Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic

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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-

er pore-forming antibacterial peptides, such as an overall positive charge and amphipathicity. However, the high bactericidal activity of nisin against Gram-positive bacteria, relative to the lower activity of the antibacterial peptides of animal origin (such as magainin), has not been accounted for in the literature. Elucidation of the cause of the activity difference may lead to the development of new classes of antibiotics with high activities.

The difference in bactericidal activity between nisin and magainin is illustrated for the Gram-positive bacterium *Micrococcus flavus* in Fig. 1B. The *M. flavus* cells were killed by nanomolar concentrations of nisin [minimal inhibitory concentration (MIC) = 3.3 nM] (*1*). In contrast, magainin (*2*) was at least two orders of magnitude less active than nisin, because even at the highest peptide concentration tested (0.4 μM) not all *M. flavus* cells were killed.

The results of the viability assay correlate with the ability of the peptides to permeabilize the membrane of *M. flavus* cells (Fig. 1C). This permeabilization caused the dissipation of vital ion gradients such as potassium, resulting in dissipation of the proton-motive force and eventually cell death. Nisin efficiently permeabilizes *M. flavus* membranes: At 5 nM nisin a permeabilization effect was detected, whereas magainin was active only at 3 μM. However, when the two peptides were tested with membranes composed of a lipid extract of *M. flavus*, the membrane-permeabilizing activity of nisin dropped markedly (now active in the micromolar range), and magainin was the more active peptide (Fig. 1D). Similar results were obtained when potassium leakage from vesicles was measured (*3*), indicating that these large differences are not a reflection of the nature of the indicator molecule. In pure phospholipid systems both peptides form short-lived transmembrane pores, and it has been shown using synthetic phospholipids that negatively charged lipids play an active role in this process (*4*–*7*). Thus, a membrane composed of an isolate from a chloroform/methanol extract (containing mostly lipids and hardly any protein), or pure synthetic phospholipids, may lack one or more specific molecules needed for high nisin activity.

