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Mediation by a CREB Family Transcription Factor of NGF-Dependent Survival of Sympathetic Neurons

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Nerve growth factor (NGF) and other neurotrophins support survival of neurons through processes that are incompletely understood. The transcription factor CREB is a critical mediator of NGF-dependent gene expression, but whether CREB family transcription factors regulate expression of genes that contribute to NGF-dependent survival of sympathetic neurons is unknown. CREB-mediated gene expression was both necessary for NGF-dependent survival and sufficient on its own to promote survival of sympathetic neurons. Moreover, expression of Bcl-2 was activated by NGF and other neurotrophins by a CREB-dependent transcriptional mechanism. Overexpression of Bcl-2 reduced the death-promoting effects of CREB inhibition. Together, these data support a model in which neurotrophins promote survival of neurons, in part through a mechanism involving CREB family transcription factor–dependent expression of genes encoding prosurvival factors.

Nerve growth factor (NGF), a member of the neurotrophin family of growth factors, is a target-derived survival factor for developing sympathetic neurons (*1*). CREB [cyclic adenosine monophosphate (cAMP) response element binding protein] is a mediator of the nuclear response to neurotrophins in PC12 cells and cortical neurons (*2*–*5*). Moreover, exposure of distal axons of sympathetic neurons grown in compartmentalized cultures to NGF results in robust phosphorylation of CREB on its transcriptional regulatory site, serine 133 (*6*). These observations support the idea that a target-derived NGF signal is propagated retrogradely from distal axons to CREB within nuclei of developing sympathetic neurons to control gene transcription. Whether CREB-dependent gene expression is required for survival of sympathetic neurons is unclear. Moreover, the identity of NGF-sensitive genes that contribute to growth and survival of sympathetic neurons is unknown.

Neonatal sympathetic neurons are completely dependent on NGF for survival in vivo and in vitro. To determine whether CREB-mediated gene expression is necessary for NGF-dependent neuronal survival, we monitored survival of sympathetic neurons after expression of either of two distinct inhibitors of CREB. One CREB inhibitor, A-CREB, is a potent and selective inhibitor of CREB DNA binding activity (*4*). The other, CREBm1, binds to CREB binding sites in DNA but is not activated because the transcriptional regulatory residue, serine 133, is mutated to alanine (*7*). Microinjection of expression vectors encoding either A-CREB or CREBm1, but not vector alone, led to nearly complete apoptotic death of sympathetic neurons grown in the presence of NGF, as assessed by Hoechst

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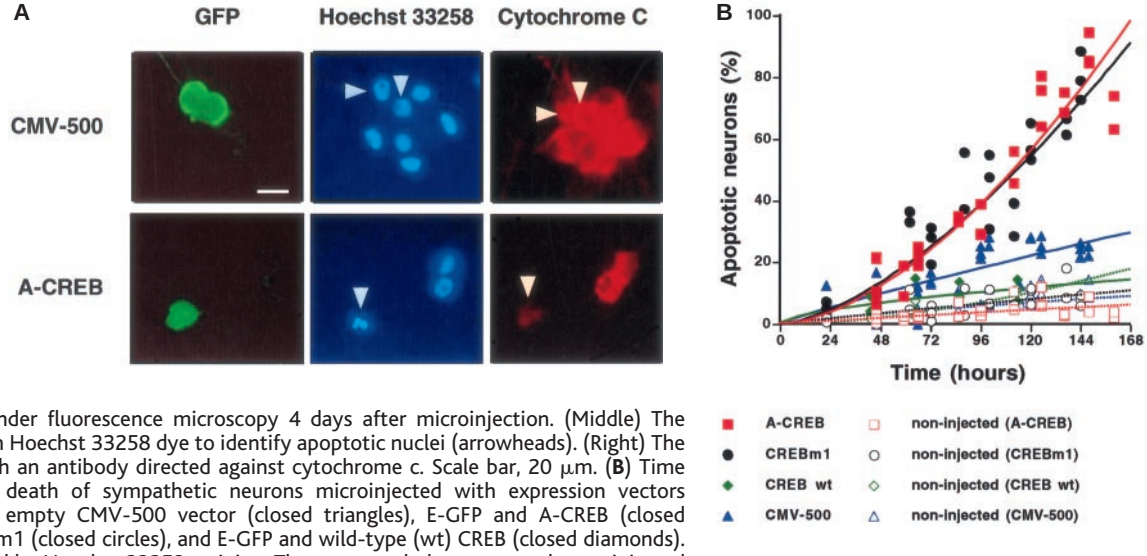

