



# LUND UNIVERSITY

## REGULATED UPTAKE OF BIOPOLYMERS Role of cell surface proteoglycans Implications for drug and gene delivery

Sandgren, Staffan

2005

[Link to publication](#)

*Citation for published version (APA):*

Sandgren, S. (2005). *REGULATED UPTAKE OF BIOPOLYMERS Role of cell surface proteoglycans Implications for drug and gene delivery*. [Doctoral Thesis (compilation), Breastcancer-genetics]. Staffan Sandgren Department of Experimental Medical Science Medical Faculty Lund University.

*Total number of authors:*

1

### General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00

# The Human Antimicrobial Peptide LL-37 Transfers Extracellular DNA Plasmid to the Nuclear Compartment of Mammalian Cells via Lipid Rafts and Proteoglycan-dependent Endocytosis\*

Received for publication, October 17, 2003, and in revised form, January 22, 2004  
Published, JBC Papers in Press, February 11, 2004, DOI 10.1074/jbc.M311440200

Staffan Sandgren, Anders Wittrup, Fang Cheng, Mats Jönsson, Erik Eklund, Susann Busch, and Mattias Belting‡

From the Department of Cell and Molecular Biology, Section of Cell and Matrix Biology, Lund University, Biomedical Center C13, SE-221 84 Lund, Sweden

**Antimicrobial peptides, such as LL-37, are found both in nonvertebrates and vertebrates, where they represent important components of innate immunity. Bacterial infections at epithelial surfaces are associated with substantial induction of LL-37 expression, which allows efficient lysis of the invading microbes. Peptide-mediated lysis results in the release of bacterial nucleic acids with potential pathobiological activity in the host. Here, we demonstrate that LL-37 targets extracellular DNA plasmid to the nuclear compartment of mammalian cells, where it is expressed. DNA transfer occurred at physiological LL-37 concentrations that killed bacterial cells, whereas virtually no cytotoxic or growth-inhibitory effects were observed in mammalian cells. Furthermore, LL-37 protected DNA from serum nuclease degradation. LL-37-DNA complex uptake was a saturable time- and temperature-dependent process and was sensitive to cholesterol-depleting agents that are known to disrupt lipid rafts and caveolae, as shown by flow cytometry. Confocal fluorescence microscopy studies showed localization of internalized DNA to compartments stained by cholera toxin B, a marker of lipid rafts, but failed to demonstrate any co-localization of internalized DNA with caveolin-positive endocytotic vesicles. Moreover, LL-37-mediated plasmid uptake and reporter gene expression were strictly dependent on cell surface proteoglycans. We conclude that the human antimicrobial peptide LL-37 binds to, protects, and efficiently targets DNA plasmid to the nuclei of mammalian cells through caveolae-independent membrane raft endocytosis and cell surface proteoglycans.**

Several *in vitro* and *in vivo* studies have provided compelling evidence for the importance of antimicrobial peptides in the innate host defense of multicellular organisms against microbial intruders (1–5). Antimicrobial peptides were initially isolated from insects and subsequently also in higher organisms in which an adaptive immune system has evolved (6, 7). A common characteristic among antimicrobial peptides (>700

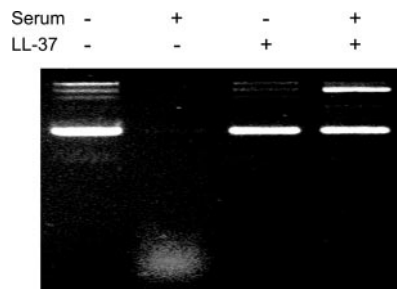
described so far) is the ability to adopt an amphipathic conformation where clusters of hydrophobic and cationic amino acids are spatially organized in discrete sections of the molecule. The relative sensitivity of bacterial cells to peptide cytotoxicity has generally been attributed to the high content of negatively charged phospholipids in the outer membrane leaflet as opposed to that of animal and plant cells, which principally consist of electroneutral lipids (8).

The defensins and the cathelicidins are the two major families of antimicrobial peptides in mammals. Cathelicidins consist of a highly conserved N-terminal cathelin domain and a more diverse antimicrobial C terminus (9, 10). LL-37, a 37-amino acid peptide with two N-terminal leucines, is the only known human cathelin-associated antimicrobial peptide. The precursor of LL-37, hCAP-18, and its mouse homolog, CRAMP, are primarily expressed in bone marrow cells but are also broadly expressed in nonmyeloid tissues, including epididymis, spermatids, and epithelial cells of a number of organs, emphasizing their role in primary host defense (11–15). The LL-37 concentration in adult sweat and the bronchoalveolar lavage fluid of infants is  $\sim 1 \mu\text{M}$  (16). Importantly, expression and excretion of LL-37 are induced severalfold upon infectious or inflammatory stimuli, both in keratinocytes and in epithelial cells at other sites (17, 18), *e.g.* the LL-37 concentration in skin lesions from patients with psoriasis reaches the impressive median value of  $304 \mu\text{M}$  (16). A wide range of Gram-negative as well as Gram-positive bacteria is sensitive to the cytotoxic activity of LL-37 at substantially lower peptide concentrations (19). The antibacterial activity is regulated by environmental changes in osmolarity or pH, which relates to the fact that LL-37 adopts an  $\alpha$ -helical oligomeric conformation in its active state (20). Accordingly, LL-37 activity was reduced in the respiratory epithelia of mice suffering from cystic fibrosis, a condition associated with increased extracellular osmolarity, and virus-mediated over-expression of LL-37 restored bacterial killing to normal levels in cystic fibrosis xenografts (21). In addition to bacterial cell lysis, LL-37 has been reported to neutralize bacterial endotoxin and to possess chemoattractive effects on leukocytes, which may provide additional mechanisms by which LL-37 combats microbial invasion (12, 23, 24).

In a recent report by Islam *et al.* (25), it was shown that in bacterial infections with *Shigella*, expression of LL-37 and  $\beta$ -defensin-1 is reduced or turned off, which could partly explain the chronic inflammatory response associated with *Shigella* infections. Interestingly, the study further demonstrated that plasmid DNA, released from lysed bacteria by the action of LL-37, was a major mediator of antimicrobial peptide down-regulation. Treatment of bacterial lysates with DNase blocked down-regulation of LL-37, whereas pure bacterial plasmid mediated the effect.

