Identification of an anti-mycobacterial domain in NK-lysin and granulysin

David ANDREU*, Cristina CARREÑO*, Charlotte LINDE†, Hans G. BOMAN‡ and Mats ANDERSSON§[1]

*Department of Organic Chemistry, University of Barcelona, E-08028 Barcelona, Spain, †Swedish Institute for Infectious Disease Control, S-105 21, Stockholm, Sweden, ‡Microbiology and Tumorbiology Centre, Karolinska Institute, S-171 77, Stockholm, Sweden, and §Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden

NK-lysin and granulysin are homologous cationic anti-bacterial peptides produced by pig and human cytolytic lymphocytes, respectively. The solution structure of NK-lysin comprises five amphipathic α-helices. To investigate the properties of a helix-loop-helix region postulated to be a membrane-docking part of NK-lysin, we synthesized 22- and 29-residue peptides reproducing this region for both NK-lysin and granulysin. CD spectroscopy of the synthetic peptides in a liposomal solution showed spectra typical of α-helical peptides. The peptides were active against Gram-positive and Gram-negative bacteria, with the two NK-lysin peptides showing higher anti-bacterial activities than the two from granulysin. One NK-lysin peptide was active against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two organisms against which NK-lysin is inactive. Granulysin peptides were inactive against these bacteria, in contrast with granulysin, which is known to be active against them. Both NK-lysin and all synthetic analogues killed *Mycobacterium tuberculosis* and K562 tumour cells, but did not display haemolytic activity. These results identify a potent anti-mycobacterial domain in NK-lysin and granulysin consisting of a 22-residue (helix 3) sequence plus a disulphide-constrained loop.

Key words: antibiotic peptide, cytolytic cell, helix-loop-helix domain, synthetic peptide.

INTRODUCTION

NK-lysin is a porcine, 78-residue, antibiotic peptide containing three disulphide bonds [1]. It has close structural similarity with granulysin, a human polypeptide [2]. Both peptides are produced by cytolytic cells and stored in intracellular granules [1,2]. A model of target-directed release of granular components (including NK-lysin/granulysin) has been proposed as a mechanism used by cytolytic lymphocytes to kill bacteria and infected cells [3]. NK-lysin is active against Gram-positive bacteria, Gram-negative bacteria and fungi [1]. Granulysin is also anti-bacterial and was shown recently to have potent activity against *Mycobacterium tuberculosis* [4]. Other cysteine-containing antibiotic peptides have been shown to possess anti-mycobacterial activity [5]. However, neither NK-lysin nor granulysin show any significant similarity to cysteine-rich peptide antibiotics such as α- and β-defensins [6], protegrins [7], bactenecin [8] or tachyplesins [9]. The three-dimensional structure of NK-lysin in aqueous solution, as determined by NMR, consists of five compact α-helical segments [10], a folding that differs from other cysteine-containing peptide antibiotics, which mostly adopt β-sheet structures [11–13]. CD spectra of NK-lysin in water and liposome solutions show similar patterns, suggesting that the secondary structure does not change in the presence of membranes [14], although NK-lysin has been shown to interact with membranes and to lyse lipid vesicles [15]. This interaction gives rise to ion fluxes but does not involve persistent pore formation. NK-lysin interacts with outer-membrane lipids (lipopolysaccharides) of Gram-negative bacteria, although this is not generally sufficient for bacterial killing [16]. Thus current data support a superficial membrane location of NK-lysin as a first step of action. This raises questions about the importance and specificity of the entire 78-residue sequence of NK-lysin and granulysin, which are roughly 2–3 times larger than most other anti-bacterial peptides. The second and third α-helices in NK-lysin define a helix-loop-helix motif that resembles the structural patterns of smaller anti-bacterial peptides such as protegrins (sheet-loop-sheet) [11] or bactenecin (coil-loop-coil) [8].