Fig. 1. Requirement of a CREB family transcription factor for survival of sympathetic neurons grown in the presence of NGF. Six to nine DIV sympathetic neurons were microinjected with expression vectors encoding GFP and either a CMV-500 expression vector or expression vectors encoding either A-CREB or CREBm1 (*23*). (**A**) Apoptotic cell death of sympathetic neurons expressing A-CREB. (Left) Neurons photographed under fluorescence microscopy 4 days after microinjection. (Middle) The same neurons stained with Hoechst 33258 dye to identify apoptotic nuclei (arrowheads). (Right) The same neurons stained with an antibody directed against cytochrome c. Scale bar, 20 μm. (**B**) Time course of apoptotic cell death of sympathetic neurons microinjected with expression vectors encoding E-GFP and the empty CMV-500 vector (closed triangles), E-GFP and A-CREB (closed squares), E-GFP and CREBm1 (closed circles), and E-GFP and wild-type (wt) CREB (closed diamonds). Cell viability was measured by Hoechst 33258 staining. The open symbols represent the noninjected cells from the same plates. Each data point represents the value from one individual plate of microinjected neurons for which viability of 50 or more microinjected or noninjected neurons was determined.

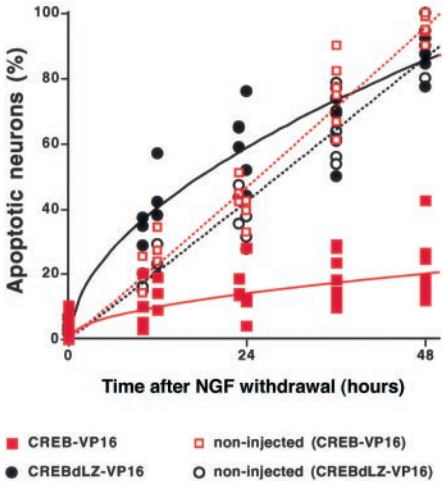

Fig. 2. Activation of CRE-mediated gene expression promotes survival of sympathetic neurons after NGF withdrawal. Time course of apoptotic cell death of sympathetic neurons microinjected with an expression vector encoding GFP and either CREB-VP16 (closed squares) or CREBdLZ-VP16 (closed circles) or noninjected neurons from the same cultures (open circles and squares). NGF was withdrawn 48 hours after microinjection (*23*). Each data point represents the value from one individual plate of microinjected neurons for which viability of 50 or more microinjected or noninjected neurons was determined.

33258 staining of sympathetic neuron nuclei (Fig. 1A). Moreover, inhibition of CREB led to a loss of cytochrome c from mitochondria (Fig. 1A), an event that precedes, and is necessary for, activation of caspases in several models of programmed cell death (*8*). After expression of either A-CREB or CREBm1, sympathetic neurons underwent apoptotic cell death over the course of several days; almost no healthy neurons were detectable 5 days after expression of either A-CREB or CREBm1 (Fig. 1B). Furthermore, expression of either A-CREB or CREBm1 enhanced the rate of apoptotic cell death after NGF withdrawal (*9*). In contrast, overexpression of wild-type CREB had no effect on NGF-dependent survival of sympathetic neurons (Fig. 1B). These observations indicate that CREB DNA binding activity and phosphorylation of CREB are necessary for NGF-dependent survival of sympathetic neurons. This is consistent with the findings that brains of *CREB*$^{-/-}$ mice are smaller than brains of their normal littermates (*10*) and mice that express CREBm1 in the anterior pituitary have few, if any, somatotrophs (*11*).

