cDNA samples were diluted 10-fold before amplification with the following protocol: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 28 cycles (*E4BP4*) or 22 cycles (actin). These cycle numbers were confirmed to correspond to the proportional phase of amplification. After Southern blot transfer, radioactive hybridisation was performed using 5′-labelled internal primers (*E4BP4*, 5′-gagctacatgggtagctctttctccac-3′; and *actin*, 5′-acctgacagactacctcatgaagatcc-3′). The intensity of hybridised PCR bands was measured using Image J software. For each individual experiment, the ratio of *E4BP4* to actin intensities in control conditions was calculated (termed R_c). The corresponding ratio for

the same cell preparation cultured with neurotrophic factors (R_n) was then expressed as a fraction of the former. This intensity ratio (R_n/R_c) was calculated for a total of 30 independent preparations and plotted as mean±s.e.m. (Fig. 5C).

Quantification of E4BP4 in primary neuronal cultures by western blotting

Dissociated E13 ventral spinal neurons were cultured as above. Following direct lysis in 300 μl SDS-gel loading buffer, 50 μl samples were subjected to conventional SDS-PAGE and transferred to nitrocellulose (Immobilon-P, Millipore). After blocking (5% milk powder in TBST), membranes were incubated with anti-E4BP4 (1/400; V-19, Santa Cruz) and anti-α-tubulin (1/7000; clone DM 1A, Sigma) antibodies. Anti-goat HRP and anti-mouse HRP (Jackson) were used as secondary antibodies, respectively. Protein bands were visualized using the ECL Plus reagent (Amersham) and quantified using Image J software after scanning. Relative intensity values were normalised with respect to the tubulin control as described for RT-PCR.

Electroporation of chicken embryo spinal cord

Fertilised chicken eggs were incubated for 55-60 hours until they reached stage 15 to 17 (Hamburger and Hamilton, 1951). After opening the shell, black ink (Waterman 1/10 in PBS, 0.22 μm filtered) was injected below the embryo and 100 μl PBS were added above. Using a sharpened tungsten needle, a small hole was made in the dorsal membrane of the spinal cord at somite level 22, providing access to the neural tube. Freshly prepared DNA solution was injected into the neural tube with a glass capillary. The DNA was prepared as follows: 15 μl cE4BP4-pCAGGS (5-7 μg/μl water) + 3.5 μl GFP-pCAGGS (5 μg/μl water) + 1.5 μl Fast Green (0.3% in water) + 2.2 μl 10×PBS. For control electroporation, cE4BP4-pCAGGS was replaced by GFP-pCAGGS. The first electrode was placed on the vitelline membrane (under PBS) and the second under the membrane (after making a small hole into it) and beside the embryo, so as to electroporate most efficiently the cells of the ventral horn. Electroporation protocol: six pulses, 22-23 V, 30 ms pulse length, 1 second interval (BTX, ECM 830). Finally, 200 μl PBS with antibiotics were layered over the embryo and after closing the shell with tape, embryos were incubated for 3-8 days.

TUNEL labelling of whole mount spinal cords and analysis

After removing viscera, the vertebral column of electroporated chicken embryos (E6.5 or E7.5) was opened from the ventral side and electroporation confirmed by visualizing co-electroporated GFP under a fluorescence binocular (Zeiss). Only when GFP was visible in nerves that projected into the wings were embryos retained for TUNEL analysis. Spinal cords were removed (if necessary the dorsal side was opened) and fixed (4% PFA overnight). Whole-mount TUNEL labelling was performed as described (Yamamoto and Henderson, 1999). The NBT/BCIP reaction was stopped in PBSE (PBS, 5 mM EDTA) and the spinal cords post-fixed in 4% PFA/PBS overnight. Finally, the spinal cords were incubated in storage solution (80% glycerol, 0.5% PFA in PBS) and flat-mounted under a dissecting microscope. The area of electroporation was visualised by detection of the remaining GFP expression with a fluorescence dissecting microscope. All TUNEL-positive nuclei in the GFP-positive area of the electroporated and the contralateral side were counted (viewing from the ventral side of the spinal cord) and compared with each other. The TUNEL-positive nuclei of an area of the same length rostral and caudal of the GFP positive area were analysed in the same way. All data were controlled by recounting the positive nuclei after turning the spinal cords (viewing from the dorsal side of the spinal cord).

Results

Expression of E4BP4 by developing motoneurons

In order to evaluate the potential role of E4BP4 in motoneuron development, we first looked for expression of *E4BP4* in spinal cord in vivo. Whole-mount in situ hybridisation on E14 rat embryo spinal cord, at the peak of developmental cell death (Yamamoto and Henderson, 1999), gave clear signal for *E4BP4* in many motoneurons (Fig. 1A), and RT-PCR on motoneurons purified at the same stage confirmed the presence of *E4BP4* mRNA (Fig. 1D, left panel; see Materials and methods). Moreover, western blots of spinal cord gave a band at ~60 kDa (Fig. 1E), as previously reported (Ikushima et al., 1997; Lai and Ting, 1999).