To evaluate the biological role of this helix-2-loop-helix-3 motif, in this article we examine the anti-microbial and cytolytic properties of synthetic peptides reproducing this region and the associated disulphide bond in both NK-lysin and granulysin.

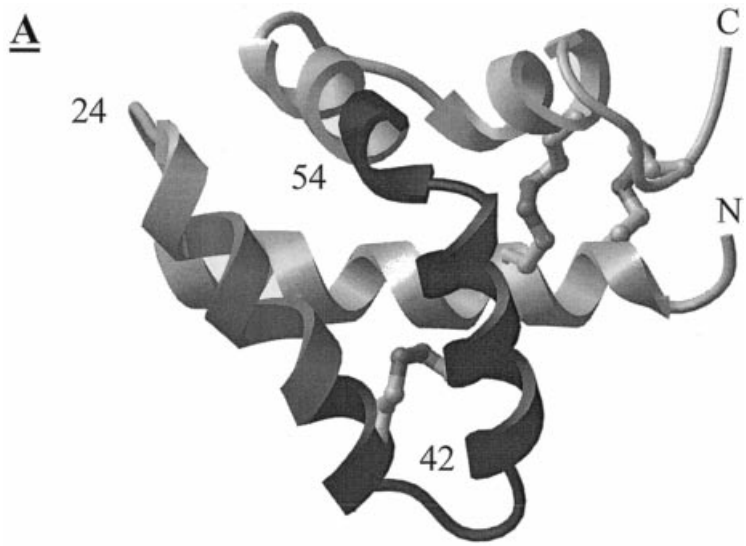
MATERIALS AND METHODS

Peptide synthesis

Two NK-lysin (NKLF1, M_r 3361; NKLF2, M_r 2692) and two granulysin (GranF1, M_r 3686; GranF2, M_r 2986) peptides (Figure 1) were synthesized by solid-phase procedures as C-terminal carboxamides, using t-butoxycarbonyl/benzyl chemistry on *p*-methylbenzhydrylamine resin [17,18]. After chain assembly, the protected peptide resins (150 mg, 30 μmol) were treated with 50% piperidine/*N*,*N*-dimethylformamide (DMF) for 2 h at 25 °C, with vigorous air bubbling, to remove the formyl- (Trp) and 9-fluorenylmethyl- (Cys) protecting groups and to promote simultaneous intramolecular disulphide-bond formation [19,20]. The resins were next washed with DMF, and then treated with 2% piperidine/DMF buffered with acetic acid to pH 8.5, with constant air bubbling, for an additional 13 h, to complete the oxidation process. This was followed by deprotection at the *N*-terminus with trifluoroacetic acid (10 min) and peptide-resin cleavage and side-chain deprotection by anhydrous hydrogen fluoride/*p*-cresol/*p*-thiocresol (18:1:1, by vol.) for 1 h at 0 °C. The resulting crude peptides were purified by preparative HPLC on C_{18}-silica using linear 0–50% acetonitrile gradients in water (both solvents containing 0.05% trifluoroacetic acid) over 1 h at 25 ml/min. For every sequence, fractions corresponding to the major peak were tested by analytical HPLC for purity and by matrix-assisted laser-desorption ionization–time-of-flight MS for the presence of the target structure.

Abbreviations used: DMF, *N*,*N*-dimethylformamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide.

[1] To whom correspondence should be addressed (e-mail mats.andersson@mbb.ki.se).



