Biological characterization of the zinc site coordinating histidine residues of staphylococcal enterotoxin C2

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The bacterial toxin staphylococcal enterotoxin C2 (SEC2) can cause staphylococcal toxic shock syndrome and food poisoning. Although the previously determined crystal structure of SEC2 revealed that some histidine residues (His47, His118 and His122) contribute to the binding of zinc ions, little is known about their biological roles in SEC2. This prompted us to investigate the role of the zinc site coordinating histidine residues in the biological activities of SEC2. The mutants with substitutions at positions 118 and 122 all retained T-cell stimulatory activity, whereas the histidine mutants at position 47 were defective in the ability to stimulate T-cell proliferation. Further toxicity assays *in vivo* indicated that mutants SEC2-H118A and SEC2-H122A were defective in emetic and febrile activities. However, mutant SEC2-H47A could cause significant emetic and febrile responses in comparison with the other two histidine mutants. These findings suggested that the zinc-coordinating histidine residues play significant roles in superantigen and toxic activities of SEC2 and further implied that superantigen and febrile activities could be separable in staphylococcal enterotoxins. The results also show that it should be possible to design new SEC2 immunotherapeutic agents that have superantigen activity and low toxicity.

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INTRODUCTION

Staphylococcal enterotoxins (SEs) belong to the family of structurally related bacterial toxins secreted by *Staphylococcus aureus* and *Streptococcus pyogenes* (Bohach *et al.*, 1990), which are responsible for several human and animal diseases, including diarrhoea, emesis, fever and clinical shock. Although the number of recognized SEs continues to grow, 16 major serological types have been characterized, namely SEA through SEE, SEG through SET, and SEU (Hovde *et al.*, 1990; Su & Wong, 1995; Munson *et al.*, 1998; Dinges *et al.*, 2000; Letertre *et al.*, 2003; Ono *et al.*, 2008). SEC was further classified into three subtypes (C1–C3) according to minor epitope differences, which differ in amino acid sequence at only 10 residues (Hovde *et al.*, 1990). It is speculated that it is the high degree of homology that makes these toxins share some important biological properties such as emesis, fever and superantigenicity (Bohach *et al.*, 1990; Dinges *et al.*, 2000). As superantigens, SEs can bind to the outside of the antigenic

groove of major histocompatibility complex class II (MHC II) molecules without being processed by antigen-presenting cells (APC), and then stimulate vigorous proliferation of T-cells bearing certain T-cell receptor (TCR) V β regions (Dellabona *et al.*, 1990; Dinges *et al.*, 2000). As a result, polyclonal T-cell expansion produces a large amount of various cytokines, which may result in acute systemic illness and clinical shock (Dinges *et al.*, 2000).

Besides the gastrointestinal symptoms in human and animals induced by SEs, superantigens also direct strong cell-mediated cytotoxicity preferentially against MHC II-positive target tumour cells. In addition, a large number of different cytokines have been shown to elicit a systemic antitumour response, and have been extensively employed for the studies of antitumour immunotherapy (Hedlund *et al.*, 1993; Ochi *et al.*, 1993; Mondal *et al.*, 2002; Xiu *et al.*, 2007). However, the emetic and febrile responses induced by SEs have seriously limited their clinical application for antitumour immunotherapy.

Recently, many investigators have focused their research on the relationship between the structure and function of SEs. A considerable amount of structural data is now available

Abbreviations: FBS, fetal bovine serum; MHC II, major histocompatibility complex class II; MTT, methylthiazol tetrazolium; SE, staphylococcal enterotoxin; SEC2, staphylococcal enterotoxin C2; TCR, T-cell receptor.