If E4BP4 were acting as a pro-apoptotic factor, its expression should be high in regions undergoing intensive cell death, and low at the end of the period of naturally occurring cell death. In fact, our expression data suggested the opposite. At sacral levels in rat and chick, cell death removes >90% of the motoneurons generated (Oppenheim et al., 1989). Sacral motoneurons are readily detected by in situ hybridisation (Coulouarn et al., 2001), but there was no detectable signal for *E4BP4* (Fig. 1A; not shown for chick). In chicken embryos, motoneuron cell death at lumbar levels is finished by E9. Nevertheless, *E4BP4* expression at this stage was prominent in

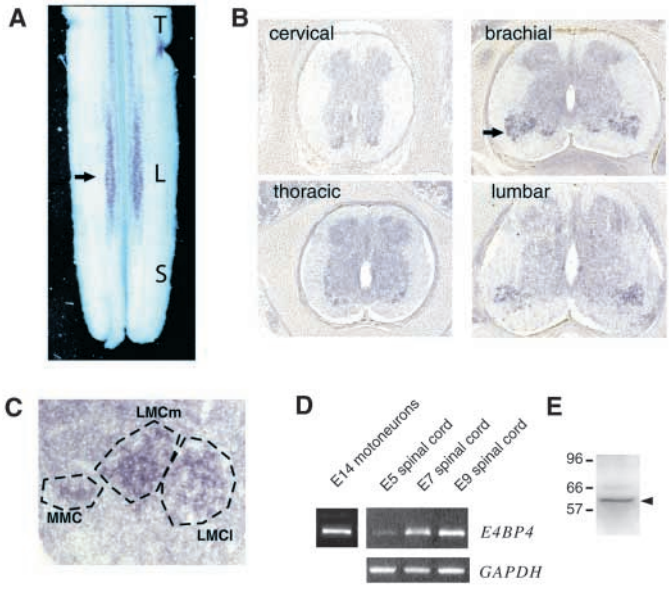

Fig. 1. Expression of E4BP4 during motoneuron development. (A) Whole-mount in situ hybridisation for *E4BP4* on a flat-mounted E14 rat spinal cord using a mouse probe shows prominent labelling in the lumbar (L) region (arrow), weaker labelling at thoracic levels (T) and no detectable signal at sacral levels (S). (B) In situ hybridisation on sections of E9 chicken embryos using a chicken probe shows prominent labelling at limb-innervating levels (arrow). (C) Higher-magnification picture of the ventral horn of E9 lumbar spinal cord. The major motor columns defined by *ISL1* labelling of an adjacent section (not shown) are indicated. MMC, medial motor column; LMCm and LMCl, medial and lateral subdivisions of the lateral motor column. (D) RT-PCR on freshly-purified E14 rat motoneurons (left panel) and chicken embryo spinal cord at indicated ages (right panel). (E) Western blot of chicken E7 spinal cord using an anti-mouse E4BP4 immune serum, performed as described (Ikushima et al., 1997).

motoneurons at lumbar and brachial levels (Fig. 1B). Staining was also apparent in some thoracic and cervical motoneurons and, with lower intensity, elsewhere in the spinal cord. Accordingly, comparison with adjacent sections stained for *ISL1* (a motoneuron marker) showed that *E4BP4* is expressed in both lateral motor columns (LMCl and LMCm), and more weakly in the medial motor column (MMC) (Fig. 1C). Time-course studies in chick revealed that *E4BP4* mRNA was only weakly detected by semi-quantitative RT-PCR at E5, at the beginning of cell death, and that relative levels steadily increased up to E9 (Fig. 1D, right panel). Taken together, our data suggested that the in vivo role of E4BP4 was unlikely to be pro-apoptotic.

Cell-autonomous survival-promoting activity of E4BP4 for motoneurons

We took a gain-of-function approach to analyse the potential role of E4BP4 expressed in motoneurons. When motoneurons are purified from rat embryos and cultured at low density without neurotrophic factors such as BDNF or GDNF, ~50% undergo programmed cell death during the first day of culture (Henderson et al., 1993; Henderson et al., 1994); this is generally considered to model cell death in vivo as neurons compete for access to trophic support. We reasoned that this would provide a means of assaying for factors that either exacerbated (pro-apoptotic) or inhibited (anti-apoptotic) cell death. In order to overexpress the transcription factor E4BP4 in motoneurons, we used a technique recently developed in our laboratory for electroporation of neurons in suspension, which gives transduction rates of at least 50% of surviving motoneurons, and a co-electroporation efficacy of 90% with reporter plasmids (Raoul et al., 2002). Only about 10% of the electroporated motoneurons survive the electroporation procedure. However, they show normal healthy morphology and retain normal survival and death responses, suggesting that they are representative of the complete population of motoneurons.