B

```
                  24                      42              54
                  *                       *               *
NK-lysin      NEDTVTQAASRVCDKMKI.LRGVCKKIMRTFLRRISKD
NKLF1             VTQAASRVCDKMKI.LRGVCKKIMRSFLRR
NKLF2                   VCDKMKI.LRGVCKKIMRSFLRR
GranF2                  VCRTGRSRWRDVCRNFMRRYQSR
GranF1            VSNAATRVCRTGRSRWRDVCRNFMRRYQSR
Granulysin    TQRSVSNAATRVCRTGRSRWRDVCRNFMRRYQSRVTQG

                  VxxAAxRVC...x...R.VCx.xMR.x..R
```

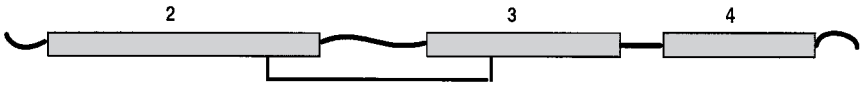


Figure 1 Solution structure of NK-lysin and primary structures of synthetic peptides

(**A**) NMR structure of NK-lysin [10]. NKLF2 (34–55) is represented in black and NKLF1 (27–55) in black plus dark grey. (**B**) Amino acid sequences of the peptides used in the assays. The asterisks indicate the first residue in each helix. The identities and similarities (x) between NK-lysin and granulysin are shown in the bottom line. Regions identified as helices are shown as well the disulphide bond between helix 2 and 3.

Fractions containing substantial levels of the desired peptide were pooled and rerun once or twice under similar chromatographic conditions to give HPLC-homogeneous products with the correct masses and amino acid compositions. Yields of purified product were in the 2–10 % range relative to initial resin substitution.

CD analysis

Peptide secondary structures were examined by CD spectroscopy (Aviv 62DS spectropolarimeter, Aviv Associates, Lakewood, NJ, U.S.A.). Liposomes of 200 nm were made from L-α-phosphatidylcholine/L-α-phosphatidyl-DL-glycerol (3:1) in phosphate buffer at 1 mg/ml [21]. All spectra were recorded at 25 °C at a resolution of 1 nm/point. The average helix-conformation content was calculated from the experimental molar ellipticities at 208 and 222 nm [22,23].

Anti-bacterial assays

Anti-bacterial activity was determined by an inhibition-zone assay in thin agarose plates [24]. Lethal concentrations were calculated from the zones observed by serial dilutions of peptide.

Anti-mycobacterial activity was tested using the Bactec radiometric assay (Becton–Dickinson, Sparks, MD, U.S.A.) [25]. Peptides were dissolved in distilled water, and a 90-μl portion was inoculated into Bactec 12B vials with 4 ml of 7H12 Middlebrook media. *M. tuberculosis* H37Rv, A.T.C.C. 25618, was suspended in sterile PBS to a bacterial density of McFarland 1.0 standard. This suspension was further diluted 1:10, and 100 μl was inoculated in the test vials to a final concentration of 7.5×10^5 colony forming units (cfu)/ml. The growth-index values were monitored for 8 days, ranging from 0 to 999. To estimate the activity of test peptides, a 10-fold dilution of the bacterial suspension was inoculated as a 10 % control and was considered equivalent to 90 % of the bacteria killed.

Cytolytic assay

The human erythroleukaemia cell line K562 was maintained as a suspension culture in RPMI 1640, supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % heat-inactivated foetal calf serum (Sigma). Cells (10×10^3 in 100 μl) were seeded into wells (Costar microtitre plates) and 100 μl of test solution was added. After 6 h, the number of viable cells in each well was assayed by the addition of 20 μl of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; 5 mg/ml) in RPMI. After 4 h, 100 μl of 0.004 M HCl/isopropyl alcohol was added. The percentage of cell survival was calculated as the absorbance at 540 nm: (A_{540} of treated wells/mean A_{540} of control wells) $\times$ 100. Assays were performed in duplicate.