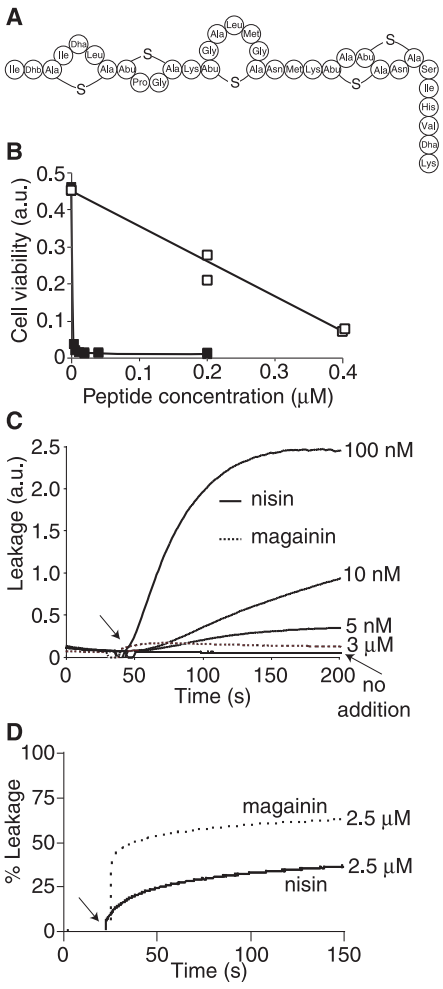

Fig. 1. Activity of nisin Z and magainin toward intact *M. flavus* cells and model membrane vesicles. (**A**) Primary structure of nisin Z. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine; S, the sulfur atom of the thioether bond. (**B**) Activity of nisin and magainin in a cell viability assay. Nisin (closed squares) and magainin (open squares) were added at the specific concentrations to *M. flavus* cells in complex growth medium (*18*), and viability was determined by measuring the absorbance at 600 nm as described (*19*). (**C**) Activity of nisin and magainin toward intact *M. flavus* cells. The peptide activity was measured by monitoring the effect on the membrane potential with the fluorescent membrane potential–sensitive probe 3,3′-diethylthiodicarbocyanine iodide [DiS-C_2(5)] (*20*). Cells were grown until mid-log phase, harvested, and washed once with a buffer solution of 250 mM sucrose, 5 mM $MgSO_4$, and 10 mM potassium phosphate (pH 7.0), then resuspended in the same buffer. Cells were added to the fluorescence cuvette at an optical density at 600 nm of 0.075 together with DiS-C_2(5) at 1 μM. (**D**) Activity of nisin and magainin toward model membranes composed of a lipid extract from *M. flavus*. The peptide activity was measured by monitoring the leakage of carboxyfluorescein from vesicles made from a lipid extract of *M. flavus* by measuring the increase in fluorescence due to dilution of the dye from self-quenching concentrations as described (*20*). The concentration of both nisin and magainin was 2.5 μM. The arrows in (C) and (D) mark the time point of peptide addition.

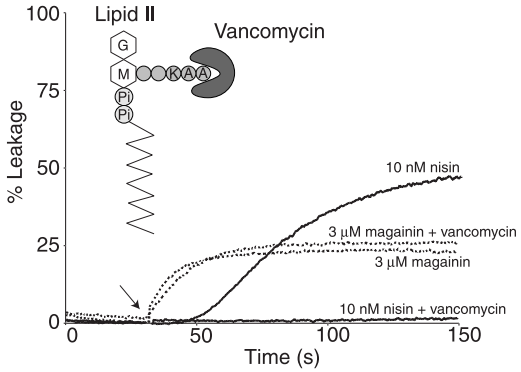

Fig. 2. Vancomycin blocks the activity of nisin against *M. flavus* cells. The activity of the peptides was monitored as described for Fig. 1. When present, vancomycin was added 2 min before the addition of nisin or magainin. Washing the cells after treatment with vancomycin gave similar results. The arrow marks the time point of peptide addition. Inset: Schematic structure of Lipid II with vancomycin bound to the COOH-terminal Lys-D-Ala-D-Ala sequence. The structure consists of a membrane-incorporated undecaprenyl moiety to which the amino sugar MurNAc is attached via a pyrophosphate. To the MurNAc (M) a pentapeptide is attached, of which the composition may slightly differ within different bacterial genera. The final subunit of Lipid II is GlcNAc (G). Pi, phosphate.

Next we examined the effect of nisin on *M. flavus* cells in the presence of vancomycin (Fig. 2). Vancomycin inhibited the membrane leakage activity of nisin against intact cells but did not affect the activity of magainin. Vancomycin kills bacteria by blocking the cell wall biosynthesis by binding to the Lys-D-Ala-D-Ala motif of the pentapeptide of the membrane-anchored cell wall precursor Lipid II (*8, 9*) (inset, Fig. 2). This suggests that Lipid II is used by nisin to permeabilize the membrane (*10*). If this is the case, then the cells should become more sensitive to nisin upon increasing the Lipid II content of their membranes. This is true for isolated cytoplasmic membranes of both *M. flavus* and *Escherichia coli* (Fig. 3A). Fusing Lipid II–containing model membranes with the bacterial membranes increased the sensitivity of both membrane types for nisin but not for magainin. The effect was the highest for the *E. coli* mem-

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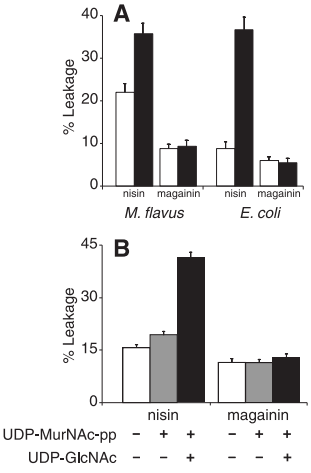

