Volume 329, number 1,2, 219–222 FEBS 12896 August 1993

The C-terminal region of the S component of Staphylococcal leukocidin is essential for the biological activity of the toxin

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Received 31 May 1993; revised version received 8 July 1993

The Staphylococcal toxin leukocidin consists of two protein components, F and S. From a culture medium of *Staphylococcus aureus* RIMD 310925, we isolated a truncated form of S (LS_2), of which the C-terminal 17-residue segment is missing. Unlike intact S, LS_2 showed neither leukocytolytic activity in the presence of F nor affinity for monosialoganglioside G_{M1} (G_{M1}). When excited at 280 nm, both S and LS_2 exhibited intrinsic tryptophan fluorescence with an emission maximum at 318 nm. Upon binding to G_{M1}, the emission maximum of S underwent a blue shift to 310 nm, whereas no change in fluorescence took place on mixing G_{M1} with LS_2. We conclude that the C-terminal region of S is essential for its biological activity as well as for its binding to G_{M1} and that this binding is accompanied by a conformational change of the S protein.

Staphylococcal leukocidin; S component; Monosialoganglioside G_{M1}

1. INTRODUCTION

Leukocidin secreted by *Staphylococcus aureus* exerts a cytotoxic action on polymorphonuclear leukocytes and this toxin activity arises from a synergistic interaction between the two protein components, F and S [1]. We have recently shown that F is identical with HγI component of γ-hemolysin from the same organism [2,3]. It is known that S binds specifically to monosialogangliosied G_{M1} (G_{M1}) on the leukocyte membrane and thus activates phospholipase A_2 [4]. However, the nature of this binding is only poorly understood. While purifying F and S from a culture medium of *S. aureus* RIMD 310925, we accidentally isolated a truncated form of S (LS_2), which had lost the C-terminal 17 amino acid residues. In this study, we examine the biological activity of the truncated protein. The results obtained indicate that the C-terminal region of S is essential for its toxin activity and binding to G_{M1}. We also report evidence that tryptophan residues of S are less exposed to the protein surface in the S–G_{M1} complex as compared to those of unbound S.

2. MATERIALS AND METHODS

2.1. *Materials*

[*galactose*-6-^{3}H]G_{M1} was purchased from ARC, USA. Purification of F and S and preparation of polyclonal anti-S antibodies were previously described [5]. LS_2 was isolated as follows. *S. aureus* RIMD 310925 [2] was grown in 2.5% heart fusion broth (Difco) at 37°C for 24 h under aeration with O_2/CO_2 (80:20, v/v) and the culture supernatant was collected by centrifugation at 4°C. The supernatant (20 liter) was diluted 3-fold with distilled water and applied to a hydroxylapatite column (3 × 10 cm) equilibrated with 80 mM potassium phosphate buffer, pH 6.8. After washing the column extensively with the same buffer, all components of leukocidin and γ-hemolysin were eluted from the column with 0.8 M potassium buffer, pH 6.8, and the eluate was dialyzed against 30 mM sodium phosphate buffer, pH 6.5 (buffer A). The dialyzate was subjected to HPLC on a TSK gel SP-5PW cation-exchange column (TOSOH, Tokyo) using a linear NaCl gradient (0–0.8 M) in buffer A. Two protein peaks, designated LS_1 and LS_2 in the order of elution, were eluted at the position where S is usually eluted (Fig. 1). These were separately purified by rechromatography on the SP-5PW column, dialyzed against buffer A, and stored at −80°C until use. As will be described below, LS_1 was identified as S itself.

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Abbreviations: S, S component of leukocidin; F, F component of leukocidin; G_{M1}, monosialoganglioside G_{M1}; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

2.2. *Assay of leukocidin activity*

Leukocidin activities of S and LS_2 were assayed in the presence of purified F as described previously [2]. Human polymorphonuclear leukocytes were separated from blood by centrifugation at 2,000 × *g* for 30 min at room temperature using a Mono–poly resolving medium (Flow Laboratories, North Ryde, Australia) and used for the assay.

2.3. *Assay of S and LS_2 binding to G_{M1}*

One nmol of purified S or LS_2 was incubated with [*galactose*-6-^{3}H]G_{M1} (1.2 nmol) in 60 μl of Locke's solution [6] at 37°C for 15 min and the mixture was centrifuged at 10,000 × *g* to remove aggregated materials. The recoveries of S and G_{M1} in the supernatant were 70 and 80%, respectively. The supernatant was then subjected to gel filtration on a Toyopearl (TOSOH, Tokyo) column (0.8 × 20 cm) using Locke's solution as solvent. Fractions (0.5 ml each) were collected and assayed for ^{3}H radioactivity in an Aloka model LSC 903 liquid scintillation spectrophotometer and for protein. The protein in the fractions was quantified as follows. The samples (50 μl each) were spotted on a nylon membrane. The nylon membrane was treated with monospecific anti-S antibodies. Antigen–antibody complexes were detected with anti-rabbit IgG (Fc)-alkaline phosphatase conjugate (Sei-

kagaku Kogyo Co., Tokyo). The spots were developed with Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrates. The stained spots were quantified by using a Bio-Rad model 620 video densitometer.

