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Mutational Analysis of the Superantigen Staphylococcal Exfoliative Toxin A (ETA)[1]

James V. Rago,* Gregory M. Vath,† Gregory A. Bohach,‡ Douglas H. Ohlendorf,† and Patrick M. Schlievert[2]*

Exfoliative toxin A (ETA) is known to be a causative agent of staphylococcal scalded skin syndrome (SSSS). Although relatively little is known about exactly how the exfoliative toxins (ETs) cause SSSS, much has been discovered recently that may help elucidate the mechanism(s) by which ETA exhibits activities such as lymphocyte mitogenicity and epidermolytic activity. Here, we have shown that highly purified ETA does have T lymphocyte mitogenic activity in that wild-type ETA induced T cell proliferation whereas several single amino acid mutants lacked significant activity. Neither wild-type ETA nor any single amino acid mutants were proteolytic for a casein substrate, yet esterase activity was detected in wild-type ETA and several mutants, but eliminated in other mutants. A mutation in aa 164 (Asp to Ala) showed a 9-fold increase in esterase activity as well. Finally, we correlated esterase activity with epidermolytic activity. All mutants that lost esterase activity also lost epidermolytic activity. Conversely, mutants that retained esterase activity also retained exfoliative activity, implicating serine protease or serine protease-like activity in the causation of SSSS. Moreover, the mutants that displayed markedly reduced T cell superantigenic activity retained their epidermolytic activity (although some of these mutants required higher doses of toxin to cause disease), which suggests an ancillary role for this activity in SSSS causation. *The Journal of Immunology,* **2000, 164: 2207–2213.**

In the early 1970s, a staphylococcal protein then known as "exfoliatin" was isolated from phage group II strains of *Staphylococcus aureus* and was shown to be the causative agent of skin peeling in staphylococcal scalded skin syndrome (SSSS)[3] (1–3). This protein, when injected into neonatal mice, elicited exfoliation of the epidermal layer of skin similar to the disease manifestations seen in human neonates. Since its discovery, many properties of exfoliative toxin A (ETA, as it came to be known) have been elucidated.

Later in that decade, it was shown that ETA possessed both edematous activity (4) and mitogenic activity toward T lymphocytes (5). It was hypothesized that these properties may account for the epidermolytic activity and edema and skin rash seen in SSSS. It is noteworthy that several other superantigens have been described that belong to a different family of toxins, namely, the pyrogenic toxin superantigen (PTSAg) family (which includes toxic shock syndrome toxin-1, the staphylococcal enterotoxins, and the scarlet fever toxins of streptococci) that also have edematous and T cell proliferative activity that result in skin rash and peeling (6).

Studies in the late 1980s and early 1990s involving the sequencing of the ETA gene (*eta*) (7) led many researchers to believe that ETA may be a member of the large family of serine protease enzymes. Several studies demonstrated that ETA had esterase activity (which is an intrinsic property of serine proteases; Refs. 8–10). The solution of the three-dimensional structure of the molecule in 1997 definitely established that ETA had structural elements similar to serine proteases (11, 12). These structural similarities were most notable in the active site region known as the catalytic triad, which consists of three residues (His^{72}, Asp^{120}, and Ser^{195} in ETA) that comprise the functional active site in a serine protease enzyme. Ser^{195} and one mutant of Ser^{195} (S195C, in which Ser^{195} was mutated to Cys) have been studied in detail by several groups already (8, 10, 13). These findings contributed to the idea that ETA causes SSSS by acting directly or indirectly as an epidermal site-specific serine protease.

More recently, ETA has been demonstrated to have T lymphocyte mitogenic activity and a specific Vβ stimulation profile (14), which has further defined the status of ETA as a superantigen.

This work is a study of the superantigenic, epidermolytic, and esterolytic activities of wild-type and mutant forms of ETA to determine the contributions of these properties to SSSS. Several amino acids in ETA were targeted for mutagenesis for a number of reasons, such as their location in the active site structure (His^{72}, Asp^{120}, Ser^{195}), maintenance of active site structure (Pro^{192}, Ser^{211}), presence in uniquely oriented regions such as the D-loop, which is comprised of residues 162 through 169 (Pro^{162}, Phe^{163}, Asp^{164}), and potential role in endowing ETA with substrate specificity (Lys^{213}). Other charged residues on exterior surfaces (R87G) that may be important in binding to various ligands, and conserved mutations (I62V) were made by random mutagenesis.