Fig. 3. Increase in the Lipid II content of isolated cytoplasmic membranes increases their nisin sensitivity. (**A**) Lipid II incorporation via vesicle fusion (solid bars, fused; open bars, control). Small unilamellar vesicles composed of 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine in an equimolar mixture with or without 1 mol % purified Lipid II were fused with isolated membrane vesicles of *E. coli* or *M. flavus* by rapidly freezing the mixture into liquid nitrogen and thawing on ice (*21*). Lipid II was purified from *Micrococcus luteus* as described (*22*). A membrane potential was generated in the membranes with the artificial electron donor/acceptor system ascorbate-phenazine methosulfate (*23*). The activity of 30 nM nisin or 400 nM magainin was monitored using the fluorescent dye DiS-C_2(5) as described (*20*). Maximum depolarization was determined by addition of a mixture of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which completely dissipates the membrane potential. (**B**) Regeneration of the Lipid II pool in *M. flavus* membranes increases the sensitivity toward nisin. The *M. flavus* membranes were subjected to eight freeze-thaw cycles in the presence of 0.1 mM UDP-MurNAc-pentapeptide and 0.1 mM UDP-GlcNAc (solid bars), or only 0.1 mM UDP-MurNAc-pentapeptide (gray bars), or with no addition (open bars). After freeze-thawing, the membranes were incubated for 1 hour at 20°C, which was essential for the enzyme reactions to take place. A membrane potential was generated, and the activity of nisin (30 nM) and magainin (400 nM) was determined as described for (A).

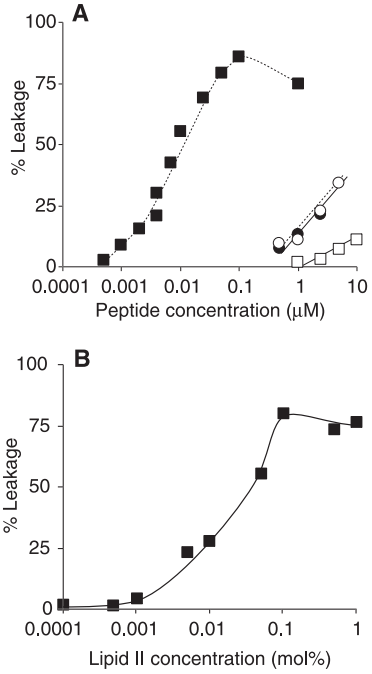

Fig. 4. (**A**) The presence of Lipid II in model membrane systems markedly increases the nisin activity. The activity of nisin (squares) and magainin (circles) was measured by monitoring the leakage of carboxyfluorescein from model membranes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine in the presence (filled symbols) or absence (open symbols) of 0.065 mol % purified Lipid II. (**B**) Nisin activity is dependent on the concentration of Lipid II in the membrane. The activity of nisin (100 nM) was measured as described for (A).

branes. One explanation for this effect lies in the intrinsic low Lipid II content of *E. coli*, which contains about 2000 molecules per cell (*11*), whereas *M. flavus* contains about 10^5 Lipid II molecules (*12*).

We observed that the *M. flavus* membranes were less sensitive toward nisin (by a factor 3 to 6) relative to the intact cells (*13*). This can be explained by a loss of part of the Lipid II pool during the isolation of the membrane vesicles, which is due to the high turnover rate of Lipid II. Because the Lipid II biosynthesis machinery is still active, this allowed us to regenerate Lipid II in the presence of biosynthetic precursors. Upon regeneration of the Lipid II pool by supplying the complete set of precursors, the sensitivity of the isolated *M. flavus* membranes to nisin increased, whereas the sensitivity for magainin was unaffected (Fig. 3B). When only one of the uridine 5′-diphosphate (UDP)–activated sugars was present, the vesicles did not become sensitive to nisin. This can be explained from current knowledge of the Lipid II biosynthesis pathway. Lipid II is synthesized at the cytosolic side of the plasma membrane. First, the UDP-activated amino sugar *N*-acetyl muramic acid (MurNAc), containing a pentapeptide, is attached to the undecaprenol carrier; then, Lipid II synthesis is completed upon attachment of the second UDP-activated amino sugar *N*-acetyl-D-glucosamine (GlcNAc). Lipid II is transported to the exterior side of the membrane and becomes available for binding by nisin only when the synthesis is completed. An additional finding was that the freeze-thawed membranes had to be incubated at 20°C for 1 hour to obtain the effect of the UDP-activated sugars (*3*). Apparently, this is one way to control the onset of the Lipid II synthesis. Thus, this experimental setup could be used in the investigation of the transport process of Lipid II, an important step in the cell wall biosynthesis.