\* This work was supported by grants from the Swedish Society of Medicine, the Swedish Research Council, the Swedish Network for Pharmaceutical Sciences (FLAK), the Crafoord Foundation, the Royal Physiographic Society of Lund, and the Zoëgas Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: The Scripps Research Institute, Depts. of Immunology and Vascular Biology, C-204, 105 50 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-9238; E-mail: belting@scripps.edu.



**FIG. 1. LL-37 protects plasmid DNA from serum nuclease degradation.** DNA plasmid (20  $\mu\text{g}/\text{ml}$ ) was incubated in minimal essential medium supplemented with 50% fetal calf serum either in the absence (-) or in the presence (+) of LL-37 (100  $\mu\text{g}/\text{ml}$ ). After incubation at 37  $^{\circ}\text{C}$  for 16 h, the DNA material was recovered for analysis by agarose gel (0.8%) electrophoresis. The results are representative of six independent experiments.

This study investigates the ability of the antimicrobial peptide LL-37 to interact with extracellular DNA and to mediate uptake and nuclear transfer of functional plasmids in mammalian cells. The involvement of cholesterol-rich plasma membrane domains (26) and cell surface proteoglycan (PG)<sup>1</sup> in LL-37-mediated DNA internalization is also explored.

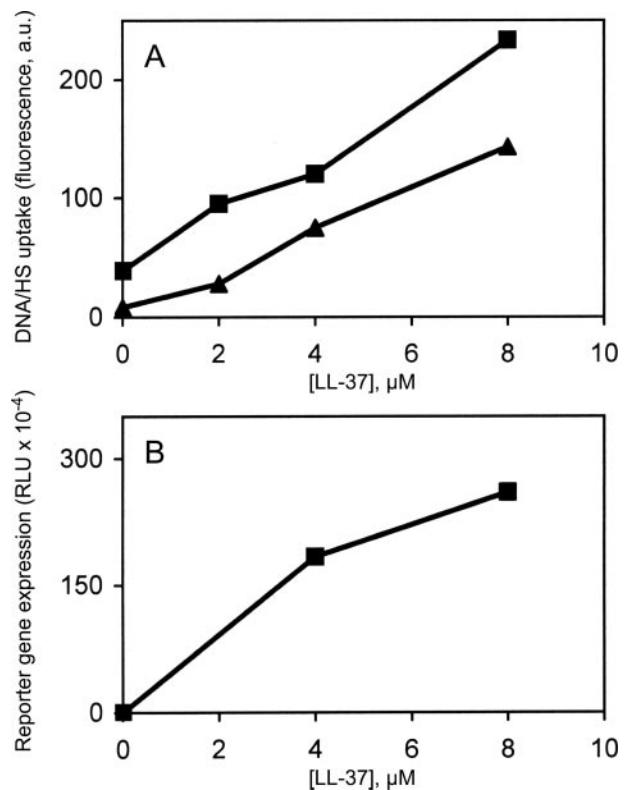
#### EXPERIMENTAL PROCEDURES

**Materials**—LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNQLV-PRTES-C-amide) was synthesized by Innovagen AB, Lund, Sweden. The peptide sequence was confirmed by reverse phase high pressure liquid chromatography and mass spectrometry. pGL3 control vector (luciferase reporter gene plasmid) was provided by Dr. Oldberg (Lund University, Sweden), and the luciferase assay system was purchased from Promega. DNA was labeled with YOYO-1 fluorophore (Molecular Probes) according to the standard protocol provided by the manufacturer. Rabbit anti-caveolin-1 antibody was from Transduction Laboratories (Lexington, KY), and goat anti-rabbit IgG antibody labeled with Texas Red fluorophore and cholera toxin subunit B (CTxB)-Alexa Fluor 594 conjugate were from Molecular Probes. Heparan sulfate (HS) preparations were the same as described previously (27). Rhodamine green (Molecular Probes)-labeled HS was prepared as described by Cheng *et al.* (28). LipofectAMINE reagent was from Invitrogen. PD-10 and Microspin S-200 HR columns were from Amersham Biosciences AB. All fine chemicals were from Sigma.

**Cell Culture**—Wild-type Chinese hamster ovary cells (CHO-K1), pgsA-745 (PG-deficient CHO mutant), pgsB-618 (PG-deficient CHO mutant), COS-7, and human embryonic lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Manassas, VA). CHO cells were routinely cultured in F12K nutrient mixture supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (growth medium) in a humidified 5%  $\text{CO}_2$  (37  $^{\circ}\text{C}$ ) incubator. HFL-1 and COS-7 cells were maintained in Eagle's minimal essential medium with the same supplements as described above.

**Nuclease Protection Assay**—pGL3 DNA plasmid (20  $\mu\text{g}/\text{ml}$ ) was incubated in minimal essential medium supplemented with 50% fetal calf serum either in the absence or in the presence of LL-37 (100  $\mu\text{g}/\text{ml}$ ). After incubation at 37  $^{\circ}\text{C}$  for 16 h, the incubation was terminated by the addition of sodium chloride (final concentration, 1 M), followed by repeated phenol/chloroform extraction and sodium acetate precipitation to recover the DNA. The samples were finally analyzed by gel electrophoresis (0.8% agarose, supplemented with ethidium bromide) and visualized using a trans-illuminator system from Techtum Laboratory.