2.4. *Tryptophan fluorescence of S and LS_2 in the presence and absence of G_{M1}*

The tryptophan fluorescence spectra of purified S and LS_2 were measured in Locke's solution using a Hitachi model 204A fluorescence spectrophotometer; excitation wavelength being 280 nm. The fluorescence spectra of S and LS_2 were also determined in the presence of G_{M1}. For this purpose, 1 nmol of S or LS_2 was incubated with varying amounts of G_{M1} in 400 μl of Locke's solution at 37°C for 20 min. The precipitate formed was removed by centrifugation at 10,000 × g, and the tryptophan fluorescence of the supernatant was recorded.

2.5. *Other analytical methods*

SDS-PAGE and Western blot analysis using monospecific anti-S antibodies were carried out as described [2]. The N- and C-terminal amino acid sequences were determined as described previously [2].

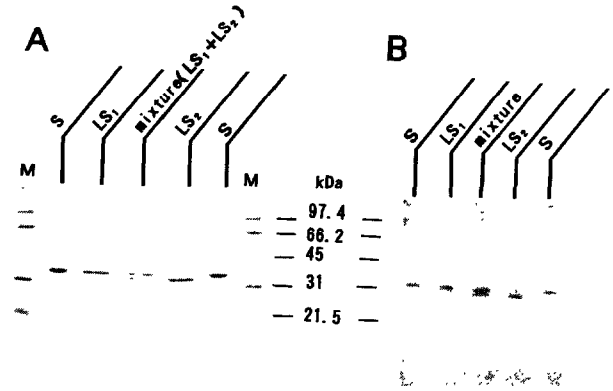

Fig. 2. SDS-PAGE (A) and Western immunoblotting (B) analyses of LS_1 and LS_2. The gel was stained with Coomassie brilliant blue R-250. Molecular mass standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa).

3. RESULTS AND DISCUSSION

3.1. *Isolation and characterization of LS_2*

In an attempt at purifying F and S from a culture medium of *S. aureus*, we noticed that two protein peaks (LS_1 and LS_2 in Fig. 1) were eluted from the HPLC column at the position where S is usually eluted.

We therefore purified these proteins to gel electrophoretic homogeneity (Fig. 2A). The molecular mass of LS_2 (31.5 kDa) was smaller than that of LS_1 (33 kDa). Monospecific anti-S antibodies reacted with both LS_1 and LS_2 in Western blot analysis (Fig. 2B). The N-terminal 40-residue sequence determined (underlined in Fig. 3) for both LS_1 and LS_2 was in complete match with that of S predicted from the DNA nucleotide sequence [7]. On the other hand, the C-terminal amino acid sequence of LS_1 and LS_2 were determined to be -His-Glu-Ile-Lys-Val-Lys-Gln-Asn-COOH and -Arg-Asn-Tyr-Thr-Val-COOH, respectively. The C-terminal 9-residue sequence of LS_1 is identical with that predicted for S, whereas the C-terminal 5-residue sequence of LS_2 corresponds to residues 265 through 269 in the predicted S sequence. From these results, it can be concluded that LS_1 is S itself and LS_2 is a truncated form of S, which has lost the C-terminal 17-residue segment (Fig. 3). Interestingly, LS_2 was eluted after LS_1 from the cation exchange column (Fig. 1), even though LS_2 is more acidic than LS_1 because of the loss of 5 basic and 2 acidic residues from the C-terminus of LS_1. This suggests the possibility that LS_1 and LS_2 are in different conformations. It appears likely that the truncated form of S was produced in this particular purification experiment by the action of a contaminating protease.