describing the molecular architecture for members of the SE family, and some active sites have been identified (Swaminathan *et al.*, 1992; Schad *et al.*, 1995; Papageorgiou *et al.*, 1995; Kumaran *et al.*, 2001; Papageorgiou *et al.*, 2004). As one of the most notable features, some histidine residues participate in the formation of one or two zinc-binding sites in some SE structures, and may play a functional role for SEs. Fraser *et al.* (1992) showed that zinc binding was required for the stabilization of the binding domain of MHC II molecules, and suggested that histidine residues in SEA might be essential in a coordination with zinc. Moreover, the crystal structure of SEA indicated that His187, His225 and Asp227 were found in zinc-binding sites, and His61 is from the neighbouring molecule that mediates the dimerization (Schad *et al.*, 1995). Site-directed mutagenesis showed that His225 was important for both superantigen and emetic activities of SEA, and His61 appeared to be responsible only for emetic activity (Hoffman *et al.*, 1996). In addition, His187 appeared to be unnecessary for both the activities (Hoffman *et al.*, 1996). These results further suggested that the superantigen and emetic activities are separable in SEs. However, it is still unclear whether there is a correlation between the superantigen and febrile activity in SEs.

Although the previously determined crystal structure showed the presence of two zinc-binding sites in SEC2 (Papageorgiou *et al.*, 1995, 2004), and that the histidine residues at positions 47, 118 and 122 mediate the formation of the two sites, the role of the histidine residues in the biological activities of SEC2 is still unclear.

In the present study, we employed site-directed mutagenesis to introduce amino acid substitutions in an attempt to assess the role of these zinc-coordinating histidine residues. The results indicated that these histidine residues play an important role in the immunological activities of SEC2, and implied that the superantigen and febrile activities could be dissociated. The data in the present study also suggested that, in the future, it should be possible to construct a promising superantigen agent with low toxicity. Furthermore, this study also provides experimental evidence that zinc binding may be functional for the biological activities in SEC2.

METHODS

Bacterial strains, plasmids and cell lines. *S. aureus* 0165 (CGMCC 0165) producing SEC2 was provided by Shenyang Xiehe Pharmaceutical Group Company (China). It was grown in trypticase soy broth (TSB) medium. For transformation and protein expression, *Escherichia coli* BL21(DE3) was cultured in Luria-Bertani (LB) medium. Expression vector pET28a was from Novagen.

Murine fibrosarcoma L929 cells (NCTC clone 929) and murine hepatoma Hepa1-6 cells were purchased from the American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 medium with 10% (v/v) fetal bovine serum (FBS).

Animals. Female BALB/c mice (6–8 weeks old, 20–25 g) and adult New Zealand White rabbits weighing 2.0–2.5 kg were supplied by the experimental animal centre, China Medical University (Shenyang,

China). The animals were maintained under specific-pathogen-free conditions on a 12 h light/dark cycle.

Healthy cats about 8–10 weeks old, with a mean body weight of 500 g, were supplied by Tianjin Institute of Pharmaceutical Research. The cats were caged separately in 12 h light/dark cycle at an ambient temperature of 22–26 °C and relative humidity of 40–70%, and food and water were available at all times. The animals were allowed to acclimate to the facility for at least 1 week before randomization into different experimental groups. All experiments were carried out following the guideline principles for the care and use of laboratory animals approved by the Animal Care Committee of China.

Chemicals and enzymes. IPTG and methylthiazol tetrazolium (MTT) were purchased from Sigma; restriction enzymes, RNase A and *Pfu* DNA polymerase were from Takara Biotechnology Co.; Ni-NTA His.Bind Resin was from Qiagen; the DNA Gel Extract kit and Mini-preparation of plasmid kit were from BioDev-Tech Co.

Site-directed mutagenesis. The full-length SEC2-encoding gene was amplified from the total DNA of *Staph. aureus* 0165 by PCR using the sense primer 5'-CGGAATTCGAGAGTCAACCAGA-3' and the antisense primer 5'-TCGCTCGAGTTATCCATTCTTTGTTG-3'. The amplified DNA fragments were purified with the DNA Gel Extract kit and verified by DNA sequencing, then used as the template for constructing the mutated SEC2 gene. Single amino acid substitutions of the SEC2 gene were introduced using sequence overlap extension (Ho *et al.*, 1989). Mutants with multiple mutations were constructed by sequential mutagenesis. All primers used for site-directed mutagenesis are listed in Table 1. All PCR-generated fragments were digested by *EcoRI* and *XhoI*, and ligated into plasmid pET28a(+) digested with the same enzymes. Each constructed plasmid was then transformed into *E. coli* BL21(DE3) separately and identified by DNA sequence analysis.