**Cell Growth Assays**—To assess mammalian cell (CHO-K1, pgsA-745, and COS-7) proliferation, the same procedure was used as described previously (29). In brief, cells were seeded in 96-well microplates at  $3 \times 10^3$  cells/well and allowed to adhere for 4 h in growth medium, followed by a starvation period of 24 h in serum-free medium. The medium was aspirated and cells were incubated for 72 h at 37  $^{\circ}\text{C}$  in growth medium



**FIG. 2. LL-37 mediates uptake and efficient expression of exogenous plasmid DNA in mammalian cells.** A, fluorophore-labeled DNA ( $\blacksquare$ ) or HS ( $\blacktriangle$ ) at 10  $\mu\text{g}/\text{ml}$  was premixed with LL-37 (0, 2, 4, and 8  $\mu\text{M}$ ) and then incubated with confluent COS-7 cells for 4 h at 37  $^{\circ}\text{C}$ . After removal of the incubation medium, the cells were detached with trypsin followed by extensive washing with ice-cold PBS, 1% BSA to remove unspecific extracellular fluorophore. The cells were then suspended in PBS, 1% BSA, and intracellular DNA and HS were determined by FCM. Results are presented as means  $\pm$  S.E. (error bars,  $n = 5$ ). a.u., arbitrary units. B, pGL3-luciferase plasmid (10  $\mu\text{g}/\text{ml}$ ) and the indicated concentrations of LL-37 were premixed for 30 min at room temperature. The DNA-peptide mixture was then added to subconfluent cell layers for 4 h at 37  $^{\circ}\text{C}$ . After an additional period of incubation for  $\sim 36$  h in growth medium, luciferase activity was determined as described under "Experimental Procedures." Results are presented as means  $\pm$  S.E. (error bars,  $n = 8$ ). RLU, relative light units.

with or without LL-37 at the indicated concentrations. Cell growth was determined from the amount of crystal violet adsorbed to the cells as measured in a Multiscan 351 photometer (Labsystems) at 595 nm. For bacterial cytotoxicity assays, *Escherichia coli* cells (Top 10, provided by Dr. Oldberg, Lund University, Sweden) in log growth phase were incubated in minimal essential medium with or without various concentrations of LL-37 for 4 h at 37  $^{\circ}\text{C}$ . The bacteria were then pelleted and redissolved in 2-YT broth and seeded on agar plates. Following another growth period of  $\sim 14$  h, colony-forming units were determined.

**Flow Cytometry (FCM)**—Fluorophore-labeled DNA plasmid or labeled HS were preincubated with LL-37 at different concentrations in F12K for 30 min at room temperature. The mixture was then added to preincubated subconfluent cell layers in 24-well plates, and the incubations were allowed to proceed for various periods of time at 37  $^{\circ}\text{C}$  or, in some cases, at 4  $^{\circ}\text{C}$ . After removal of the incubation medium and rinsing with PBS, the cells were detached with trypsin followed by extensive washing with ice-cold PBS, 1% (w/v) BSA to remove unspecific extracellular fluorophore. Finally, the cells were suspended in PBS, 1% BSA and analyzed for fluorescence in a fluorescence-activated cell sorter instrument (FACSCalibur, BD Biosciences) operated by Cell-Quest software.

**Luciferase Gene Expression Assay**—Cells were plated on 24-well plates at  $1 \times 10^5$  cells/well in 0.5 ml of growth medium for 24 h before peptide-DNA incubation. To prepare LL-37-DNA complexes, pGL3-luciferase plasmid DNA (10  $\mu\text{g}/\text{ml}$ ) and LL-37 (5–100  $\mu\text{g}/\text{ml}$ ) were mixed by inversion and incubated for 30 min at room temperature. The DNA-peptide mixture was then added to subconfluent cell layers. At the end of the 4-h incubation at 37  $^{\circ}\text{C}$  in 5%  $\text{CO}_2$ , the medium was aspirated and replaced with 0.5 ml of growth medium. After an additional period of

<sup>1</sup> The abbreviations used are: PG, proteoglycan; CHO, Chinese hamster ovary; CTxB, cholera toxin subunit B; FCM, flow cytometry; HS, heparan sulfate; MCD, methyl- $\beta$ -cyclodextrin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HIV, human immunodeficiency virus; CDTA, 1,2-cyclohexylenedinitrotetraacetic acid.

TABLE I  
Effects of LL-37 on the growth of *E. coli* bacteria and mammalian cells

LL-37 was added to the growth medium of *E. coli*, COS-7, and wild-type (CHO-K1) or PG-deficient (*pgsA*-745) CHO cells. Cell growth was determined as described under "Experimental Procedures." Results are expressed as % of control growth (no LL-37 added)  $\pm$  S.D. ( $n = 6$ ). ND, not determined.

| [LL-37]<br>$\mu$ M | Growth         |             |             |              |
|--------------------|----------------|-------------|-------------|--------------|
|                    | <i>E. coli</i> | CHO-K1      | PgsA-745    | COS-7        |
|                    | % of control   |             |             |              |
| 0                  | 100 $\pm$ 32   | 100 $\pm$ 5 | 100 $\pm$ 2 | 100 $\pm$ 16 |
| 0.5                | 93 $\pm$ 16    | 94 $\pm$ 3  | 99 $\pm$ 4  | 115 $\pm$ 10 |
| 1.5                | 39 $\pm$ 18    | ND          | ND          | 122 $\pm$ 6  |
| 3                  | 0.1 $\pm$ 0.1  | 95 $\pm$ 2  | 100 $\pm$ 4 | 126 $\pm$ 6  |
| 5                  | ND             | ND          | ND          | 122 $\pm$ 9  |
| 10                 | ND             | 85 $\pm$ 12 | 97 $\pm$ 6  | 122 $\pm$ 7  |

incubation for  $\sim$ 36 h, the cells were washed with PBS and solubilized in 200  $\mu$ l of cell lysis reagent containing 25 mM Tris-HCl, pH 7.8, 2 mM CDTA, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100 for 1 h at 4  $^{\circ}$ C. Luciferase expression was quantified in 25  $\mu$ l of the cell lysate supernatant using a luciferase assay kit. Light emission was measured by integration over 15 s at 25  $^{\circ}$ C using BMG Fluostar Optima equipment (Labvision). To investigate the involvement of cholesterol-rich membrane domains in LL-37-DNA complex uptake, cells were pre-treated with either nystatin (50  $\mu$ g/ml), filipin (5  $\mu$ g/ml), 1 mM methyl- $\beta$ -cyclodextrin (MCD), or 1–5 mM MCD-cholesterol for 30 min at 37  $^{\circ}$ C. The cells were then incubated with DNA or HS and LL-37 in the presence of the respective drug at the indicated concentrations, after which FCM and luciferase experiments were conducted as described above. Under these conditions, no cytotoxicity or changes in cell morphology as a result of drug treatment were observed.