RESULTS

Peptide design and synthesis

It was first predicted and later shown experimentally that NK-lysin adopts a secondary structure that consists of five amphipathic α-helices [10,14]. Based on the NMR structure of NK-lysin (Figure 1A), it was proposed that helix 3 interacts with membranes [10,15]. We therefore decided to synthesize two short versions of NK-lysin: a 29-residue peptide covering both α-helices 2 and 3 (NKLF1, NK-lysin residues 27–55), linked by an internal disulphide bond, and a 22-residue peptide (NKLF2, NK-lysin 34–55) corresponding to α-helix 3 with an N-terminal extension up to Cys-34 at the end of α-helix 2, to allow formation of the disulphide bond (Figure 1B). We also synthesized the homologous granulysin peptides (GranF1 and GranF2). The sequences, as well as the helical regions identified by NMR and the position of the disulphide bond, are shown in Figure 1(B). Alignment of NK-lysin and granulysin shows a high degree of identity at helical regions 2 and 3, but differences in the loop region. The overall charge distribution is similar in both NK-lysin and granulysin fragments.

Secondary structure

The solution conformation of NK-lysin and the four synthetic peptides was examined by CD in both aqueous and liposome environments. In phosphate buffer, CD spectra of NK-lysin and NKLF2 were in accordance with the α-helix content of the peptides calculated from the NMR structure of NK-lysin [1], whereas the other fragments displayed large minima at 203 nm,

Table 1 Inhibition of bacterial growth by NK-lysin and granulysin fragments

Bacteria were grown in agarose and Luria–Bertani broth plus medium E or, if indicated (LB), in Luria–Bertani broth only. NKLF1, NK-lysin residues 27–55; NKLF2, NK-lysin 34–55; GranF1, granulysin 26–55; GranF2, granulysin 33–55; nt, not tested.

		Lethal concentration (μM)				
Strain	Peptide...	NK-lysin	NKLF1	NKLF2	GranF2	GranF1
B. megaterium Bm11		1	0.9	0.3	1.3	1
E. coli D21		0.9	2.3	0.2	23	46
E. coli D21 (LB)		8	2	nt	10	12
P. aeruginosa OT97		> 190	13	nt	300	300
S. aureus Cowan I (LB)		> 190	13	nt	300	300
Erythrocytes		> 170	> 354	nt	> 390	> 320

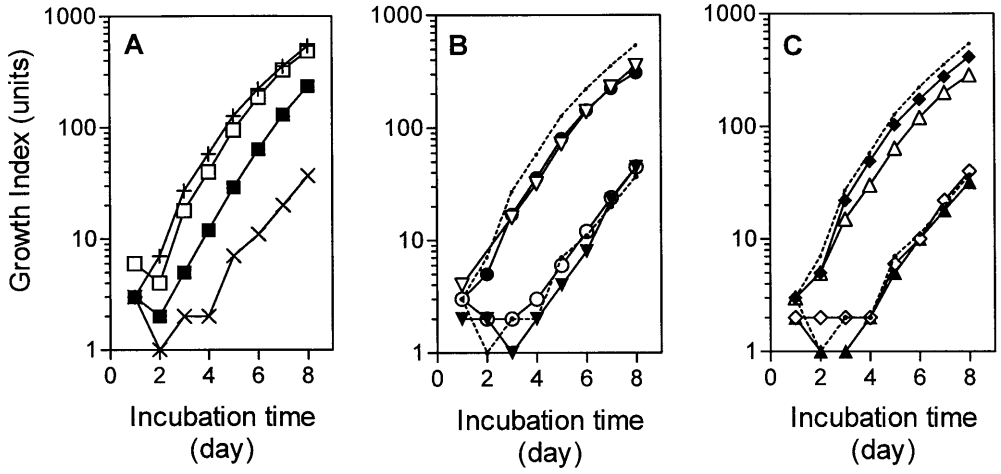


Figure 2 Anti-mycobacterial activity of NK-lysin, NK-lysin fragments and granulysin fragments

Growth index of *M. tuberculosis* H37Rv inoculated in Bactec 12B vials was monitored. Peptide-treated suspensions were compared with the control. (**A**) Control (+); control diluted 10 times (×); NK-lysin, 3 μM (□) and 30 μM (■). (**B**) NKLF1, 3 μM (▽) and 30 μM (▼); NKLF2, 3 μM (●) and 30 μM (○). (**C**) GranF1, 3 μM (◆) and 30 μM (◇); GranF2, 3 μM (Δ) and 30 μM (▲). Controls are marked with dotted lines in (**B**) and (**C**). The graphs show one experiment representative of two independent assays.