We tested whether activation of expression of CREB-regulated genes was sufficient to promote survival of sympathetic neurons. We used an expression vector encoding a CREB-VP16 fusion protein (CREB-VP16), which binds to cAMP response elements (CRE) through the basic-leucine zipper (B-ZIP) domain of CREB and activates transcription through the transcriptional activation domain of the herpes virus VP16 protein (*12*). Sympathetic neurons were microinjected with expression vectors encoding either CREB-VP16 or CREBdLZ-VP16, which serves as a negative control because it lacks the CREB B-ZIP domain and cannot bind to CRE sequences. The effects of the microinjected constructs were determined for sympathetic neurons grown in the absence of NGF. To ensure complete removal of NGF from the cultures, we replaced growth medium containing NGF with medium containing antibody to NGF 2 days after microinjection. Nearly all sympathetic neurons that expressed CREBdLZ-VP16 and noninjected neurons underwent apoptotic cell death within 36 to 48 hours after NGF withdrawal. In contrast, the majority of sympathetic neurons microinjected with the CREB-VP16 expression vector survived more than 48 hours after NGF withdrawal (Fig. 2). Many sympathetic neurons expressing CREB-VP16 survived and had large cell bodies and long healthy processes more than 4 days after NGF withdrawal, despite the fact that essentially all of their noninjected neighbors had long since died and the culture plates contained much cellular debris. Taken together, our results indicate that CREB-mediated gene expression is both necessary and sufficient for NGF-dependent survival of sympathetic neurons.

We used cDNA array analyses to identify NGF-sensitive genes in sympathetic neurons. We identified several genes whose expression increased in response to exposure of sympathetic neurons to NGF (*9*). Among the genes with the greatest increase in expression in response to NGF is *bcl-2*. The *bcl-2* gene family includes proapoptotic and antiapoptotic members that control survival of many cell types, including neurons. Overexpression of Bcl-2 prevents programmed cell death of sympathetic neurons (*13*). Moreover, many sympathetic neurons from *bcl-2*$^{-/-}$ mice die when grown in either the presence or absence of NGF in vitro (*14*), and 40% of sympathetic neurons in the superior cervical ganglion die during postnatal development of *bcl-2*$^{-/-}$ mice in vivo (*15*). Thus, Bcl-2 is both necessary and sufficient for survival of sympathetic neurons. Amounts of Bcl-2 mRNA increased after exposure of sympathetic neurons to NGF (Fig. 3A). Exposure of PC12 cells to NGF, or of cortical neurons to the related neurotrophin brain-derived neurotrophin factor (BDNF), also led to increased expression of Bcl-2 (Fig. 3A). Neurotrophin treatment also increased amounts of Bcl-2 protein, as determined by protein immunoblotting (Fig. 3B). An increase in the amount of Bcl-2 protein was detected within 3 hours of exposure of sympathetic neurons to NGF. Exposure of distal axons of sympathetic neurons grown in compartmentalized cultures to medium containing a high concentration of NGF also led to an accumulation of Bcl-2 mRNA (Fig. 3C). This result indicates that NGF activates a signal that propagates retrogradely from distal axons to cell bodies and regulates expression of Bcl-2. A retrograde NGF signal is carried from distal axons