**Fluorescence Microscopy**—Cells were seeded at low density in 4-well chamber slides and allowed to adhere overnight and were then incubated with YOYO-1-DNA and LL-37 according to the figure legends. When used, fluorophore-conjugated CTxB (10  $\mu$ g/ml) was co-incubated with YOYO-1-DNA and LL-37 for 1 h. The cells were then cleared from unspecific extracellular fluorophore by brief rinsing with 1 M NaCl in PBS followed by extensive rinsing with PBS. The cells were then fixed in 4% (w/v) paraformaldehyde in PBS for 20 min. After fixation, the slides were washed with PBS and distilled water and then air-dried.

For caveolin-1 immunostaining, cells were permeabilized using PBS, 0.2% Triton X-100 (v/v) for 20 min or 1 mM saponin in PBS for 5 min and then blocked with 1% goat serum (v/v) in PBS, 1% BSA. The cells were then rinsed (three times for 5 min with PBS, 1% BSA), incubated for 3 h with 1:500 rabbit anti-caveolin-1 antibody in PBS, 1% BSA, rinsed again, and finally incubated for 2 h with 1:500 Texas Red-labeled goat anti-rabbit IgG antibody in PBS, 1% BSA. The slides were then washed with PBS and distilled water and air-dried. The fixed cells were visualized using a Nikon Eclipse E800 microscope and a Bio-Rad MRC 1024 confocal laser scanning microscopy system. The collected data were analyzed using PC-compatible Laser-Sharp software.

**Statistical Analyses**—Each data point in the dose activity and time course experiments is the mean  $\pm$  S.E. ( $n = 5$ –8) from at least two separate experiments. In some cases the error bars were smaller than the drawn symbols.

## RESULTS

**LL-37 Peptide Targets Intact DNA Plasmid to the Nuclear Compartment of Mammalian Cells**—In the absence of LL-37, DNA plasmid was degraded by serum nucleases, whereas the presence of LL-37 efficiently protected plasmid DNA against serum nuclease activity (Fig. 1). The data suggest significant complex formation between polyanionic DNA and the polybasic peptide LL-37 in 50% serum. LL-37-DNA complex formation may enable efficient internalization of DNA to mammalian cells, as has been shown previously for other polybasic peptides, e.g. polylysine and HIV-Tat (30, 31). Therefore, the effect of LL-37 on the uptake of plasmid DNA in COS-7 cells was studied (Fig. 2A, filled squares). Under the conditions used, LL-37 increased DNA plasmid uptake up to 6-fold. The same results were obtained with CHO cells, human embryonic fibroblasts, and bladder carcinoma cells (results not shown). The

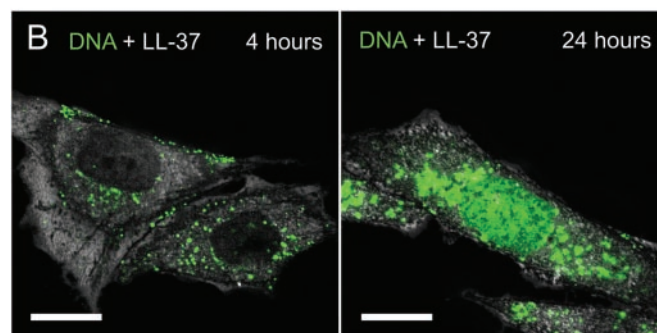
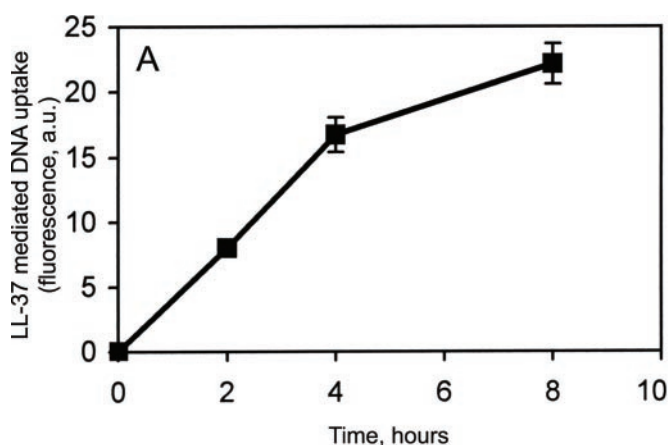
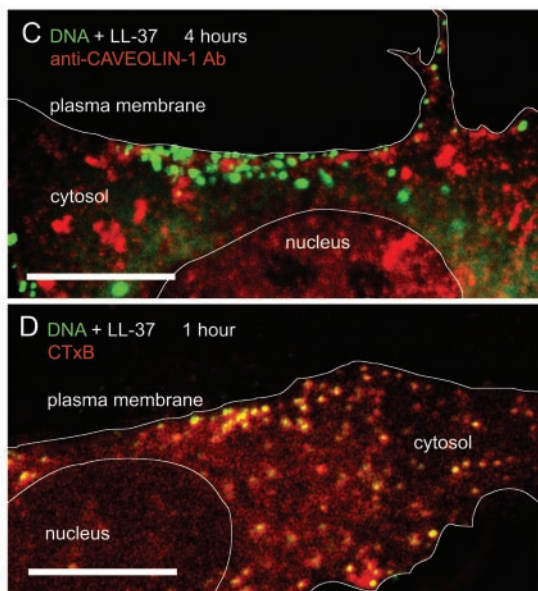
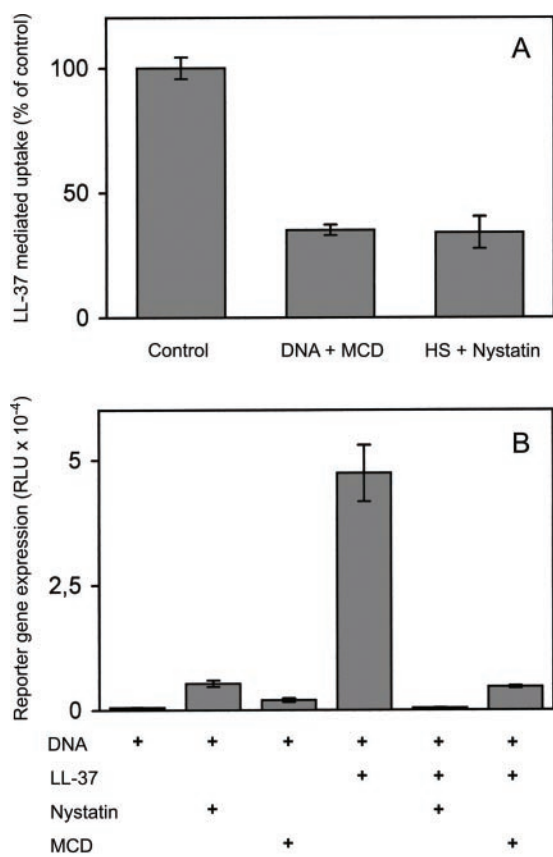