Survival values were expressed as a percentage of the number of neurons surviving with neurotrophic support at the same time. When electroporated with GFP vector alone, more than 40% of motoneurons died after 1 day in culture in the absence of trophic support (Fig. 2A), as with non-electroporated neurons. Co-electroporation with the E4BP4 plasmid reduced this figure to less than 10%, even after 2 days (Fig. 2A). Thus, forced expression of E4BP4 has similar effects to those of exogenous trophic factors. In long-term cultures (5 days in vitro), the survival effect was barely significant (Fig. 2A), but it was not clear whether this was due to loss of E4BP4 expression or to limited duration of the E4BP4 effect. To determine whether E4BP4 and BDNF were acting on the same motoneuron population, we electroporated motoneurons with either GFP vector alone, or with both GFP and E4BP4 vectors and cultured them in optimal concentrations of BDNF. The ratio of the number of neurons expressing GFP alone to that expressing GFP plus E4BP4 was 0.95±0.05 (mean±s.e.m., n=6 independent experiments). Very similar results were obtained using other potent trophic factors for motoneurons such as GDNF or a combination of BDNF, GDNF and CNTF (see Materials and methods). These data strongly suggest that E4BP4 and neurotrophic factors act on the survival of the same population of motoneurons, although they do not formally exclude the possibility that E4BP4 keeps alive a different population while having a pro-apoptotic effect on BDNF-responsive cells.

In order to determine whether survival effects of E4BP4 were limited to trophic deprivation, we exposed motoneurons cultured with optimal trophic support to a specific death inducer: soluble Fas ligand (sFasL) (Raoul et al., 1999). As

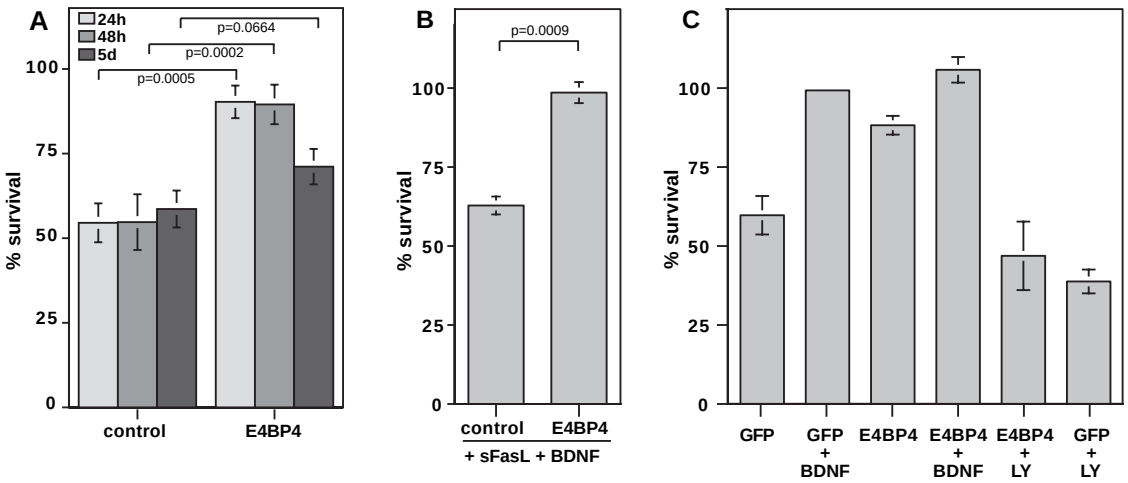

Fig. 2. Survival-promoting activity of E4BP4 over-expressed in motoneurons. (A) Effects on motoneuron survival following trophic deprivation. E14 rat motoneurons electroporated with a plasmid encoding GFP (control) or with GFP and E4BP4 were seeded in the absence or the presence (not shown) of the neurotrophic factor BDNF. Survival values were expressed as a percentage of the value in BDNF at the same stage (see text). E4BP4 provided nearly complete protection against cell death at 24 hours or 48 hours after seeding. In long-term cultures (5 days in vitro, 5 d), the survival promoting effect was barely significant. Values are means ± s.e.m.; n=3-5 independent experiments. Statistical significance was calculated by Student's t-test (two-tailed). (B) Protection by E4BP4 against cell death triggered by Fas death receptor. Motoneurons electroporated with control and E4BP4 plasmids were exposed to soluble Fas ligand (sFasL) in the presence of BDNF. Survival is expressed as a percentage of the value in controls without sFasL (mean±s.e.m., n=3). (C) Effects of the PI3K inhibitor LY294002 (LY) on motoneuron survival induced by E4BP4. Survival is expressed as a percentage of the value for control neurons in BDNF. Mean±s.e.m.; n=4.

with untransfected motoneurons, addition of sFasL in the presence of BDNF led to death of 35% of motoneurons electroporated with GFP (Fig. 2B). Here, overexpression of E4BP4 provided complete protection against Fas-triggered cell death (Fig. 2B).