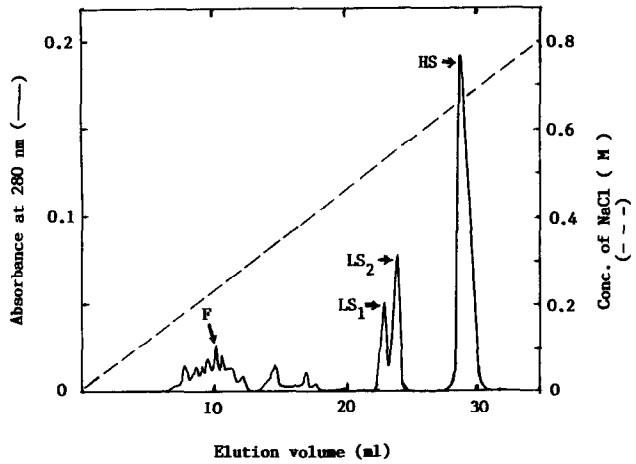

Fig. 1. Elution profile of leukocidin and γ-hemolysin components from the SP-5PW cation-exchange column. Leukocidin and γ-hemolysin fractions eluted from a hydroxylapatite column were subjected to HPLC (TOSOH, Tokyo) equipped with a TSK gel SP-5PW column. HS represents HγII fraction of γ-hemolysin.

3.2. *Leukocidin activities of S and LS_2*

The concentration of S (= LS_1) required for 100% leukocytolytic activity toward 10^6 human polymorphonuclear leukocytes in 30 min in the presence of an excess amount of F was determined to be 1 μg/ml. In contrast, LS_2 at all the concentrations tested showed no leukocidin activity in the presence of F, indicating that the C-terminal 17-residue segment of S is essential for leukocidin activity.

3.3. *Binding of S and LS_2 to G_{M1}*

Gel filtration was used to assess the binding of S and LS_2 to G_{M1}. Gel filtration of [^{3}H]G_{M1} alone resulted in the elution of 74% of the radioactivity at the inclusion volume (fraction A in Fig. 4a) indicating that this amount of G_{M1} was monomeric under the conditions used, whereas 26% of G_{M1} was eluted as large micelles with an apparent molecular mass of about 50 kDa (fraction B in Fig. 4a). S and LS_2 were eluted at the positions corresponding to molecular masses of about 35 and 70 kDa, respectively, suggesting that LS_2 was dimeric in Locke's solution. Upon gel filtration of a mixture of S

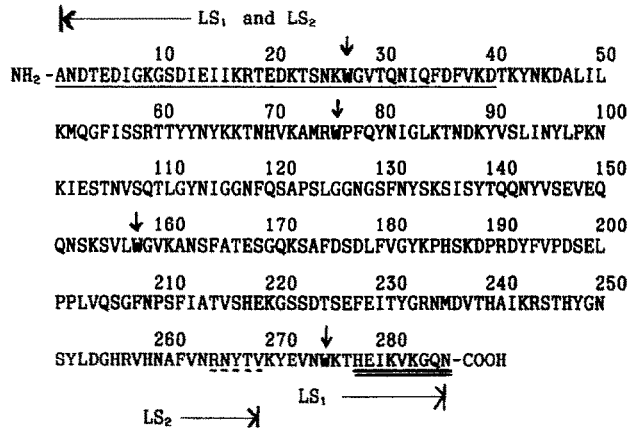

Fig. 3. Amino acid sequence of S, which was deduced from the DNA sequence reported previously [5]. N- and C-terminal amino acid sequences of LS_1 and LS_2 preparations determined are shown by single-, double- and dotted underlines, respectively. Intrinsic Trp residues in S are marked by vertical arrows.

and [^{3}H]$G_{M\,1}$, two radioactive peaks corresponding to about 30 and 68 kDa (fractions C and D, respectively, in Fig. 4b) were eluted and both peaks contained S. The recoveries of S in fractions C and D were 55 and 45%, respectively. The S to $G_{M\,1}$ molar ratios in fractions C and D were calculated to be about 1:1 and 1:1.1, respectively. Although the reason why two types of the S–$G_{M\,1}$ complex were formed is still to be elucidated, this finding indicates that S does bind to $G_{M\,1}$. On the other hand, the gel filtration profile of a mixture of LS_2 and $G_{M\,1}$ (Fig. 4c) provides no evidence for association of the two components. It can thus be concluded that the C-terminal 17 amino acid residues of S play a pivotal role in the binding of the protein to $G_{M\,1}$. F did not bind to $G_{M\,1}$ (data not shown).