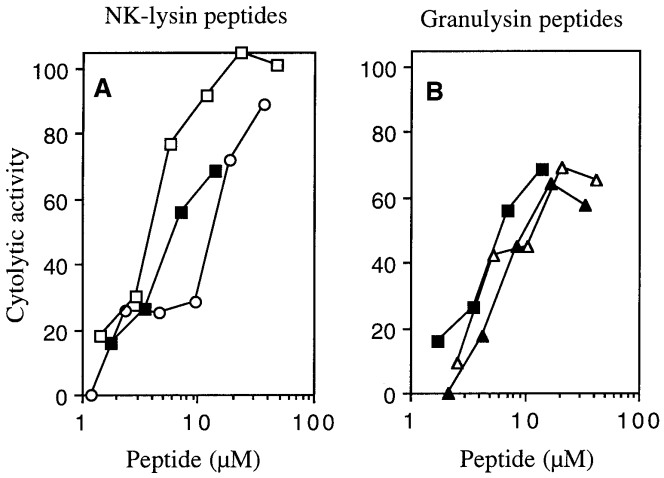


Figure 3 Cytolytic activity of NK-lysin, NK-lysin fragments and granulysin fragments

K562 cells were plated (20000 cells/well) and incubated in the presence or absence of the peptides. After 6 h, viable cells were analysed using the MTT assay. (**A**) NK-lysin (■); NKLF1 (○); NKLF2 (□). (**B**) NK-lysin (■); GranF1 (▲); GranF2 (Δ).

indicating a high degree of aperiodic conformation. The calculated α-helical contents of NKLF1, NKLF2, GranF1 and GranF2 in liposome solution (63, 40, 36 and 67 % respectively) fit well with the values derived from the NMR structure (72, 45, 43 and 70 % respectively).

Biological activity

The anti-bacterial properties of NK-lysin and the synthetic peptides are summarized in Table 1. Against *Bacillus megaterium* and *Escherichia coli*, both NKLF1 and NKLF2 had potencies comparable with NK-lysin on a molar basis. In addition, NKLF1 was more potent than NK-lysin against *Psuedomonas aeruginosa* and *Staphylococcus aureus*. The dose-dependent activity was similar for NK-lysin and NKLF1 (results not shown), suggesting a similar mechanism of killing. The granulysin peptides, GranF1 and GranF2, were very active against *B. megaterium* and moderately so against *E. coli*, but showed no activity against *P. aeruginosa* or *S. aureus*.

All four synthetic peptides were capable of inhibiting *M. tuberculosis* growth. Using a radiometric assay, 90 % inhibition was observed after 8 days at 30 μM peptide concentration (Figures 2B and 2C), compared with 60 % for native NK-lysin (Figure 2A).

The cytolytic activities of NK-lysin and the synthetic peptides against K562 cells were not very different (Figure 3), with all peptides killing 30 % of the cells in the 3–7 μM range.

DISCUSSION

Antibiotic peptides are believed to play an important role in innate host defence against microbes [26,27]. Although diverse in size, sequence and folding pattern, anti-microbial peptides can be grouped into a few structural categories [26]. NK-lysin and granulysin belong to the cysteine-rich group and have three disulphide bonds that are essential for activity [10,14,28]. Our results show that smaller (22- and 29-residue-long) peptides based on the known structures of NK-lysin and granulysin retain the anti-bacterial activities of the parent molecules and even expand their anti-bacterial spectra in certain cases, while maintaining their low haemolytic activities. Since the only structural features retained from the parent molecules were the loop between α-helices 2 and 3, helix 3 and variable lengths of helix 2, it would appear that a substantial part, if not all, of the activities of the native structures can be associated with these regions. Bactericidal domains involving loop structures have been identified previously in lactoferrin, an iron-binding antibiotic protein, and defensin [29,30]. Loop structures are also common among other small ($\sim$ 20-residue-long) natural disulphide-containing anti-bacterial peptides [8,9,11,31].