Many neurotrophic factors act through PI3 kinase to mediate their survival effects, and in pro-B cells it has been shown that PI3K can act upstream of E4BP4 to enhance survival (Kuribara et al., 1999). Low concentrations (10 μM) of the PI3K inhibitor LY294002 were added to electroporated motoneurons in different conditions. LY294002 only slightly reduced survival of GFP-expressing motoneurons cultured in basal medium (Fig. 2C) but completely inhibited the trophic effect of BDNF (not shown). Inhibition of PI3 kinase completely blocked the anti-apoptotic effect of E4BP4 (Fig. 2C).

Effects of E4BP4 on cell size

In order to determine whether E4BP4 affected other aspects of motoneuron development, we performed quantitative analysis of cell body area (Fig. 3). As expected, addition of BDNF tended to increase cell size. Surprisingly, the effect of E4BP4 on cell size was even greater (Fig. 3A). Results from five independent experiments were combined by calculating a median value (area attained by 50% of individual neurons) from each data set (Fig. 3B). Whereas BDNF increased neuronal area by 16%, E4BP4 expression led to a 44% increase. As BDNF and E4BP4 act on the same motoneurons (see above), this must reflect the increase in size of individual neurons induced by E4BP4, and not the survival of a subpopulation of large neurons. Addition of BDNF to cells overexpressing E4BP4 did not further increase cell body size (7.3±0.3 pixels; n=3), suggesting that cells that responded to BDNF represented a subpopulation of the E4BP4-sensitive neurons. As in the case of the survival experiments, the effect of E4BP4 on cell size was blocked by 10 μM LY294002 (not shown).

Effects of E4BP4 on axonal growth

Inspection of electroporated neurons revealed another clear effect of E4BP4: an increase in neurite length (Fig. 4A). For all neurons in a given condition, we therefore quantified total neurite length and length of the longest neurite. As these gave identical conclusions, only results with the latter are shown (Fig. 4B). Given the interneuron variation in all conditions, we used cumulative plots in which the percentage of neurons that had attained a given length was plotted as a function of neurite length; these provide an overall view of the neurons in each population (Bloch-Gallego et al., 1993).

Addition of BDNF to GFP-electroporated neurons produced a clear shift to the right (i.e. longer neurites) when compared with the cells from the same preparation cultured in basal medium (Fig. 4B). As there were survival differences between the two conditions (Fig. 2A), this could arguably reflect the saving of a population of large neurons by BDNF. However, E4BP4 once again produced a much stronger shift than BDNF (Fig. 4B), indicating that this was not only a survival effect. When median values were calculated, they revealed that over the first day in culture, E4BP4 produced more than a threefold increase in axonal growth compared with basal medium, and a twofold increase compared with BDNF (Fig. 4C). This effect was also PI3 kinase-dependent (not shown). Addition of BDNF