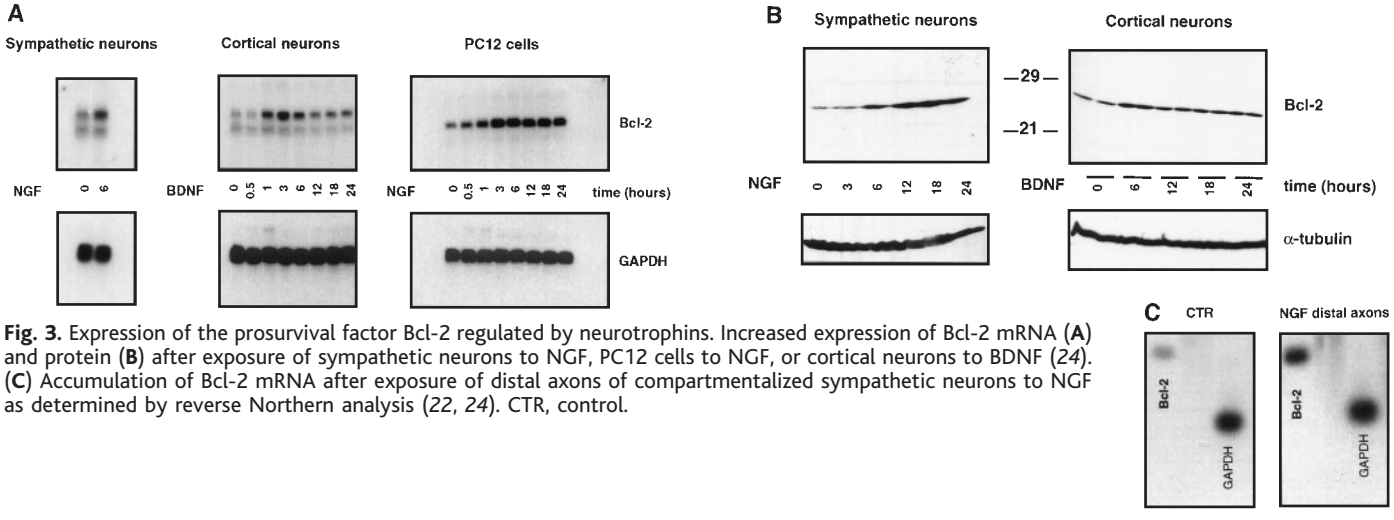

Fig. 3. Expression of the prosurvival factor Bcl-2 regulated by neurotrophins. Increased expression of Bcl-2 mRNA (**A**) and protein (**B**) after exposure of sympathetic neurons to NGF, PC12 cells to NGF, or cortical neurons to BDNF (*24*). (**C**) Accumulation of Bcl-2 mRNA after exposure of distal axons of compartmentalized sympathetic neurons to NGF as determined by reverse Northern analysis (*22*, *24*). CTR, control.

to activate CREB in the nucleus (*6*). Thus, an NGF-mediated retrograde signal may increase expression of Bcl-2 through phosphorylation and activation of CREB.

CREB and Bcl-2 are both necessary and sufficient for survival of sympathetic neurons, and CREB is a mediator of NGF-dependent gene transcription. Therefore, we sought to determine whether CREB mediates NGF induction of expression of Bcl-2 in neuronal cells. To test this possibility, we performed promoter analysis experiments with constructs that contained various regions of the *bcl-2* promoter region fused to a luciferase reporter gene (*16*). These constructs were introduced into PC12 cells, an NGF-responsive cell line. Two days later, cells were exposed to NGF for various times, and luciferase activity was monitored as an indicator of transcriptional activity of the *bcl-2* reporter genes. A region of the *bcl-2* gene between −1640 and −1337 relative to the translation start site was required for NGF-sensitive transcription (Fig. 4A). This region contains a near-perfect consensus CRE (Fig. 4B). Activated CREB can bind to this region of the *bcl-2* promoter, and this interaction is critical for expression of Bcl-2 in a B lymphocyte cell line (*16*). Thus, we tested whether the integrity of the *bcl-2* CRE was necessary for the NGF-induced expression of *bcl-2*. A *bcl-2* reporter construct harboring a two–base pair mutation of the CRE, rendering it unable to bind CREB, was impaired in its responsiveness to NGF (Fig. 4B).

Because CREB is the major CRE binding protein in PC12 cells and sympathetic neurons (*9*), we suggest that CREB is a mediator of neurotrophin-induced expression of Bcl-2. Consistent with this idea, A-CREB and CREBm1 completely blocked NGF-induced expression of a *bcl-2* reporter gene in PC12 cells (Fig. 4C). Moreover, expression of CREB-VP16, but not CREBdLZ-VP16, led to an accumulation of a *bcl-2*–luciferase reporter gene in PC12 cells (Fig. 4D) and endogenous Bcl-2 mRNA in PC12 cells and HEK 293T cells (Fig. 4E). Finally, amounts of Bcl-2 protein were reduced in brains of *CREB*$^{-/-}$ embryonic day (E) 16 to E18 embryos (*10*) as compared with brains from control heterozygous and wild-type littermates (Fig. 4F). Together, these results indicate that CREB is a