All four synthetic peptides displayed significant levels of α-helical conformation in the presence of liposomes, suggesting that in this environment they are structurally very similar to the parent molecules. NKLF1 showed activity against *Pseudomonas* and *Staphylococcus*, to which NK-lysin is inactive. Against *E. coli*, the shorter NKLF2 peptide was more active than NKLF1, a fact that may be related to the higher tendency of NKLF2 to form helices in phosphate buffer. Stability of an α-helical conformation is important for the activity of many anti-bacterial peptides, as recently shown for LL-37 [32]. The granulysin peptides, in contrast, were inactive against *Pseudomonas* and *Staphylococcus*, whereas the recombinant protein had high activity [4]. One explanation might be that additional residues are required to reproduce the native folding of granulysin in this region, which is predicted to be highly helical (results not shown). Since both NK-lysin/granulysin peptides have identical global charge, a simple cationic effect can be ruled out, in favour of conformation-dependent action.

Both NK-lysin and granulysin were active against *M. tuberculosis* (Figure 2 and [4]). Killing of intracellular pathogens may be an important function for these peptides. One mechanism put forward suggests that perforin (co-stored with granulysin in granula) drills holes in the infected cell, which allow granulysin-mediated killing of intracellular *M. tuberculosis* [3,4]. These peptides are therefore natural anti-mycobacterial agents and it is important to understand their function. All our synthetic NK-lysin and granulysin peptides were as potent as the parental peptides against *M. tuberculosis* (Figure 2 and [4]), which suggests that residues 34–55, involving helix 3 and the disulphide loop, define an important anti-mycobacterial domain in NK-lysin and granulysin. Finally, the synthetic peptides were cytolytic against tumour cells (K562), similar to the full-size peptides [1,2,28].

In conclusion, we have shown that the main properties of a relatively large anti-bacterial peptide can be reasonably reproduced by a short (about one-third of the original sequence) synthetic version designed on the basis of the native three-dimensional structure, preserving significant conformational features and retaining, even improving, some of the antibiotic functions of the parental molecule. In particular, this motif appears to define the structural requirements essential for anti-mycobacterial activity.

We thank Dr. Catrina Sergiu Bogdan for performing the cytolytic assay. This work was supported by the Swedish Society of Medicine, Magnus Berwall's Foundation, the Scandinavian Society of Chemotherapy, the Swedish Cancer Foundation and the Swedish Medical Research Council. Work at the University of Barcelona was funded by Centre de Referencia de Biotecnologia (Generalitat de Catalunya, Spain).

REFERENCES

1 Andersson, M., Gunne, H., Agerberth, B., Boman, A., Bergman, T., Sillard, R., Jörnvall, H., Mutt, V., Olsson, B., Wigzell, H. et al. (1995) EMBO J. **14**, 1615–1625
2 Pena, S. V., Hanson, D. A., Carr, B. A., Goralski, T. J. and Krensky, A. M. (1997) J. Immunol. **158**, 2680–2688
3 Tschopp, J. and Hofmann, K. (1996) Trends Microbiol. **4**, 91–94