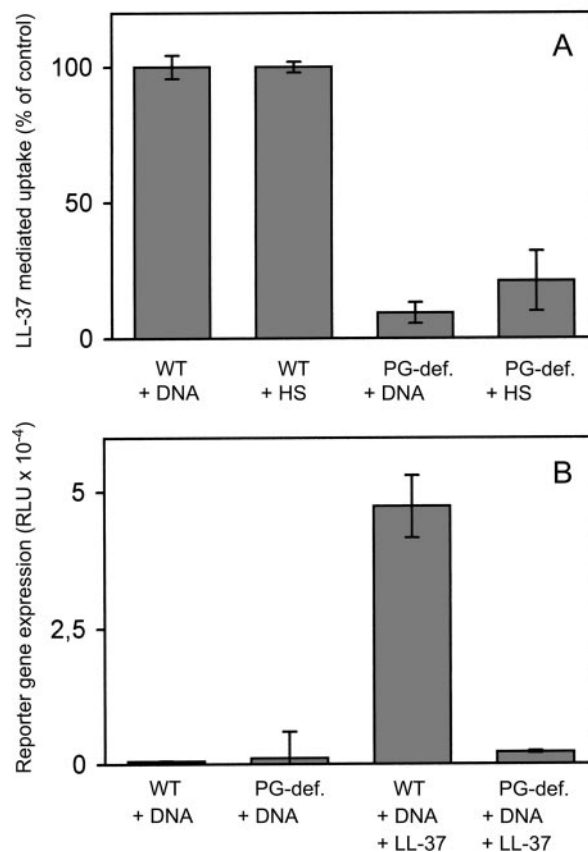
FIG. 3. LL-37 targets exogenous plasmid DNA to the nuclear compartment. A, CHO-K1 cells grown to confluence were incubated with YOYO-1-DNA (10  $\mu$ g/ml) and LL-37 (8  $\mu$ M) for 0, 2, 4, and 8 h. The cells were then analyzed for intracellular DNA by FCM as described in Fig. 2A. Results are presented as means  $\pm$  S.E. (error bars,  $n = 6$ ). a.u., arbitrary units. B, CHO-K1 cells were sparsely seeded on chamber slides and allowed to adhere for a period of 24 h followed by incubation with YOYO-1-DNA (10  $\mu$ g/ml, green) and LL-37 (8  $\mu$ M) for 4 h (left panel) and 24 h (right panel). The cells were then processed for confocal fluorescence microscopy as described under "Experimental Procedures." Bars indicate 20  $\mu$ m.

effect of LL-37 was not specific for DNA, as cellular uptake of HS (a major extracellular polyanion) was also substantially increased by LL-37 (up to  $\sim$ 20-fold; Fig. 2A, filled triangles). The data indicate that LL-37 binds to DNA via nonspecific electrostatic interactions that result in the protection of DNA from serum nuclease cleavage and transfer of DNA over the plasma membrane. To test the possibility that increased DNA uptake in the presence of LL-37 was simply a result of membrane rupture and cytotoxicity, cell proliferation studies, with several of the cell lines included in this study, were performed. None of the cell lines was affected at relevant LL-37 concentrations (up to 10  $\mu$ M), whereas *E. coli* bacteria, as expected, were efficiently killed by the LL-37 preparation used in this study (Table I).

In the next series of experiments, a luciferase reporter gene expression assay was employed to gain more insight into the intracellular fate of internalized LL-37-DNA complexes. As shown in Fig. 2B, LL-37 peptide substantially increased the expression of luciferase reporter plasmid in CHO cells (up to  $\sim$ 1600-fold). The data indicate that LL-37 mediates significant nuclear transfer of intact DNA plasmid in cultured mammalian cells with no concomitant cytotoxic effects. This notion was supported by confocal fluorescence microscopy studies that demonstrated rapid nuclear accumulation of internalized DNA in the presence of LL-37 (Fig. 3B). At 4 h of incubation, fluorescence was restricted to well defined vesicular structures at the cell membrane and in the cytoplasm (Fig. 3B, left panel). At 24 h, internalized DNA displayed a more diffuse staining pat-



**FIG. 4. LL-37-mediated gene transfer involves membrane rafts.** A, subconfluent CHO-K1 cells were either untreated (*Control*) or pretreated with MCD (1 mM) or nystatin (50  $\mu$ g/ml) for 30 min at 37  $^{\circ}$ C followed by incubation with LL-37 (8  $\mu$ M) and YOYO-1-DNA (10  $\mu$ g/ml) or rhodamine green-HS (10  $\mu$ g/ml), respectively, for 4 h. Cholesterol-depleting agents were present during the whole course of the experiments. The cells were then analyzed for intracellular DNA or HS by FCM. B, subconfluent CHO-K1 cells were pretreated with nystatin (50  $\mu$ g/ml) or MCD (1 mM) for 30 min, as indicated by the table below panel B, followed by another incubation period of 4 h with pGL3-luciferase plasmid (8  $\mu$ g/ml) and LL-37 (5  $\mu$ M), as indicated, in the continued presence of the respective cholesterol-depleting drugs. The media were then replaced with growth medium, and the cells were incubated in the absence of cholesterol-depleting drugs for 36 h and then finally analyzed for luciferase activity. Results are presented as means  $\pm$  S.E. (*error bars*,  $n = 8$ ). *RLU*, relative light units. C, sparsely seeded CHO-K1 cells were incubated with YOYO-1-DNA (10  $\mu$ g/ml, *green*) and LL-37 (8  $\mu$ M) for 4 h. The cells were stained for caveolin-1 (*red*) and