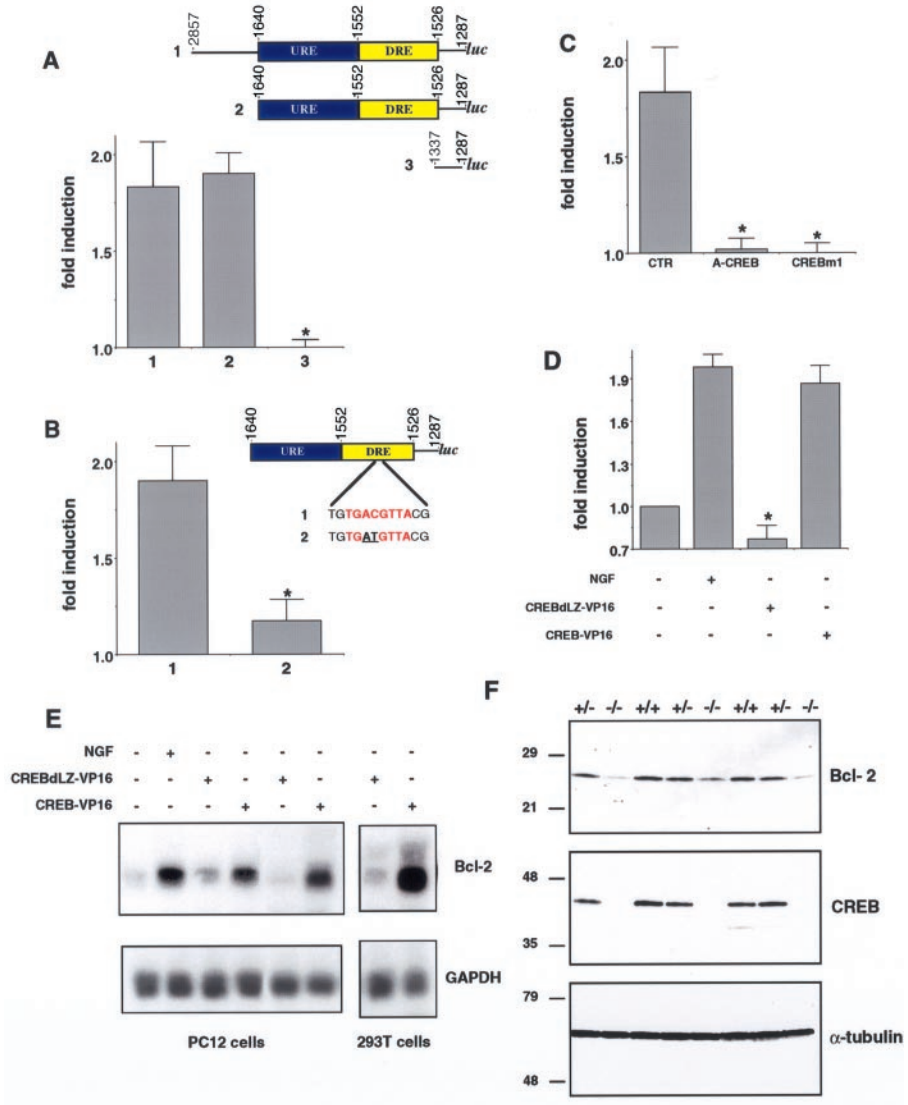

Fig. 4. NGF-induced expression of Bcl-2 through a CREB-dependent mechanism. (**A** to **D**) *bcl-2*–luciferase reporter constructs (*16*) were introduced into PC12 cells by transfection. After 2 days, cells were treated without or with NGF (100 ng/ml) for 6 hours, and luciferase activity was determined (*25*). For experiments shown in (C) and (D), the −2857/−1287 *bcl-2* luciferase reporter gene was cotransfected into PC12 cells with either a control vector (CTR) or expression vectors encoding either A-CREB or CREBm1 (C) or CREBdLZ-VP16 or CREB-VP16 (D). All values were normalized for expression of TK-*renilla* luciferase, which served as a control for transfection efficiency. Each experiment was done at least four times in either duplicate or triplicate. Shown are means ± SEM of normalized luciferase measurements for extracts from cells treated with NGF relative to luciferase activities for untreated cells (*, $P < 0.05$), except for values shown in (D), which are relative to untreated cells. (**E**) PC12 cells (left) or HEK 293T cells (right) were either untransfected (left two lanes) or transfected with either CREB-VP16 or CREBdLZ-VP16 (*25*). Two days later, cells were treated without or with NGF for 3 hours, RNA was isolated, and Northern blotting was done to assess expression of Bcl-2 and GAPDH mRNAs (*24*). (**F**) Expression of Bcl-2, CREB, and tubulin protein was assessed in extracts from brains of wild-type (+/+), heterozygous (+/−), and *CREB*$^{-/-}$ embryos by immunoblotting (*24*).