Materials and Methods

Site-directed mutagenesis

The ETA gene was first inserted into the plasmid vector pCE104 (amp^r, erm^r) resulting in pCE117 (11). Most mutants of plasmid pCE117 (ETA^+, amp^r, erm^r) were constructed by use of a variation of a whole-plasmid PCR

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[3] Abbreviations used in this paper: SSSS, staphylococcal scalded skin syndrome; ETA, exfoliative toxin A; TFA, trifluoroacetic acid; ED, epidermolytic dose.

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Table I. *Amino acid residues affected and primers used to make mutants of ETA*

Type	Amino Acid	Mutant(s)	Mutant Primer(s) Used
Active site mutants	His^{72}	H72A	5′-TCTAACAAATAGAGCTATCGCTAAATTTGC-3′
	Asp^{120}	D120A	5′-TGGTGCAGGTGTTGCTTTAGCATTAATCAG-3′
	Ser^{195}	S195C	5′-AGTTCCGGGAAATTGTGGATCAGGTATATT-3′
Active site structure maintenance mutants	Pro^{192}	P192A	5′-TGGATTTACAGTTGCGGGAAATTCTGGATC-3′
	Ser^{211}	S211A	5′-AGTTGGTATACATGCTAGCAAAGTGTCTCA-3′
D-loop mutants	Pro^{162}	P162A	5′-ATTAATAGGCTATGCATTCGATCATAAAGT-3′
	Phe^{163}	F163G	5′-AATAGGCTATCCAGGCGATCATAAAGTTAA-3′
	Asp^{164}	D164A	5′-AGGCTATCCATTCGCTCATAAAGTTAACCA-3′
		D164G	5′-GGCTATCCATTCGGTCATAAAGTTAAC-3′
Substrate specificity mutants	Lys^{213}	K213X[a]	5′-TATACATTCTAGCRVNGTGTCTCATCTTGA-3′
Other mutants	Glu^{12}	E12A	5′-AAAAAACATGAAGCGAAATGGAATAAG-3′
	Ile^{62}	I62V	Generated by random mutagenesis
	Arg^{87}	R87G	Generated by random mutagenesis

[a] Where "X" refers to any amino acid change, and in nucleotide changes, where R = A or G, V = A or C or G, and N = A or C or G or T. Underlined bases are areas in the primer that have been mutated to elicit the corresponding amino acid mutation. A complementary primer for each primer listed here was also made to perform the mutagenesis method outlined in *Materials and Methods*.

technique based on the procedure described in the Quik-Change site-directed mutagenesis protocol (Stratagene, La Jolla, CA). The mutants made and primers used are listed in Table I. The positions of the residues targeted for mutagenesis are shown on the ribbon diagram of the three-dimensional structure of ETA in Fig. 1. Individual residues with more than one mutation (e.g., K213A, K213E, and K213T) employed the use of mutant primers with degenerate codons at the position of the mutation. Two mutants were constructed via random mutagenesis (i.e., were created by using limiting amounts of a nonmutagenized primer in a standard PCR reaction) reaction (15, 16). Sequencing of mutant DNA was performed by a modification of the dideoxynucleotide chain termination method for dsDNA (Sequenase T7 DNA polymerase version 2.0 kit; United States Biochemicals, Cleveland, OH). Randomly mutagenized ETA was sequenced along the entire gene to insure no aberrant second-site mutations had occurred. Mutants made by the Quik-Change method were sequenced across the region of the desired mutation to insure the change had been made.

Cloning

Synthesized mutant DNA was transformed into DH5α *Escherichia coli* made competent with 50 mM $CaCl_2$ (pH 6.4) for the purposes of sequencing and plasmid propagation. For toxin production, DNA was transformed into *S. aureus* strain RN4220 via protoplast transformation (17). Positive clones were tested for ETA expression in a double immunodiffusion assay (18) by use of a rabbit polyclonal antisera raised against wild-type ETA.