Expression and purification of mutant proteins. For expressing mutant proteins, overnight cultures of transformed *E. coli* BL21(DE3) were inoculated at a ratio of 1:100 (v/v) into LB medium supplemented with 50 µg kanamycin ml⁻¹. Cells were incubated at 37 °C with vigorous shaking until the OD₆₀₀ reached 0.5; then generation of the mutant protein was induced by the addition of 1.0 mM IPTG and the cells were grown at 30 °C for 4 h with vigorous shaking. The cells were then harvested by centrifugation at 3000 g for 10 min and stored at -70 °C until use.

The cell pellet was resuspended in ice-cold lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 10 mM imidazole, pH 7.9), followed by sonication on ice. The cell lysate was clarified by centrifugation at 12 000 g for 30 min. The supernatants were collected and loaded onto a Ni-saturated chelating Sepharose column pre-equilibrated with lysis buffer. After non-specifically bound host proteins were washed off with wash buffer (50 mM NaH₂PO₄, 500 mM NaCl, 40 mM imidazole, pH 7.9), the mutant SEC2 protein bound to the resin specifically was eluted with wash buffer containing 250 mM imidazole and dialysed against PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) at 4 °C for 48 h. Purified protein concentrations were determined by Bradford assay using BSA as the standard (Bradford, 1976). Relative protein purity was estimated by SDS-PAGE and Coomassie brilliant blue R-250 staining.

Proteolytic lability studies. Purified preparations of SEC2 or its mutant proteins were incubated with trypsin to assess their general stability. Each protein (100 µg ml⁻¹) was incubated at 37 °C with trypsin at a trypsin:protein ratio of 1:25 (w/w). After desired periods of time, aliquots were removed, and the reaction was terminated by boiling in SDS-PAGE sample buffer for 5 min. The extent of tryptic digestion was assessed by SDS-PAGE.

Table 1. Summary of SEC2 mutants used in this study

Site-directed mutation(s)*	Amino acid substitution†	Protein designation	Primers‡
C139→G; A140→C; T141→A	His-47→Ala	SEC2-M01	P1: 5'-TTTGGCAGC AG ATTTAATTTATAAC3-' P2: 5'-GTTATAAAATTAATCT GC TGCCAAA3-'
C352→G; A353→C	His-118→Ala	SEC2-M02	P3: 5'-GGAATAACAAA AGCT GAAAGAAACCAC-3' P4: 5'-GGTTTCCTT CAGCT TTTGTTATTCC-3'
C352→T	His-118→Tyr	SEC2-M03	P5: 5'-CTATGTAAACATATATTTTTCATCCAA-3' P6: 5'-GGATGAAAAATATATGTTTACATAGTAA-3'
C364→G; A365→C	His-122→Ala	SEC2-M04	P7: 5'-GGAAAC GCCT TTTGATAATGGGAAC-3' P8: 5'-CCATTATCAA AGGCG TTTCCTTC-3'
C352→G; A353→C; C364→G; A365→C	His-118→Ala; His-122→Ala	SEC2-M05	P3, P4; P7, P8
C139→G; A140→C; T141→A; C352→G; A353→C; C364→G; A365→C	His-47→Ala; His-118→Ala; His-122→Ala	SEC2-M06	P1, P2; P3, P4; P7, P8

*Specific point mutation produced in particular amino acid codon. Numbers refer to the positions of nucleotide bases within the SEC2 gene.

†Specific amino acid substitution.

‡The primers were designed for amino acid substitution. The sequences of mutant residues are indicated in boldface.

Cellular proliferation assays. Splenocytes from 6- to 8-week-old BALB/c female mice were used in T-cell proliferation assays (Hufnagle *et al.*, 1991). Splenocytes were seeded into the wells of 96-well microtitre plates at a density of 8×10^5 cells per well in RPMI 1640 medium containing 10% FBS. Serial 10-fold dilutions of SEC2 and mutants made in RPMI 1640 were added to each well, starting with $10 \mu\text{g ml}^{-1}$, to 1 ng ml^{-1} . RPMI 1640 medium served as negative control. The splenocytes were grown at 37°C with 5% CO_2 for 72 h.