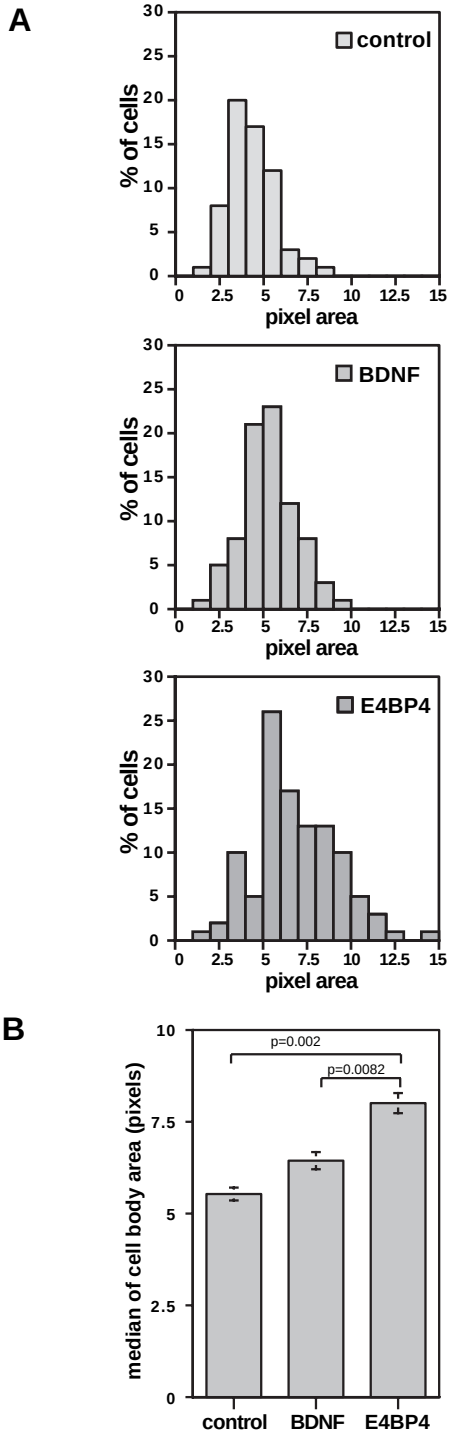

Fig. 3. E4BP4 enhances cell body growth. (A) Histograms for cell body area from a single experiment. Motoneurons electroporated with GFP were cultured without trophic factors (control), or with neurotrophic factor (BDNF). Motoneurons expressing E4BP4 were cultured without BDNF. Cell body area for 80-200 neurons in each condition was measured 1 day later and expressed in arbitrary pixel units. There is a clear shift to larger sizes in motoneurons expressing E4BP4. (B) Median cell body areas in each condition (mean±s.e.m.; n=5 independent experiments). Statistical significance was calculated by Student's t-test (two-tailed). The addition of BDNF to E4BP4 electroporated motoneurons did not further increase cell body size (7.3±0.3 pixels; n=3).

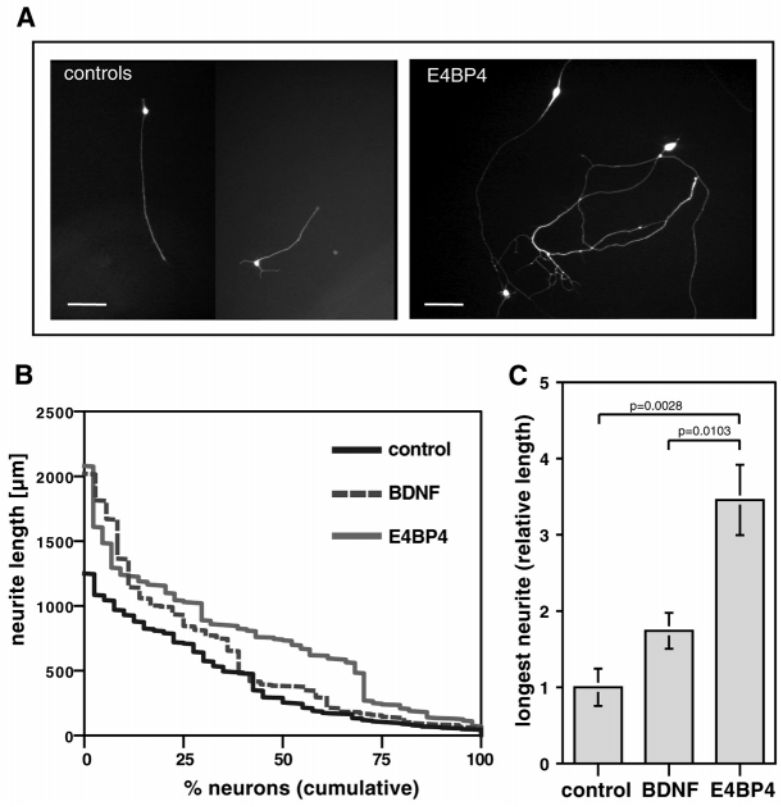

Fig. 4. E4BP4 potently stimulates axonal outgrowth. (A) Typical images of control and E4BP4-expressing motoneurons after 1 day in culture, as used for subsequent image analysis. Scale bar: 20 µm. (B) Cumulative plot of length of longest neurite from one representative experiment. For each value of neurite length, the percentage of the total number of neurons having a neurite at least that length is plotted. A shift of the curve to the right thus indicates increased axonal growth. Values from control neurons (black line), control neurons with BDNF (broken line) and E4BP4-expressing neurons without BDNF (grey line) are shown. (C) Median values for length of the longest neurite, expressed relative to control neurons with BDNF, fixed as 1 (mean±s.e.m.; *n*=5 independent experiments).

to E4BP4-expressing neurons produced no further increase in outgrowth (see legend to Fig. 4).

In vitro, therefore, E4BP4 has profound effects on neuronal growth. In our experiments, these were greater than those of BDNF or GDNF (not shown). However, this may simply reflect stronger stimulation of growth pathways by overexpression of *E4BP4* than by application of exogenous growth factors. The non-parallelism of the E4BP4 and BDNF curves in Fig. 4C may reflect the fact that E4BP4 is active in all electroporated neurons, whereas only a fraction of them respond to BDNF.