Selection of mutants

Active site residues. The catalytic triad of a serine protease enzyme consists of a conserved structural motif (19) consisting of a histidine residue, an aspartic acid residue, and a serine residue. His^{72}, Asp^{120}, and Ser^{195} of ETA comprise what is sequentially and structurally believed to be the putative active site of ETA (11, 12). In serine protease hydrolysis of peptide (and ester) bonds, the Ser^{195} residue initiates a nucleophilic attack on the substrate, whereas His^{72} accepts a proton from Ser^{195} and Asp^{120} orients His^{72} to accept the proton from Ser^{195}.

Active site structure maintenance residues. Crystallographic analyses have shown that several residues in ETA are important for the maintenance of the conformation of the putative active site (11, 12, 19). The carbonyl oxygen of Pro^{192} occupies the oxyanion hole in serine protease enzymes. Ser^{211} is also believed to stabilize the active site by hydrogen bonding to and ensuring the proper orientation of Asp^{120}. Ser^{211} is also thought to stabilize the charge that builds up on the Asp-His pair during catalysis. Although Asp^{164} is also believed to be important in maintaining the structure of the active site, it will hereafter be described with the other D-loop residues.

D-loop residues. Although most serine protease enzymes have a relatively conserved structure, ETA has several unique structural characteristics and motifs, such as the amphipathic N-terminal α-helix and the orientation of what is known as the D-loop. Pro^{162}, Phe^{163}, and Asp^{164} are located in this D-loop region and are believed to contribute to the unique orientation of the D-loop. Asp^{164} is involved in the stabilization of the uniquely oriented bond between Pro^{192} and Gly^{193} by hydrogen bonding to the main chain nitrogen of Gly^{193}. Asp^{164} is also located in and probably involved in the unique orientation of the D-loop of ETA. Two mutants of D^{164} were made, one with an alanine substitution (to analyze the change while maintaining main-chain structure and rigidity), the other with a glycine substitution (to allow main-chain flexibility and thusly, more flexibility in the D-loop).

Substrate specificity mutants. Lys^{213} is believed to endow ETA with specificity for a cleavage site that follows glutamic acid (and possibly, aspartic acid) in its putative substrate. The positively charged lysine residue is thought to stabilize a negatively charged side chain in a substrate (i.e., aspartic acid and glutamic acid) (11, 12). It also appears to be integral in the biological activity of ETA. For this reason, several different mutations of Lys^{213} were used, namely, K213A, K213E, and K213T.

N-terminal α-helix residues. Glu^{12} is located in the highly charged N-terminal α-helix. An N-terminal α-helix deletion mutant was also made, where residues 5–22 of ETA, which comprise the actual α-helical portion of the N terminus, were deleted. The first four amino acids remained in the mutant to ensure proper synthesis and secretion.

Other mutants. Two mutants were made via random mutagenesis and were also used in our studies. I62V was used to study the effects of a small-scale amino acid substitution in a putative "unimportant," interior area of the protein. R87G was also made and is a mutation in a charged residue on the exterior surface of the first of the two β-barrels near the active site.

Toxin production and purification

Wild-type and mutant toxins were produced in RN4220 *S. aureus* grown to stationary phase in 1.2 liters of a pyrogen-free, dialyzable beef-heart medium with 5 μg/ml erythromycin at 37°C and the proteins precipitated in four volumes of ethanol for 48 h (20). Precipitates were resuspended in pyrogen-free water and cleared by centrifugation. Supernatants were dialyzed against deionized water for 2 days (changing water each day), and ETA and mutants forms of ETA were purified via flatbed isoelectric focusing (which involves separating proteins based on their isoelectric points) in pH gradients of 3.5–10, and then 6–8. Toxin-positive fractions were collected and dialyzed against deionized water for 4 days (changing water each day). Purified toxin was quantified via a double immunodiffusion dilution assay and lyophilized until needed (18). On average, 2–10 mg toxin or mutants were obtained per liter of medium. Multiple preparations of toxin were used to insure reproducibility among toxin preparations. Toxin purity was assessed by reverse-phase HPLC (14) and SDS PAGE (21). For reverse-phase HPLC, toxins were loaded onto a Hewlett Packard HP1090 apparatus (Waldbronn, Germany) equipped with a 15-cm C18 column (VYDAC, Hesperia, CA) in 0.1% trifluoroacetic acid (TFA) and eluted in a gradient of 0–60% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. ETA and mutants were pure as tested by these techniques.