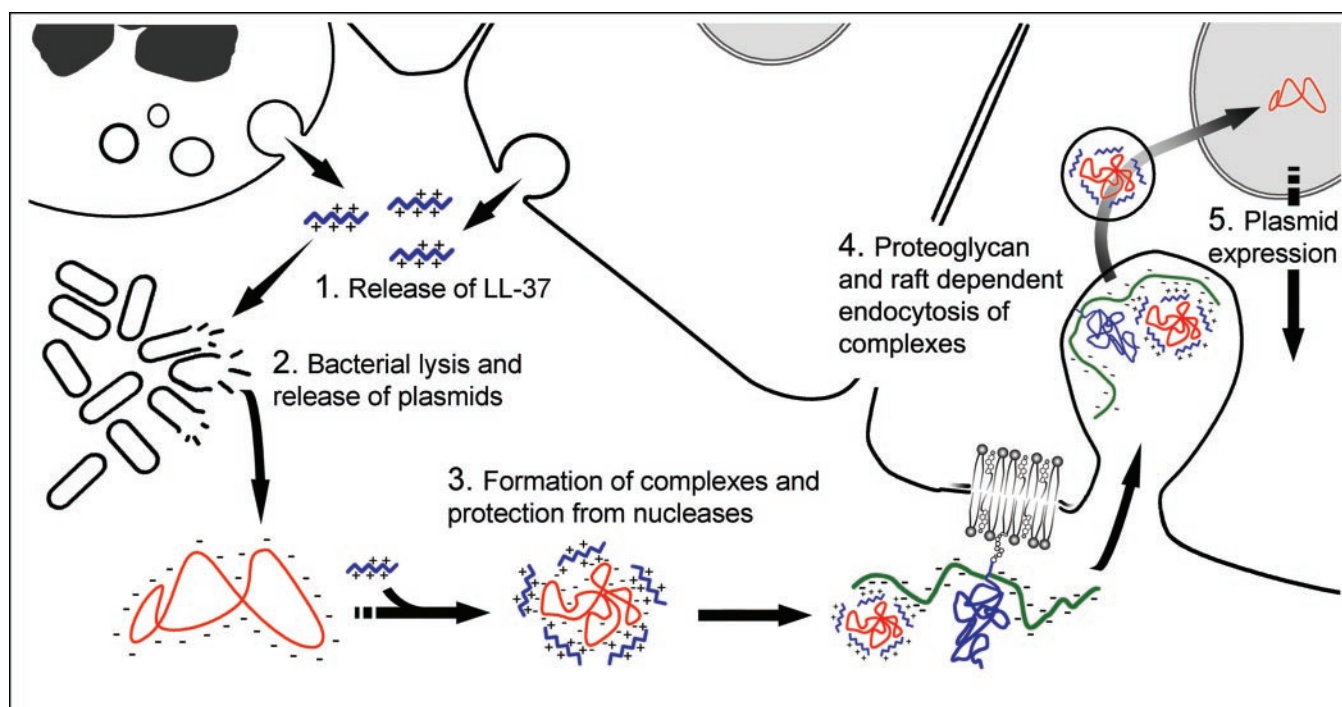


**FIG. 5. LL-37-mediated gene transfer requires cell surface proteoglycans.** A, subconfluent wild-type CHO-K1 cells (*WT*) or mutant PG-deficient (*PG-def.*) *pgsA-745* cells were incubated with YOYO-1-DNA (10  $\mu$ g/ml) or rhodamine green-HS (10  $\mu$ g/ml) in the presence of LL-37 (8  $\mu$ M) for 4 h and then analyzed by FCM. B, WT and PG-deficient (*PG-def.*) CHO cells were incubated with pGL3-luciferase plasmid (8  $\mu$ g/ml) in the absence or presence (+) of LL-37 (5  $\mu$ M) for 4 h, followed by another incubation period of 36 h, and then analyzed for luciferase activity. Results are presented as means  $\pm$  S.E. (*error bars*,  $n = 8$ ). *RLU*, relative light units

tern in the cytosol, consistent with release from endocytotic vesicles, and could also be observed to be largely redistributed to the nuclear compartment (Fig. 3B, right panel). In the absence of peptide, a much weaker DNA signal was observed in relatively few and small vesicular structures (results not shown). LL-37-mediated DNA uptake was time-dependent (Fig. 3A), saturable, and temperature-dependent (no DNA uptake at 4  $^{\circ}$ C, results not shown), which is consistent with an endocytotic uptake mechanism.

**LL-37-mediated Gene Transfer Occurs via Membrane Rafts and Cell Surface PGs**—We next sought to determine the molecular mechanism of LL-37-DNA complex uptake. For this purpose, cells were treated with either MCD or nystatin, both of which represent well established cholesterol-depleting agents employed for studying the involvement of rafts/caveolae in cellular transport (32–34). Cholesterol- and sphingolipid-enriched membrane domains may exist either as small phase-separated “rafts” or, when associated with caveolin, may form flask-shaped invaginations called caveolae, which are involved in special forms of nonclathrin-dependent endocytosis (26). As

processed for confocal fluorescence microscopy as described under “Experimental Procedures.” D, sparsely seeded CHO-K1 cells were incubated with YOYO-1-DNA (10  $\mu$ g/ml, *green*) and LL-37 (8  $\mu$ M) for 1 h in the presence of CTxB (10  $\mu$ g/ml, *red*) and then processed for confocal fluorescence microscopy as described under “Experimental Procedures.” Bars indicate 10  $\mu$ m.