Regulation of E4BP4 in cultured motoneurons by neurotrophic factors

Levels of E4BP4 in pro-B cells are upregulated by survival factors. To determine if this was the case in motoneurons, we looked for regulation of E4BP4 mRNA and protein in cultured ventral spinal neurons from E13 rat as a function of neurotrophic support. The choice of embryonic age reflected our findings (Fig. 1) that levels of E4BP4 are lower at the beginning of the cell death period. Cells were cultured (or not) with a cocktail of growth factors composed of BDNF, CNTF and GDNF (see Materials and methods). We analysed expression levels of *E4BP4* in these cultures 6 hours after plating by semi-quantitative RT-PCR (Fig. 5A). To avoid high PCR cycle numbers, we then performed specific radioactive hybridisation to detect RT-PCR products. Under these conditions, the expression level of *E4BP4* in motoneurons was not changed by treatment with trophic factors (Fig. 5C). At later time points (16, 18 or 24 hours after plating; data not shown) levels remained constant. Similarly, no change in *E4BP4* levels was observed when we tested BDNF (10 ng/ml), CNTF (10 ng/ml), GDNF (1 ng/ml), HGF (10 ng/ml), NT3 (3.8 ng/ml), BMP7 (10 ng/ml) or CT1 (10 ng/ml) alone (data not shown). To look for regulation of E4BP4 protein, we performed western blot analysis on dissociated rat E13 spinal neurons. Again, E4BP4 levels were not altered in the presence of a cocktail of BDNF, CNTF and GDNF (Fig. 5B,C). We therefore conclude that E4BP4 expression levels in motoneurons are not regulated by neurotrophic factors in vitro.

E4BP4 is a neuronal survival factor in vivo

The potency of these unexpected functional effects of E4BP4 led us to ask whether E4BP4 might affect the survival of motoneurons during the cell death period in vivo. For this, we used electroporation in ovo to transduce motoneuron precursors at early stages, with the aim of analysing effects on survival at later stages (Fig. 6A; see Materials and methods). When expression plasmids encoding GFP were introduced into one side of the brachial neural tube at E2.5, distinct fluorescence in motoneurons could still be detected up to 6 days later. Although this may in part reflect the intrinsic stability of the GFP protein, it also suggested that unintegrated plasmids are sufficiently stable in these conditions to have functional effects during the motoneuron cell death period. Therefore this approach, which has been much used for studies of early development, will be of general interest for studying later phenomena such as cell death.

To quantify the effects of E4BP4 overexpression on motoneuron survival, we needed to be able to visualise simultaneously those cells that had been transduced and those that were undergoing programmed cell death. For this, we used the whole-mount TUNEL labelling technique that allows

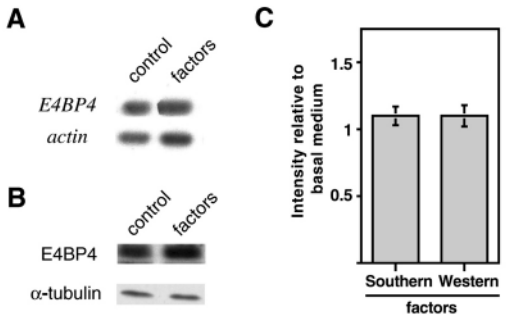

Fig. 5. E4BP4 is not regulated by survival signalling pathways in motoneurons. Semi-quantitative RT-PCR and western-blot analysis of E4BP4 expression in cultured motoneurons in the presence or absence of trophic support 6 hours after plating. (A) Radioactive Southern-blot analysis of RT-PCR amplified transcripts. (B) Western blots showing E4BP4 and α-tubulin expression. (C) Relative band intensities of RT-PCR and Western-blot analysis. Each band was scanned and its intensity analysed by Image J software. Each single E4BP4 band was normalised with respect to its corresponding actin or tubulin control band and then the values for parallel cultures with or without trophic factors were expressed as an intensity ratio (see Materials and methods). Mean values are plotted (mean±s.e.m.; n=30 independent RT-PCR experiments and n=10 western blots).

apoptotic nuclei to be detected and counted in 'open-book' preparations of whole spinal cord (Yamamoto and Henderson, 1999). Only motoneurons undergo cell death during the period examined in this study. Embryos were electroporated at E2.5, either with the GFP vector alone or with a combination of vectors encoding GFP and full-length chicken E4BP4. They were maintained until E7 and spinal cords were dissected. Only embryos showing fluorescence in the ventral roots when examined under the dissecting microscope were processed for TUNEL analysis (Fig. 6B).

Overexpression of GFP alone did not affect motoneuron cell death (Fig. 6D). However, E4BP4 clearly modified the pattern of dying motoneurons. In the single embryo illustrated (Fig. 6C), three representative zones are apparent. In zone 1, expression of E4BP4 in a position that was dorsal to motoneurons undergoing cell death did not affect the TUNEL signal (asterisk). By contrast, in zone 2, E4BP4 expressed in motoneurons that were actively undergoing cell death significantly reduced the number of TUNEL-positive profiles (compare black and open arrowheads). Last, in zone 3, where no PCD was normally detected by TUNEL, overexpression of E4BP4 in motor columns did not induce apoptosis (black arrow).