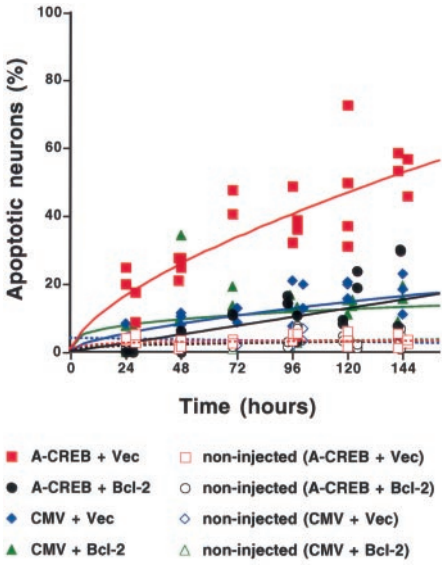

Fig. 5. Rescue by Bcl-2 of sympathetic neurons from apoptotic cell death due to inhibition of CREB. Six to eight DIV sympathetic neurons were subjected to microinjection with an expression vector encoding E-GFP and either the CMV-500 control vector (CMV) or the A-CREB expression vector in the presence or absence of an expression vector encoding Bcl-2 or empty vector (Vec) (*23*). Cell viability was measured by Hoechst 33258 staining. Each data point represents the value from one individual plate of microinjected neurons for which viability of 50 or more microinjected or noninjected neurons was determined.

mediator of expression of Bcl-2 in vitro and in vivo.

CREB appears to mediate NGF-dependent neuronal survival and expression of Bcl-2. We tested whether Bcl-2 would overcome the proapoptotic effects of inhibition of CREB-dependent gene expression in sympathetic neurons. We introduced the A-CREB expression construct into sympathetic neurons by microinjection in the presence of either an expression vector encoding Bcl-2 or an empty expression vector. Expression of A-CREB led to apoptotic death of sympathetic neurons that was prevented by overexpression of Bcl-2 (Fig. 5). Taken together, our results support a model in which NGF promotes transcription of antiapoptotic factors, such as Bcl-2, and promotes sympathetic neuron survival through a mechanism requiring CREB family transcription factors.

Note added in proof: It was recently reported that CREB mediates survival of granulosa cells and cerebellar granule neurons (*17*) and that NGF regulates Bcl-2 expression through a p42/p44 MAPK cascade in PC12 cells (*18*).