Lymphocyte mitogenicity assay

Splenocytes from American Dutch-belted rabbits were isolated and resuspended in RPMI 1640 medium (supplemented with penicillin-streptomycin and 2% FCS) and dispensed into 96-well microtiter plates at a concentration of 2×10^5 cells/well in 200-μl volumes (22). Varying dilutions of ETA (10 μg, 1.0 μg, 0.1 μg, 10 ng, 1.0 ng, and 0.1 ng) were added to each well (in quadruplicate) and incubated for up to 8 days at 37°C in 7% CO_2. Cells were then pulsed for 18 h with [^{3}H]thymidine (1.0 μCi). DNA was

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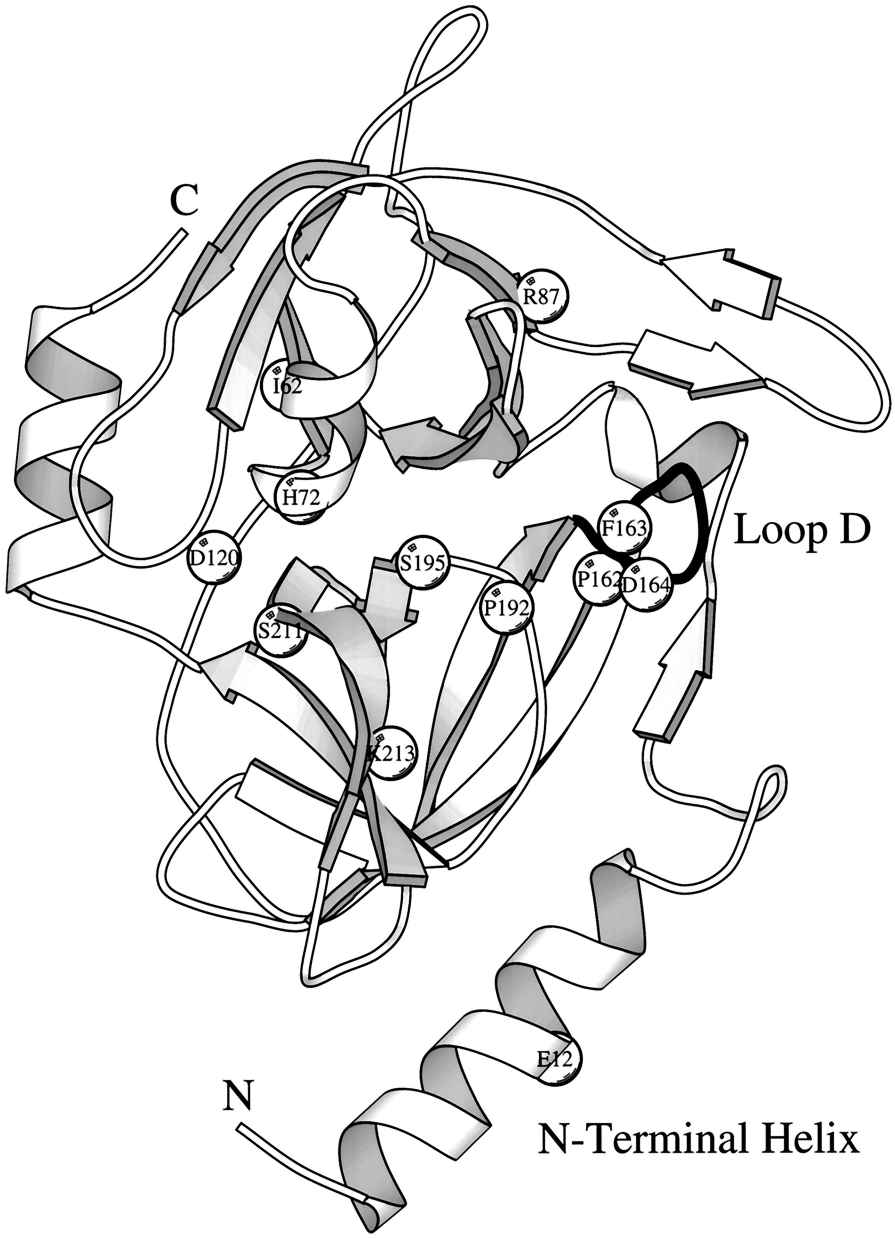