To quantify effects of E4BP4 on motoneuron death, we defined for each embryo a zone of apparently continuous GFP expression in the ventral horns ('electroporated area' in Fig. 6A,D) and counted the number of TUNEL-positive nuclei on each side. The result was expressed as a ratio of treated to

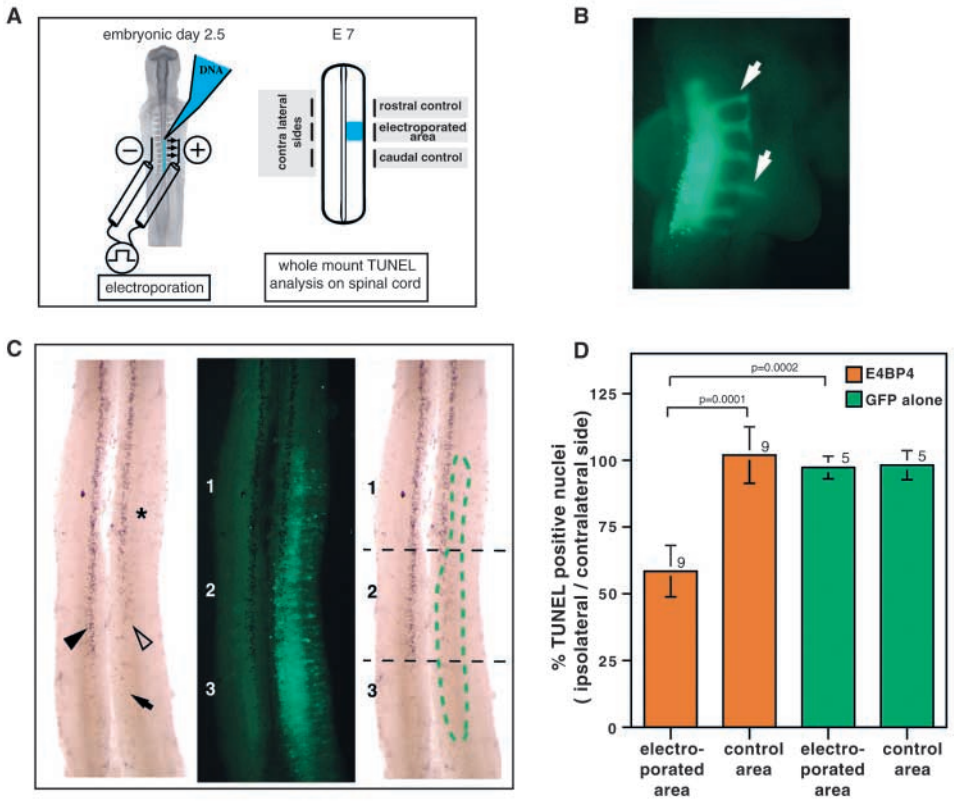

Fig. 6. E4BP4 reduces programmed cell death of motoneurons in vivo. (A) Scheme showing the electroporation protocol and areas used for TUNEL quantification. (B) High-magnification view of an E5 chicken embryo expressing GFP in ventral roots (arrows), used as a criterion for selecting embryos in which motoneurons were successfully electroporated. (C) Images of one representative spinal cord co-electroporated with GFP and E4BP4, and viewed at E7 using transmission (left panel) or fluorescence (centre panel) optics. In the right panel, the zone of GFP expression has been traced onto the transmission image. The spinal cord has been opened and dorsal regions appear at the left and right of the images, while the midline runs vertically through the tissue. The position of the ventral horns is visualised by the TUNEL signal, as only motoneurons undergo PCD at this stage. Within the area of GFP expression, three zones are apparent. In zone 1, E4BP4 is expressed dorsally to motoneurons and does not affect the TUNEL signal (asterisk). In zone 2, E4BP4 expressed in the motor columns reduces the number of TUNEL-positive cells compared with the contralateral side (compare black and open arrowheads). In zone 3, E4BP4 expressed in motoneurons not already undergoing cell death does not trigger apoptosis (arrow). (D) In each area indicated in A, numbers of TUNEL-positive neurons at E7 were expressed as a percentage of the value on the contralateral side in each spinal cord. Values are shown for embryos electroporated with GFP alone (green bars) or with GFP and E4BP4 (orange bars). Means±s.e.m. are indicated; numbers of embryos analysed are indicated above the bars. Overexpression of E4BP4 reduces the number of dying motoneurons by 45%, whereas GFP alone has no effect.

untreated sides. To eliminate the possibility of asymmetric TUNEL labelling resulting from other causes, two GFP-negative regions immediately rostral and caudal to this zone in each spinal cord were also counted (Fig. 6A; 'control area' in Fig. 6D). Overexpression of E4BP4 led to a 45% reduction in the number of TUNEL-positive profiles (Fig. 6D), demonstrating its potent anti-apoptotic activity in vivo. Given that probably not all motoneurons in the 'electroporated zone' expressed GFP, this may correspond to saving of an even higher percentage of those motoneurons effectively transduced.

Discussion

Our results provide the first evidence for a role of the E4BP4 transcription factor in repressing programmed cell death of neurons during development. E4BP4 is expressed by motoneurons at increasing levels during the period of naturally-occurring cell death, and gain-of-function studies show that it has potent anti-apoptotic activity both in vitro and in vivo. Moreover, it robustly enhances growth of neuronal cell bodies and axons, suggesting that it or its targets may play other roles during the crucial period of axonal growth and target innervation.