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23. Sympathetic neurons were isolated from superior cervical ganglia of neonatal rats (*19*) and grown in normal growth medium (*6*). Nuclei of neurons grown for 6 to 9 days in vitro (DIV) were microinjected with plasmids with an Eppendorf Transjector and Micromanipulator System. Before microinjection, neurons were placed in serum-free Hanks' balanced salt solution containing bovine serum albumin (1%), penicillin, and streptomycin. Neurons were injected with microinjection solution [48 mM K_2HPO_4, 14 mM NaH_2PO_4, and 0.45 mM KH_2PO_4 (pH 7.2)] containing pEGFP (50 μg/ml), an expression vector encoding enhanced green fluorescent protein (GFP; Clontech), Rhodamine-dextran (4 mg/ml; Sigma; this was excluded in experiments done for cytochrome c immunocytochemistry), and the indicated expression vectors. The concentrations of all other DNAs in the microinjection solutions were 50 μg/ml, except for HYC–Bcl-2 or the empty HYC vector (*20*), which were 700 μg/ml. Expression of GFP reached a maximum within 3 or 4 days after microinjection. A-CREB potently and specifically inhibits the DNA binding activity of CREB and closely related family members ATF-1 and CREM (*4*). Even at very high concentrations, A-CREB does not block the DNA binding activity of any other B-ZIP transcription factor tested, including ATF-2, c-Jun, Fos, VBP, JunD, C/EBP, and VBP (*4*). CREB is the most abundant member of the CREB subfamily of B-ZIP transcription factors in sympathetic neurons as determined by electrophoretic mobility shift assays (*9*). Thus, the effects of A-CREB are probably through inhibition of DNA binding activity of CREB, rather than that of ATF-1 or CREM. For NGF withdrawal experiments, neurons were placed in a medium containing antibody to NGF (1:1000, Sigma) 2 days after microinjection. Neurons were stained with Hoechst 33258 (Molecular Probes), microinjected neurons were identified by GFP fluorescence microscopy, and injected and noninjected neurons were scored for apoptotic nuclei as described by S. B. Maggirwar *et al.* [*J. Neurosci.* **18**, 10356 (1998)]. Cytochrome c immunocytochemistry was done essentially as described (*8*).
24. Cortical neurons were dissociated from E18 rat embryos and grown in culture (*3*). PC12 cells were grown in Dulbecco's modified Eagle's medium in the presence of fetal bovine serum (10%) and horse serum (5%). Cultures were treated with medium containing BDNF or NGF, and total RNA was collected and analyzed by Northern (RNA) blot analysis (*2*). For reverse Northern experiments, sympathetic neurons were grown in 1-mm barrier compartmentalized cultures, which allows for separation of neuronal cell bodies and proximal axons from their distal axons, as described (*6*, *21*). After 7 days of growth to develop compartmentalization of cell bodies and distal axons, growth medium in the cell body compartments was replaced with a medium containing antibody to NGF for 6 days. Then, growth medium bathing distal axons was replaced with medium containing a low concentration of NGF (2 ng/ml) for 2 days. Distal axons were then exposed to a high concentration of NGF (100 ng/ml) for 3.5 hours. Messenger RNA was isolated with Microfast Track Kit (Invitrogen), and double-strand cDNA was obtained with the Superscript Choice System (Gibco). Reverse Northern analysis of Bcl-2 and GAPDH was performed as described (*22*). Protein immunoblot analysis was performed as described (*3*). Protein extract (25 μg) prepared from forebrains of mouse embryos was resolved by SDS–polyacrylamide gel electrophoresis and immunoblotted with a Bcl-2 monoclonal antibody (1 μg/ml; Santa Cruz Biotechnology), a CREB polyclonal antibody (1:500; NEB), and an α-tubulin monoclonal antibody (1:10,000; Sigma).
25. PC12 cells were transfected with plasmids with Lipofectamine Plus Reagent (LTI), and reporter gene activity was assessed 2 days later with the Dual Luciferase Assay System (Promega). Plasmids amounts used per 60 mm plate of cells were as follows: Bcl-2 reporter constructs, 2 μg; A-CREB and CREBm1 expression plasmids, 4 μg; and GFP, 0.5 μg. Luciferase expression values were normalized for transfection efficiency, which was determined by the expression of the TK-*renilla* luciferase expression vector (Promega), 0.05 μg of which was included in all transfections. About 30% of PC12 and 80% of HEK 293T cells were transfected as determined by counting the number of GFP-positive cells.

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Use of the Cell Wall Precursor Lipid II by a Pore-Forming Peptide Antibiotic

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Resistance to antibiotics is increasing in some groups of clinically important pathogens. For instance, high vancomycin resistance has emerged in enterococci. Promising alternative antibiotics are the peptide antibiotics, abundant in host defense systems, which kill their targets by permeabilizing the plasma membrane. These peptides generally do not act via specific receptors and are active in the micromolar range. Here it is shown that vancomycin and the antibacterial peptide nisin Z use the same target: the membrane-anchored cell wall precursor Lipid II. Nisin combines high affinity for Lipid II with its pore-forming ability, thus causing the peptide to be highly active (in the nanomolar range).

Nisin Z is a member of the lantibiotic family (lanthionine-containing antibiotics) and is produced by certain strains of *Lactococcus lactis*. Because of its nontoxicity for humans and its high bactericidal activity, it is used as a food preservative. Nisin is posttranslationally modified (Fig. 1A). Characteristic features of nisin are the ring systems formed by thioether bonds and the dehydrated amino acids. Nisin shares some properties with oth-