The proliferation of splenocytes was determined by MTT assay (Mosmann, 1983). Briefly, after incubation for 72 h, $25 \mu\text{l}$ MTT (5 mg ml^{-1}) dissolved in PBS was added to each well, and the plate was incubated at 37°C for 4 h. The cells were collected by centrifugation for 10 min at 500 g. The pellet was redissolved in $120 \mu\text{l}$ DMSO at room temperature for 20 min, and the absorbance was measured with a microplate reader at 570 nm, using a reference wavelength of 630 nm. The final absorbance is the difference between these two readings. The proliferation effects were reported as a proliferation index, which is the absorbance values for the experimental groups divided by those from negative control groups.

In vitro growth inhibition assay. Cell death induced by redirected T-cell cytotoxicity (superantigen-dependent cellular cytotoxicity) was measured using the MTT assay. Briefly, splenocytes were used as effector cells. Murine fibrosarcoma cell line L929 and murine hepatoma cell line Hepa1-6 were used as target cells. Dilutions of SEC2 or mutant SEC2s were separately added to 96-well plates at 0.01, 0.1, or $1 \mu\text{g}$ per well in triplicate. Each set of target cell (2.5×10^4 cells per well) was mixed with splenocytes at an effector:target ratio of 20:1 in each well, and incubated for 48 h at 37°C in a humidified 5% CO_2 atmosphere. The blank wells (RPMI 1640 only), unsettled cell control wells (L929 cells or Hepa1-6 cells only) and lymphocyte-releasing wells (lymphocytes and proteins) were used as control. The negative control was BSA.

After incubation for 48 h, $25 \mu\text{l}$ MTT (5 mg ml^{-1}) was added to each well and incubated for another 4 h. The formazan crystals formed were redissolved in $120 \mu\text{l}$ DMSO at room temperature for 20 min, and the absorbance was measured with a microplate reader at a test wavelength of 570 nm against a reference wavelength of 630 nm. The

final absorbance is the difference in absorbance read at 570 nm compared with that read at 630 nm. Cell growth inhibition (%) was calculated with the equation $100 - [(\text{Abs value in protein-treated cells well} - \text{Abs value in lymphocytes-releasing well}) / (\text{Abs value in unsettled cells control wells} - \text{Abs value in blank control wells})] \times 100$.

Emesis assays in vivo. The cat model described previously (Martin & Marcus, 1964; Clark & Page, 1968) was used to compare diarrhoea and emetic capability induced *in vivo* by SEC2 and several representative SEC2 mutants. Purified SEC2 and mutants were diluted in PBS (PBS, pH 7.4), and sterilized by passage through a $0.22 \mu\text{m}$ pore-size filter. Two-millilitre volumes of each protein at an appropriate dilution were administered intraperitoneally to the cats. Once injected, they were disturbed as little as possible. The animals were observed for emesis and diarrhoea for up to 6 h after the intraperitoneal administration. The number and times of vomiting and diarrhoea, and time to the first response episode, were recorded.

Rabbit model experiments. The rabbit model was used to compare febrile response *in vivo* induced by SEC2 and mutants. The rectal temperature of each animal was allowed to stabilize for at least 90 min before any injections. Only rabbits whose body temperatures were stable and in the range $38.6\text{--}39.5^\circ\text{C}$ were used to determine the effect of protein application. Fever has been defined as a mean increase in body temperature $>0.5^\circ\text{C}$ over a 4 h period (Anonymous, 2002). Each animal (adult New Zealand White rabbit) was given an initial intravenous injection containing SEC2 or mutant SEC2 [$10 \mu\text{g}$ ($\text{kg body weight}^{-1}$)] dissolved in PBS. Three rabbits were injected with each protein. Positive and negative control groups were given injections of SEC2 or PBS, respectively. Rectal temperatures of rabbits were measured with indwelling rectal thermometers and recorded for 4 h after pyrogen administration. The rectal temperature changes (ΔT) of rabbits were calculated by subtracting the temperature immediately before injection from the temperature at each time point after the pyrogen injection.