In common with E4BP4 in pro-B lymphocytes, but in contrast to CES-2 in *C. elegans*, E4BP4 in motoneurons is anti-apoptotic in vivo and in vitro. The complete resistance to Fas-triggered cell death conferred by E4BP4 provides a particularly striking demonstration of this. The different functions of CES-2 in NSM neurons and E4BP4 in pro-B cells or motoneurons most probably result from different genetic interactions with downstream effectors: *CES-2* inhibits the anti-apoptotic *CES-1* transcription factor and thereby triggers cell death, whereas the fusion protein E2A-HLF, which is thought to act through a similar mechanism to E4BP4, activates the anti-apoptotic CES-1 homolog Slug and therefore promotes survival (Ikushima et al., 1997; Inukai et al., 1999; Metzstein et al., 1996; Metzstein and Horvitz, 1999). No expression of Slug, Snail or their direct homologs has been described in motoneurons, and so the pathway downstream of E4BP4 may be novel.

The actions of E4BP4 are most probably cell-autonomous, as expected for a transcription factor. It can act in low-density cultures to enhance the survival of those cells that express it, and in vivo when expressed more dorsally in the spinal cord it does not affect motoneuron survival at the same rostrocaudal level. Thus, E4BP4 is probably an intrinsic determinant of cell survival. Indeed, the role of transcriptional events in controlling the response of neurons to death and survival factors is becoming progressively more apparent (Brunet et al., 2001; Wiese et al., 1999). Two examples from our laboratory include the requirement for transcription of *REG-2* in the CNTF survival pathway (Nishimune et al., 2000) and the upregulation of *nNOS* in a motoneuron-specific death pathway triggered by the Fas receptor (Raoul et al., 2002). Of particular interest in the present context is the report that NGF-mediated neuronal survival requires CREB (Lonze et al., 2002; Riccio et al., 1999), which participates in similar transcription factor complexes to E4BP4 and is also required in the IL3 survival pathway (Chen et al., 2001). Neurons, which are essentially irreplaceable cells, may have developed slower, but more tightly controlled, mechanisms to regulate their numbers.

The effects of E4BP4 on growth of cell bodies and axons were at least as striking as those on survival. What determines the final size of a neuron, or indeed any particular cell type, has been little studied (Conlon and Raff, 1999). However, recent reports point to a crucial involvement of the PI3 kinase pathway (Backman et al., 2001; Groszer et al., 2001; Heumann et al., 2000; Kwon et al., 2001; Markus et al., 2002; Namikawa et al., 2000). This fits well with our observations that all effects of E4BP4 in motoneurons were completely inhibited by the PI3K inhibitor LY294002, suggesting that either E4BP4 activates the PI3K pathway, or PI3K needs to be activated by other means in order to cooperate with E4BP4 in promoting survival and growth. Indeed, E4BP4 can itself be phosphorylated in certain situations (Chen et al., 1995; Zhang et al., 1995). Unfortunately, the biochemical studies required to distinguish between these possibilities are not accessible with the low quantities of material available using electroporated motoneurons.

The literature suggested another potential level of involvement of PI3K: in pro-B cells, PI3K acts upstream of E4BP4: survival factors activate PI3K and thereby upregulate E4BP4 (Kuribara et al., 1999). However, unexpectedly, E4BP4 levels in motoneurons did not change following treatment with classical growth and survival factors. Our results suggest that E4BP4 is not simply a read-out or signalling intermediate for exogenous survival signals, but rather may have significant cell-autonomous functions, as in the nematode.

Our gain-of-function studies in vitro and in vivo gave results consistent with our deduction from the expression pattern in vivo. Nevertheless, it will obviously be of interest to determine by loss-of-function studies to what extent this pathway is active during normal development. We ruled out the use of a dominant-negative approach because of the non-specificity of heterodimerisation of transcription factors, and because interpretation would be complicated by the ability of E4BP4 to function either as a trans-repressor or a trans-activator (Cowell et al., 1992; Lai and Ting, 1999; Zhang et al., 1995). Null-mutant mice for E4BP4 have not been reported.

In conclusion, the parallels between the expression of CES-2 in neurosecretory motoneurons of the *C. elegans* pharynx and of E4BP4 in rat and chicken spinal motoneurons provide another example of the evolutionary conservation of elements of cell death and survival pathways. However, as in many other cases, the function of E4BP4 seems to have been modified during development. In particular, E4BP4 functions in vertebrate neurons as an intrinsic survival factor, in accordance with its expression pattern in developing spinal cord and its regulation. The pathways activated by E4BP4 therefore represent potentially interesting targets for therapeutic strategies aimed at nervous system regeneration and repair. Given its function in other systems as a clock gene (Doi et al., 2001; Mitsui et al., 2001), it is also interesting to speculate that E4BP4 may also be involved in the timing of cell death during development.

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