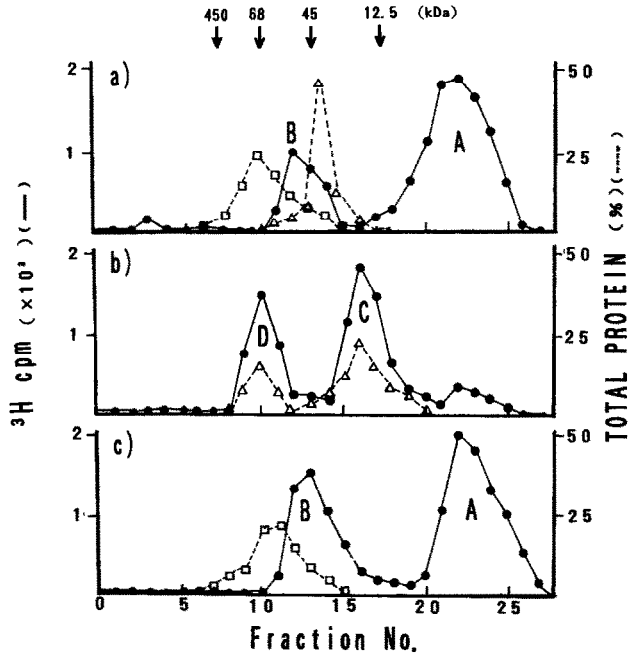

Fig. 4. Gel filtration chromatography of S–$G_{M\,1}$ complex. (a) elution profiles of $G_{M\,1}$ (●), S (△) and LS_2 (□). The three samples were subjected to gel filtration separately, but the elution profiles are illustrated in the same panel. (b) complex formation of S with $G_{M\,1}$. (c) elution profile of a mixture of LS_2 and $G_{M\,1}$. Protein amounts are given in percentage of total one applied to the column. Molecular mass standards used were ferritin (450 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and cytochrome *c* (12.5 kDa).

3.4. *Conformational change of S upon binding to $G_{M\,1}$*

Since S contains 4 tryptophan residues per molecule (Fig. 3), it was of interest to examine if the conformation of S changes upon binding to $G_{M\,1}$ by measuring intrinsic tryptophan fluorescence. When excited at 280 nm, S showed fluorescence with an emission maximum at 318 nm (Fig. 5a). The addition of increasing amounts of $G_{M\,1}$ to the solution resulted in the reduction of fluorescence intensity and a blue shift of the emission maximum (Fig. 5b–e). At a S to $G_{M\,1}$ molar ratio of 1:1, the intensity reduction was 33% and the emission maximum was 310 nm (Fig. 5f). Further addition of $G_{M\,1}$ did not affect the fluorescence. Since it has been reported that decreased exposure of tryptophan residues to the protein surface is accompanied by a blue shift of tryptophan fluorescence [8], the above finding indicates that tryptophan residues in S become less exposed to the protein surface upon binding to $G_{M\,1}$. The decreased fluorescence intensity might originate from the accessibility of Trp residues to the quenching amino acid residues in S by moving of the Trp residues in proximity to the hydrophobic area in the interior of the S molecule. The tryptophan fluorescence of LS_2 also showed an emission maximum at 318 nm, but the fluorescence intensity was considerably lower than that of S probably because LS_2 contains only 3 tryptophan residues (Fig. 5g). Moreover, this fluorescence was not affected by the addition of $G_{M\,1}$ at any concentration, providing further evidence that LS_2 does not bind to $G_{M\,1}$. The trypto-

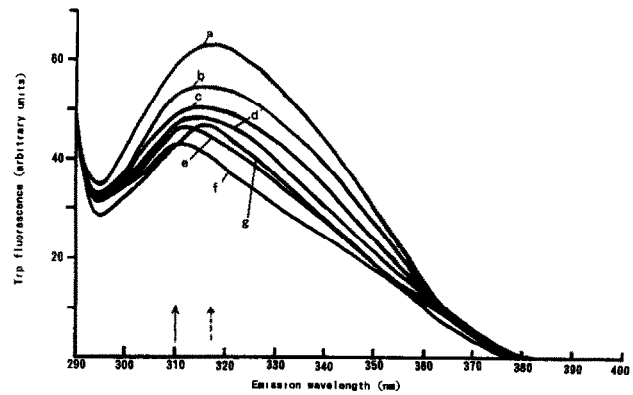

Fig. 5. Tryptophan fluorescence of S (1 nmol) and LS_2 (1 nmol) in the absence and presence of varying amounts of $G_{M\,1}$. a, S alone; b, S + 0.2 nmol $G_{M\,1}$; c, S + 0.4 nmol $G_{M\,1}$; d, S + 0.6 nmol $G_{M\,1}$; e, S + 0.8 nmol $G_{M\,1}$; f, S + 1.0 (or 1.2, 1.4) nmol $G_{M\,1}$; g, LS_2 alone or LS_2 + 0.2 to 1.4 nmol $G_{M\,1}$. Fluorescence intensity is given in arbitrary units per mol of protein.

phan fluorescence of F, which contains 6 tryptophan residues, has an emission maximum at 330 nm, but $G_{M\,1}$ had no effect on the fluorescence (data not shown) [5]. These findings clearly indicate that a conformational change of S takes place on mixing $G_{M\,1}$ with S.

Acknowledgements: This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan (to Y.K.); and Kuribayashi Foundation (to Y.K.).

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