Statistical analysis. Results are presented as the mean $\pm$ SD. Statistical analysis was performed using Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

RESULTS

Construction and expression of SEC2 mutants

To investigate the roles of the zinc-coordinating histidine residues involved in the biological activities of SEC2, a series of SEC2 mutants with the substitution of histidine residues by alanine or tyrosine residues was constructed (Table 1). Expression vectors constructed for those mutants were confirmed by DNA sequencing. All of the mutant proteins were induced by IPTG for 4 h at 30 °C. The soluble recombinant expression of SEC2 mutants were detected in supernatants by SDS-PAGE. After purification with Ni-saturated chelating Sepharose, all of the mutant proteins, appearing as a single band on SDS-PAGE, were of >95 % purity (data not shown), which could be used to further study the biological activities.

Proteolytic lability studies

To further investigate the possibility that the mutant SEC2s had altered conformations, each mutant SEC2 was tested for susceptibility to degradation by trypsin. The results showed that each of the mutant SEC2s was resistant to digestion by trypsin *in vitro*, like wild-type SEC2 (Fig. 1), which was a further indication that the substitutions did not result in significant conformational change in SEC2 mutants that would expose buried trypsin cleavage sites.

Superantigen activity assays *in vitro*

SEC2 and the mutant proteins were tested for the ability to stimulate murine T-cell proliferation. Dilutions of each protein were tested in triplicate wells. The results showed

Fig. 1. Comparison of the susceptibility of SEC2 and SEC2 mutants to degradation by trypsin *in vitro*. Purified proteins (100 µg ml⁻¹) (designated on the left) were incubated with trypsin. At various time points (indicated at the top), approximately 0.75 µg of each sample was boiled with SDS-PAGE sample buffer for 5 min, and analysed by SDS-PAGE.

Fig. 2. Induction of murine splenocyte proliferation by SEC2 and SEC2 mutants. SEC2 or mutants were incubated with murine splenocytes for 72 h before the proliferation of splenocytes was determined by MTT assay. Value on the y-axis represents the proliferation index (mean ± SD of triplicate values). Each purified protein was tested in at least three separate assays.

that mutants SEC2-M02, SEC2-M03, SEC2-M04 and SEC2-M05 were comparable to SEC2 in their ability to stimulate T-cell proliferation (Fig. 2). However, SEC2-M01 and SEC2-M06 induced a significantly lower level of proliferative response than did SEC2 ($P < 0.01$) at all concentrations (Fig. 2).

In vitro growth-inhibition assay

The inhibition of the growth of L929 and Hepa1-6 cells by SEC2 and mutants with splenic lymphocytes was examined by MTT assay. The cytotoxic effects of SEC2 and mutated SEC2s on L929 and Hepa1-6 cells were all dose-dependent, with significant cytotoxicity at a concentration of 10 ng per well and a maximum effect at 1000 ng per well ($P < 0.01$); representative results are shown in Fig. 3. Mutants SEC2-M02, SEC2-M03, SEC2-M04 and SEC2-M05 were comparable to SEC2 in their ability to inhibit the growth of L929 and Hepa1-6 cells (Fig. 3). However, SEC2-M01 and SEC2-M06 induced a significantly lower level of cytotoxic response than did SEC2 ($P < 0.01$) at all concentrations, but cytotoxic activity was statistically significant ($P < 0.01$) compared with the negative control (Fig. 3), which is consistent with the results obtained from the murine T-cell proliferation assays.

Toxicity of the mutant SEC2s *in vivo*

Mutants SEC2-M01, SEC2-M02 and SEC2-M04 were tested for their ability to induce emetic activity in a cat model. Initial experiments showed that the minimal emetic dose of SEC2 for cat was 1 mg. In order to assess the emetic ability of the SEC2 mutants, each cat was administered 2 mg to ensure an excess over the minimal emetic dose.