**FIG. 6. LL-37 targets bacterial DNA to the nuclear compartment of mammalian cells via proteoglycan-dependent membrane raft endocytosis.** Components with antimicrobial activity are exocytosed from mammalian host cells upon inflammatory and infectious stimuli (*step 1*). LL-37 and, conceivably, other microbicidal cationic peptides release bacterial DNA upon lysis of the microbe (*step 2*) followed by binding to and protection of bacterial DNA from inactivation by nucleases in the extracellular environment (*step 3*) and the transfer of intact bacterial DNA to the nuclei of mammalian host cells in a process that involves cholesterol-rich rafts and cell surface PGs (*steps 4 and 5*).

shown in Fig. 4A, cholesterol depletion resulted in an ~65% reduction of LL-37-mediated internalization of both DNA and HS. Next, the effect of cholesterol depletion on the expression of internalized reporter gene plasmid was studied (Fig. 4B). Both MCD and nystatin dramatically reduced reporter gene activity after incubations in the presence of LL-37 (~90 and 99% inhibition with MCD and nystatin, respectively). Notably, both treatments enhanced reporter gene expression following incubation of naked DNA plasmid (an ~4.5- and 11-fold increase with MCD and nystatin, respectively). Overall, the data indicate that disruption of raft/caveolar structures by cholesterol depletion inhibits LL-37-DNA complex uptake and, accordingly, expression of internalized DNA plasmid. Similar results were obtained with filipin (results not shown), another known inhibitor of raft/caveolar endocytosis. The fact that drug treatment enhanced the expression of naked DNA plasmid makes it highly unlikely that drug effects were simply a result of cellular toxicity. We next used the method described by Roepstorff *et al.* (35) to investigate the possible enhancing effect of cholesterol loading on LL-37-mediated DNA uptake. With the concentrations of MCD-cholesterol used in this study (1–5 mM), no further enhancement was observed (results not shown).

Confocal fluorescence studies were then performed to examine possible co-localization of internalized DNA with caveolin, a well established marker of caveolae (26). As shown in Fig. 4C, there was no co-localization between DNA-positive vesicles (green) and caveolae or caveosomes (red) under the conditions used. This finding was consistent irrespective of the method of cell permeabilization (mild Triton X-100 or saponin treatment) or the time point in the interval of 10 min to 4 h. However, confocal microscopy demonstrated strong co-localization of internalized DNA with CTxB in vesicular structures (Fig. 4D, yellow). Although debated regarding its specificity (36), CTxB is a widely accepted marker for lipid rafts because it interacts with ganglioside M1, which is largely excluded from clathrin-positive membrane domains (37).

PGs constitute a family of glycosaminoglycan-substituted proteins found in the extracellular matrix at the cell surface and in intracellular granulae and are known to be involved in the uptake of polyamines, viruses, and polycation-nucleic acid complexes (38). In the next series of experiments, we therefore investigated the possible involvement of cell surface PGs in LL-37-DNA complex uptake. Using two different mutant CHO cell lines genetically deficient in PG biosynthesis (*pgsA-745* and *pgsB-618*, Ref. 39), we were able to demonstrate a requirement of PG expression in LL-37-mediated DNA internalization (Fig. 5A). PG dependence was not specific for DNA, as LL-37-HS complex uptake also required PG (Fig. 5A). Accordingly, reporter gene expression was almost abolished in PG-deficient cells incubated with LL-37-DNA complexes as compared with wild-type cells (Fig. 5B).

#### DISCUSSION

Here, we show that physiologically relevant concentrations of a human antimicrobial peptide, widely expressed in bone marrow and epithelial cells, protects plasmid DNA against serum nuclease degradation and efficiently targets DNA to the nuclear compartment of mammalian cells. Furthermore, mechanistic data indicate that LL-37-DNA complexes enter mammalian cells via endocytosis that involves noncaveolar lipid raft domains as well as cell surface PG. To our knowledge, this is the first study to provide evidence for an involvement of both rafts and PGs in nonviral gene delivery. Moreover, this study adds important data to the ongoing controversy (38) on the entry mechanism of polybasic “membrane-penetrating peptides” (*e.g.* HIV-1 transactivator (Tat), *Antennapedia* protein of *Drosophila*, and herpes simplex VP22). Are these peptides truly membrane-penetrating or do they enter via temperature-dependent endocytotic mechanisms? Our data strongly speak in favor of endocytosis when peptides are complexed to DNA or HS. Notably, several viruses depend on cell surface PG attachment and nonclathrin-mediated endocytosis for efficient infec-

tion, which points toward a common entry pathway for genetic material delivered by specific viral and nonviral vehicles (34, 38). As such, peptide-nucleic acid complexes may be regarded as primitive virus particles. It has been shown previously that polylysine and HIV-Tat mediate DNA internalization via a PG-dependent pathway (30, 31). However, these and other DNA-delivering peptides have not been described as harboring antibacterial activity, as opposed to LL-37, which efficiently kills numerous bacterial species. Interestingly, the porcine homologue of LL-37 (PR-39) was shown to induce the expression of syndecan, a major cell surface PG involved in cell-cell and cell-matrix interactions (40). Ongoing studies in our laboratory investigate the possible effects of LL-37 on PG-expression. Furthermore, it is of interest to study whether a particular membrane raft-resident PG is involved in peptide-mediated gene transfer.

It was recently suggested that synthetic histidine-rich antimicrobial peptides with gene transfer activity could have potential in the treatment of cystic fibrosis, as they would eradicate bacterial infection and introduce the correcting gene of this monogenic trait simultaneously (41). Our data raise the possibility that LL-37 and other antimicrobial peptides may cause the release of plasmid DNA from lysed bacteria followed by protection of bacterial DNA from inactivation in the extracellular environment and finally the transfer of intact bacterial DNA to the nuclei of mammalian host cells (see Fig. 6 for a hypothetical model). Considering the high diversity and abundance in polybasic antimicrobial peptides and other polycations (such as the polyamines) in the reproductive tract, the transfer of bacterial genes to germ cells may also occur. Indeed, it has been demonstrated that spermatozoa are decorated with high amounts of LL-37 peptide and that hCAP-18 (the precursor of LL-37) is constitutively expressed in the epithelial lining of the female reproductive system (42, 43). The International Genome Group initially reported on the existence of between 113 and 223 genes that were present in the human genome but were absent in the lower eukaryotes (44). It was concluded that this set of genes had been subjected to direct bacterial to vertebrate transmission, *i.e.* lateral gene transfer. This has been the subject of lively debate ever since, in which gene loss in the lower eukaryotes represents the main argument against lateral gene transfer (12, 45, 46). Although provocative, our data may provide a molecular mechanism for bacterial to vertebrate lateral gene transfer.

**Acknowledgments**—We thank Profs. Catharina Svanborg and Klaus Edvardsson (both at Lund University, Sweden) for use of microscope and flow cytometry facilities and Susanne Persson for technical assistance.