These results show not only that Lipid II functions in the activity of nisin, but also that it appears to be the sole target of nisin (Fig. 4A). Incorporation of purified Lipid II in small amounts (one Lipid II molecule per 1500 phospholipid molecules) to model membrane systems composed of pure lipids markedly increased the nisin activity, whereas the magainin activity remained unchanged. In the absence of Lipid II, nisin induced leakage from this system only at concentrations above 1 μM. In the presence of Lipid II, leakage was detected at 1 nM nisin, which suggests that nisin has high affinity for Lipid II (*14*). The same effects of Lipid II were present when membranes containing negatively charged lipids were used (*3*). Because the activity of nisin in these model systems is in the range of the activity against intact cells, nisin appears to use Lipid II as its sole target. Nisin is dependent on Lipid II concentrations in the range of 0.001 to 0.1 mol % (Fig. 4B). This suggests that the diverse sensitivities to nisin displayed by different bacteria are caused by different concentrations of Lipid II in the membrane, although, in the case of intact cells, differences in the accessibility of Lipid II for nisin should also be considered.

The effect of Lipid II in all tested systems was specific for nisin, which suggests a specific interaction of Lipid II with one or more of the structural elements of nisin. Moreover, mutagenesis experiments with nisin showed that relatively subtle variations in the three NH_2-terminal rings had strong influences on the bactericidal activity of these mutants as well as on the interaction with Lipid II–containing membranes. For instance, a $Ser^3 \rightarrow$ Thr (S3T) mutation (actually, changing the first lanthionine residue into a β-methyl lanthionine) made the peptide less active by an order of magnitude (MIC for S3T = 39 nM, versus 3.3 nM for the wild type). Changing the thioether bond of the third ring into a disulfide bond had even larger effects (MIC > 60 nM). These findings suggest that at least the NH_2-terminal rings of nisin are involved in the interaction with Lipid II (*15*).

The high activity of nisin (in the nanomolar range) compared to magainin is the result of a combination of high-affinity binding to Lipid II and permeabilization of the plasma membrane, resulting in cell death. In contrast to recent reports on resistance or tolerance to vancomycin (*16*), no resistance to nisin has been reported, despite its prolonged use as a preservative for almost 50 years. This combination of factors makes nisin an attractive antimicrobial agent. The insights into its mode of action uncovered in this study can now be used as a blueprint for the development of a new class of highly efficient antibiotics.

References and Notes

1. Similar high sensitivities to nisin are found for other Gram-positive bacteria. For instance, *Lactococcus lactis* and *Streptococcus thermophilus* have MIC values of 4.5 nM and 1.8 nM, respectively.

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10. The observation that vancomycin inhibits nisin activity in vivo suggests that vancomycin and nisin bind to the same site at Lipid II. However, enterococci expressing the *van*A-type resistance gene cluster showed high vancomycin resistance relative to an isogenic strain not expressing the gene cluster (by a factor of >250), whereas the vancomycin-resistant strain remained as sensitive to nisin as the nonresistant strain (I. Wiedemann and H.-G. Sahl, data not shown). Thus, vancomycin and nisin do not share the same binding site on Lipid II.
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13. For instance, about 45% leakage could be observed for intact *M. flavus* cells with 10 nM nisin (Fig. 2), whereas about 20% leakage could be observed for the isolated membranes with 30 nM nisin (Fig. 3A).
14. The binding constant of the nisin–Lipid II interaction is 2×10^7 M^{-1}. This was determined as described (*17*) using vesicles composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine containing 0.5 mol % Lipid II. Under the assumption that only the Lipid II molecules on the outside of the vesicles were available for binding, nisin bound to Lipid II with a 2:1 stoichiometry. However, if nisin can translocate across the lipid bilayer in the presence of Lipid II, as was reported for pure phospholipid systems (*4*), then the stoichiometry would become 1:1.
15. Other mutations in the NH_2-terminal region—for instance, changing the Dhb at position 2 to Val, Ala, or Dha—did not result in loss of activity. The reduced activity of the S3T mutant was paralleled by a reduced affinity (0.04×10^7 M^{-1}) for Lipid II, which demonstrates the importance of the NH_2-terminus for Lipid II binding.
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