Fig. 3. Effect of SEC2 and mutants on inhibition of growth of Hepa1-6 cells (a) and L929 cells (b) by activating lymphocytes *in vitro*. BSA was used as negative control. Data shown represent at least three separate experiments, and the values represent the mean $\pm$ SD.

After intraperitoneal administration, the mutants SEC2-M02 and SEC2-M04 did not provoke emetic response and diarrhoea (Table 2). However, a weak but significant emetic response was observed for SEC2-M01 mutant tested at 2 mg (Table 2).

In addition, the rabbit model was used to compare the febrile response *in vivo* to SEC2 and several selected mutants. The results showed that all the histidine mutants were depressed in their ability to induce fever in rabbits from 1 to 4 h compared with SEC2, and mutant SEC2-M04 did not induce a significant fever response compared with other mutants ($P < 0.01$) (Fig. 4). However, it is notable that mutant SEC-M01 exhibited a significantly higher level of fever response than the other two mutants ($P < 0.01$) (Fig. 4).

DISCUSSION

To our knowledge, this is the first study examining the biological activities of mutant SEC2 with substitutions of histidine residues. A series of histidine mutants of SEC2 were constructed, and expressed in *E. coli*. These mutant proteins were purified with Ni-NTA affinity chromatography via an N-terminal His-tag which showed no effect on the biological activity of wild SEC2 in our previous study (Xu *et al.*, 2005). It was found that substitution of a histidine residue at position 47 dramatically affects the ability of SEC2 to stimulate murine T-cells in comparison with wild SEC2. A previous study showed that His47 in SEC2 is involved in MHC II binding (Schad *et al.*, 1997); thus it is possible that the substitution of alanine for histidine at position 47 may impair the binding of SEC2 to

Table 2. Emetic activities of SEC2 and mutants in cats after intraperitoneal administration

Toxin or control	No. of cats			Latency period to first emesis (min)*	Latency period to first diarrhoea (min)*	No. of vomiting episodes†	No. of diarrhoea episodes†
	Tested	Vomited	Diarrhoea				
SEC2	5	5	4	62 $\pm$ 20.9	50 $\pm$ 20.1	2–5	1–4
SEC2-M01	5	2	2	9, 10	132, 67	2, 2	1, 1
SEC2-M02	4	0	0	–	–	0	0
SEC2-M04	4	0	0	–	–	0	0
PBS	3	0	0	–	–	0	0

*Values for three or more cats with vomiting or diarrhoea, respectively, are means $\pm$ SD, whereas individual values are given for one or two cats with vomiting or diarrhoea, respectively.

†Values for three or more with vomiting or diarrhoea, respectively, are given as ranges, whereas individual values are given for one or two vomiting cats with vomiting or diarrhoea, respectively.

Fig. 4. Pyrogenicity of SEC2 and mutated proteins in a rabbit model. SEC2 and mutants with single histidine substitution were separately injected intravenously at a dose of $10 \mu\text{g kg}^{-1}$ in PBS. The mean rectal temperature rise (ΔT) of the rabbits (three per group) was monitored for 4 h. PBS was used as negative control. The values presented represent the mean $\pm$ SD.

MHC II molecules, and influence its superantigen activity. Moreover, because SEC2-H47A exhibited a reduced, but significant ability to induce emesis and fever, it is possible that His47 is not essential for toxicity of SEC2. In addition, a recent study of spatial structure showed that a secondary zinc-binding site (Zn II site) was found in the SEC2 structure, and His47 was involved in the interaction with zinc atoms at this site (Papageorgiou *et al.*, 2004); thus the reduced superantigen activity of the mutant SEC2-H47A suggested that the Zn II site could play a role in superantigen activity, confirming and extending the earlier finding that the Zn II site is important for the binding of SEC2 to MHC II molecules, and may regulate the activity of SEC2 (Papageorgiou *et al.*, 2004).