4 Stenger, S., Hanson, D. A., Teitelbaum, R., Dewan, P., Niazi, K. R., Froelich, C. H., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C. et al. (1998) Science **282**, 121–125
5 Miyakawa, Y., Ratnakar, P., Gururaj Rao, A., Costello, M. L., Mathieu-Costello, O., Lehrer, R. I. and Catanzaro, A. (1996) Infect. Immun. **64**, 926–932
6 Lehrer, R. I., Ganz, T. and Selsted, M. E. (1991) Cell **64**, 229–230
7 Kokryakov, V. N., Harwig, S. S., Panyutich, E. A., Shevchenko, A. A., Aleshina, G. M., Shamova, O. V., Korneva, H. A. and Lehrer, R. I. (1993) FEBS Lett. **327**, 231–236
8 Romeo, D., Skerlavaj, B., Bolognesi, M. and Gennaro, R. (1988) J. Biol. Chem. **263**, 9573–9575
9 Iwanaga, S., Muta, T., Shigenaga, T., Seki, N., Kawano, K., Katsu, T. and Kawabata, S. (1994) Ciba Found. Symp. **186**, 160–174
10 Liepinsh, E., Andersson, M., Ruysschaert, J.-M. and Otting, G. (1997) Nat. Struct. Biol. **4**, 793–795
11 Fahrner, R. L., Diekmann, T., Harwig, S. S. L., Lehrer, R. L., Eisenberg, D. and Feigon, J. (1996) Chem. Biol. **3**, 543–550
12 Hill, C. P., Yee, J., Selsted, M. E. and Eisenberg, D. (1991) Science **251**, 1481–1485
13 Zimmermann, G. R., Legault, P., Selsted, M. E. and Pardi, A. (1995) Biochemistry **34**, 13663–13671
14 Andersson, M., Curstedt, T., Jörnvall, H. and Johansson, J. (1995) FEBS Lett. **362**, 328–332
15 Ruysschaert, J.-M., Goormaghtigh, E., Homblé, F., Andersson, M., Liepinsh, E. and Otting, G. (1998) FEBS Lett. **425**, 341–344
16 Andersson, M., Girard, R. and Cazenave, P.-A. (1999) Infect. Immun. **67**, 201–205
17 Merrifield, R. B. (1963) J. Am. Chem. Soc. **85**, 2149–2154
18 Barany, G. and Merrifield, R. B. (1979) in The Peptides - Analysis, Synthesis, Biology, vol. 2 (Gross, E. and Meienhofer, J., eds.), pp. 1–284, Academic Press, New York
19 Ponsati, B., Giralt, E. and Andreu, D. (1990) Tetrahedron **46**, 8255–8266
20 Andreu, D., Albericio, F., Sole, N. A., Munson, M. C., Ferrer, M. and Barany, G. (1994) Methods Mol. Biol. **35**, 91–169
21 Refai, E., Jonsson, C., Andersson, M., Jacobsson, H., Larsson, S., Kogner, P. and Hassan, M. (1999) Nucl. Med. Biol., in the press
22 Greenfield, N. and Fasman, G. D. (1969) Biochemistry **8**, 4108–4116
23 Morrisett, J. D., David, J. S., Pownall, H. J. and Gotto, A. M. J. (1973) Biochemistry **12**, 1290–1299
24 Hultmark, D., Engström, Å., Andersson, K., Steiner, H., Bennich, H. and Boman, H. G. (1983) EMBO J. **2**, 571–576
25 Middlebrook, G., Reggiardo, Z. and Tigrett, W. D. (1977) Am. Rev. Respir. Dis. **115**, 1066–1069
26 Boman, H. G. (1995) Annu. Rev. Immunol. **13**, 61–92
27 Andreu, D. and Rivas, L. (1998) Biopolymers (Peptide Sci.) **47**, 415–433
28 Andersson, M., Holmgren, A. and Spyrou, G. (1996) J. Biol. Chem. **271**, 10116–10120
29 Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. and Tomita, M. (1992) Biochim. Biophys. Acta **1121**, 130–136
30 Thennarasu, S. and Nagaraj, R. (1999) Biochem. Biophys. Res. Commun. **254**, 281–283
31 Kreil, G. (1994) Ciba Found. Symp. **186**, 77–90
32 Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. and Agerberth, B. (1998) J. Biol. Chem. **273**, 3718–3724

Received 23 April 1999/10 September 1999; accepted 11 October 1999