#### REFERENCES

- Boman, H. G. (1998) *Scand. J. Immunol.* **48**, 15–25
- Bals, R., Weiner, D. J., Mosconio, A. D., Meegalla, R. L., and Wilson, J. M. (1999) *Infect. Immun.* **67**, 6084–6089
- Lehrer, R. I., and Ganz, T. (1999) *Curr. Opin. Immunol.* **11**, 23–27
- Hancock, R. E., and Scott, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8856–8861
- Zasloff, M. (2002) *Nature* **415**, 389–395
- Boman, H. G. (1995) *Annu. Rev. Immunol.* **13**, 61–92
- Gudmundsson, G. H., and Agerberth, B. (1999) *J. Immunol. Methods* **232**, 45–54
- Ganz, T. (2002) *J. Clin. Investig.* **109**, 693–697
- Zanetti, M., Del Sal, G., Storici, P., Schneider, C., and Romeo, D. (1993) *J. Biol. Chem.* **268**, 522–526
- Zanetti, M., Gennaro, R., and Romeo, D. (1995) *FEBS Lett.* **374**, 1–5
- Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H. G., and Gudmundsson, G. H. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 195–199
- Larrick, J., Hirata, M., Balint, R., Lee, J., Zhong, J., and Wright, S. (1995) *Infect. Immun.* **63**, 1291–1297
- Cowland, J. B., Johnsen, A. H., and Borregaard, N. (1995) *FEBS Lett.* **368**, 173–176
- Gallo, R. L., Kim, K. J., Bernfield, M., Kozak, C. A., Zanetti, M., Merluzzi, L., and Gennaro, R. (1997) *J. Biol. Chem.* **272**, 13088–13093
- Bals, R., Wang, X., Zasloff, M., and Wilson, J. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9541–9546
- Davidson, D. J., Currie, A. J., Reid, G. S., Bowdish, D. M., MacDonald, K. L., Ma, R. C., Hancock, R. E., and Speert, D. P. (2004) *J. Immunol.* **172**, 1146–1156
- Frohm, M., Agerberth, B., Ahangari, G., Ståhle-Bäckdahl, M., Lidén, S., Wigzell, H., and Gudmundsson, G. H. (1997) *J. Biol. Chem.* **272**, 15258–15263
- Dorschner, R. A., Pestonjamas, V., Tamakuwala, S., Ohtake, T., Rudisill, J., Nizet, V., Agerberth, B., Gudmundsson, G. H., and Gallo, R. L. (2001) *J. Investig. Dermatol.* **117**, 91–97
- Lehrer, R. I., and Ganz, T. (2002) *Curr. Opin. Hematol.* **9**, 18–22
- Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998) *J. Biol. Chem.* **273**, 3718–3724
- Bals, R., Weiner, D. J., Meegalla, R. L., and Wilson, J. M. (1999) *J. Clin. Investig.* **103**, 1113–1117
- Ponting, C. P. (2001) *Trends Genet.* **17**, 235–237
- De Yang, B., Chen, Q., Schmidt, A. P., Anderson, M., Wang, J. M., Wooters, J., Oppenheim, J. J., and Chertov, O. (2000) *J. Exp. Med.* **192**, 1069–1074
- Agerberth, B., Charo, J., Werr, J., Olsson, B., Idali F., Lindbom, L., Kiessling, R., Jörnvall, H., Wigzell, H., and Gudmundsson, G. H. (2000) *Blood* **96**, 3086–3093
- Islam, D., Bandholtz, B., Nilsson, J., Wigzell, H., Christensson, B., Agerberth, B., and Gudmundsson, G. H. (2001) *Nat. Med.* **7**, 180–185
- Nichols, B. J., and Lippincott-Schwartz, J. (2001) *Trends Cell Biol.* **11**, 406–412
- Belting, M., and Petersson, P. (1999) *J. Biol. Chem.* **274**, 19375–19382
- Cheng, F., Petersson, P., Arroyo-Yanguas, Y., and Westergren-Thorsson, G. (2001) *J. Cell. Biochem.* **83**, 597–606
- Belting, M., Havsmark, B., Jönsson, M., Persson, S., and Fransson, L.-Å. (1996) *Glycobiology* **6**, 121–129
- Mislick, K. A., and Baldeschwieler, J. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 12349–12354
- Sandgren, S., and Belting, M. (2002) *J. Biol. Chem.* **277**, 38877–38883
- Kilsdonk, E. P., Yancey, P., Stoult, G. W., Bangerter, F. W., Johnson, W. J., Phillips, M. C., and Rothblat, G. H. (1995) *J. Biol. Chem.* **270**, 17250–17256
- Ono, A., and Freed, E. O. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13925–139230
- Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001) *Nat. Cell Biol.* **3**, 473–483
- Roepstorff, K., Thomsen, P., Sandvig, K., and van Deurs, B. (2002) *J. Biol. Chem.* **277**, 18954–18960
- Nichols, B. (2003) *J. Cell Sci.* **116**, 4707–4714
- Nichols, B. J. (2003) *Curr. Biol.* **13**, 686–690
- Belting, M. (2003) *Trends Biochem. Sci.* **28**, 145–151
- Esko, J. D., Stewart T. E., and Taylor W. H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3197–3201
- Gallo, R. L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsbrun, M., and Bernfield, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11035–11039
- Kichler, A., Leborgne, C., März, J., Danos, O., and Bechinger, B. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1564–1568
- Malm, J., Sørensen, O., Persson, T., Frohm-Nilsson, M., Johansson, B., Bjartell, A., Lilja, H., Ståhle-Bäckdahl, M., Borregaard, N., and Egesten, A. (2000) *Infect. Immun.* **68**, 4297–4302
- Frohm-Nilsson, M., Sandstedt, B., Sørensen, O., Weber, G., Borregaard, N., and Ståhle-Bäckdahl, M. (1999) *Infect. Immun.* **67**, 2561–2566
- International Human Genome Sequencing Consortium (2001) *Nature* **409**, 860–921
- Stanhope, M. J., Lupas, A., Irlaia, M. J., Koretke, K. K., Volker, C., and Brown, J. R. (2001) *Nature* **411**, 940–944
- Salzberg, S. L., White, O., Peterson, J., and Eisen, J. A. (2001) *Science* **292**, 1903–1906