The substitutions of histidine residues at positions 118 and 122 had no effect on the superantigen activity of SEC2, suggesting that these two histidines are not required for this activity. Histidine 118 is the only histidine that is conserved among all the enterotoxins characterized (His114 in SEA, His121 in SEB) (Schad *et al.*, 1997). Our results showed that both SEC2-H118A and SEC2-H118Y are active in murine T-cell stimulation and inhibition of tumour cell growth, which is consistent with the previous result that a SEA mutant with the substitution of His114 retained the ability to induce T-cell proliferation (Hoffman *et al.*, 1996). Thus, the conserved histidine is not required for the superantigen activity of SEs. However, it was noteworthy that SEC2-H118A and SEC2-H122A did not induce an emetic response at a high dose of 2 mg per animal, although they retained the superantigen activity. These findings suggest that the histidine residues at positions 118 and 122 play a crucial role in the emetic activity of the molecule. Some investigators have suggested that the emetic and superantigen activities of SEs can be

dissociated (Harris *et al.*, 1993; Hoffman *et al.*, 1996), which was consistent with our results.

In addition, a surprising finding was that the fever response induced by SEC2-H118A and SEC2-H122A was significantly reduced compared with SEC2, and the mutant SEC2-H122A did not cause a significant fever response. Moreover, another phenomenon is that the superantigen activity of SEC2-H47A was severely defective but it still retained a high febrile activity. These results strongly suggest that the superantigen and febrile activity of SEC2 can be separated, which is inconsistent with the previous theory that the febrile activity of SE is a direct consequence of its ability to stimulate T-cell proliferation. Further investigation of other SEs would help to prove the hypothesis.

The previously published crystal structure data showed that the primary zinc-binding site (Zn I site) in SEC2 was formed by Asp83, His118 and His122, and analysis of mutants showed that zinc binding required the presence of all three native residues at positions 83, 118 and 122 (Papageorgiou *et al.*, 1995). Because the mutants SEC2-H118A, SEC2-H122A and SEC2-H118A/H122A all retained similar superantigen activity to that of wild SEC2, it is possible that the zinc binding at this site is not necessary for T-cell stimulation. However, the severely reduced emetic and febrile activities of the mutants with the substitutions at positions 118 and 122 suggest that zinc binding could play a crucial role in the emetic and fever activities of SEC2. Because the zinc-binding site of SEC2 is not conserved in SEs, we cannot rule out the possibility that the presence of the Zn I site may play an important role in the pathogenicity induced by SEC2 during the process of evolution.

Recently, much attention has focused on the therapeutic potential of SEs to treat malignant tumours in clinic because the ability of SEs to induce a tremendous expansion of T lymphocytes bearing certain TCR V β regions has given rise to strong T-cell-mediated immune responses and elicited systemic antitumour effects (Hedlund *et al.*, 1993; Ochi *et al.*, 1993; Mondal *et al.*, 2002; Xiu *et al.*, 2007). Interestingly, SEC2 has been used in clinical trials as an effective therapeutic agent for malignant tumour patients in China because of its low toxicity treatment, and some encouraging results have been reported (Chen, 2001). However, the clinical application of superantigen SEC2 is seriously restricted by the adverse effect produced by its emetic and pyrogenic toxicity (Llewelyn & Cohen, 2002). Therefore, it is necessary to investigate the structural basis for toxic activity of SEC2, and provide theoretical support for designing a low-toxicity superantigen. Our finding that the histidine residues at positions 118 and 122 are important for the emetic and fever activities of SEC2 but are not required for the ability to stimulate T-cell proliferation may provide a feasible strategy to construct a low-toxicity antitumour agent.

In summary, this study has identified three histidine residues in SEC2 that play important roles in the emetic, febrile and superantigen activities of the enterotoxin,

providing direct experimental evidence that both emetic and febrile activities may be incompletely correlated with the superantigen activity of SEC2, and extending earlier findings which suggested that only the superantigen and emetic activities are separable in SEs (Harris *et al.*, 1993). This is also the first examination of the role of the metal-binding site in the biological activities of SEC2, and the results suggest that the two zinc-binding sites in SEC2 may play different roles in the biological activities of this toxin.

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