FIGURE 1. Ribbon diagram of ETA showing the locations of residues selected for mutagenesis.

harvested onto fiberglass filters, and cpm due to [^{3}H]thymidine incorporation into the DNA of proliferating cells were measured in a standard scintillation counter. Background cpm were measured by taking counts on cells not treated with toxin. Potential contaminating factors that may have been contributed by the plasmid vector for ETA (pCE104) or cell line used for toxin purification (RN4220) were taken into account by growing RN4220 transformed with pCE104 and purifying culture fluids in the same manner as described for wild-type ETA. These purified fractions (which came from the same fractions from the isoelectric focusing (IEF) plates as did wild-type ETA) were used as a negative control in mitogenicity assays. The highest cpm for ETA were seen on day 6 at a concentration of 1.0 μg/well. ETA at 0.1 μg/well also caused significant but lower mitogenic activity that peaked on day 6. All subsequent assays of mitogenicity of wild-type and mutant ETAs were therefore harvested after 6 days, and dose response curves to several different concentrations of these same proteins (10, 1.0, and 0.1 μg, and 10, 1.0, and 0.1ng) were performed. Data shown for mitogenicity assays are representative of several different experiments, graphs are representative of at least 2 different wild-type or mutant toxin preparations, and each assay was done with splenocytes from different rabbits.

Epidermolytic assay

A standard Nikolsky test for assaying the epidermolytic capacity of each toxin was performed (1). Varying concentrations of each toxin were resuspended in PBS (PBS, 0.15 M NaCl, 5.0 mM $NaPO_4$, pH 7.1) in 50-μl amounts and injected subcutaneously into 1- to 3-day-old BALB/c mice-(Harlan Sprague Dawley, Indianapolis, IN). If given an epidermolytically active toxin, exfoliation of epidermal layers of skin occurs after 1–3 h when the skin is gently stroked. Each toxin was first assayed at 10 μg/mouse, and two to four mice were used per concentration of toxin. If active, lower doses of toxin were used until the minimal dose at which epidermolysis occurred was reached. Likewise, if inactive, the concentration of toxin was raised.

Protease assay

An azocasein substrate (Sigma, St. Louis, MO) was used in the following colorimetric assay. A proteolytically active protein will liberate azo-dye-labeled amino acids from the substrate, resulting in a color change that can be detected spectrophotometrically. One hundred microliters of 50 μg/ml toxin (in sterile, pyrogen-free water) was added to 350 μl of a protease assay buffer (0.1 M $NaPO_4$, 0.01 mM EDTA, 0.01 mM DTT, pH 7.6). Azocasein (50 μl of a 2% w/v solution) was then added, and the mixture was incubated at 37°C for varying time intervals. The remaining intact protein was precipitated with 200 μl of 10% TCA, incubated overnight at 4°C, and removed via centrifugation. The remaining supernatant was assayed for liberated azo-labeled amino acids at 450 nm in a standard spectrophotometer.

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Esterase assay

N-t-Boc-L-glutamic acid α-phenyl ester (Sigma) was diluted to 500 mM in 1–4 dioxane and used as a substrate (13). Five microliters of 500 mM substrate was added to 3.0 μg toxin suspended in 0.5 ml of 50 mM HEPES buffer (pH 7.4). Catalytic reactions were performed in 20 mM Tris/phosphate buffer (pH 7.8). Readings were taken at 60 s at 270 nm with a molar absorption coefficient of 1.5 $mM^{-1}cm^{-1}$. Data were fitted to a standard Henderson-Hasselbach equation using a least square-fitting program (Kaleidagraph, Version 3.0) to generate values for K_m, V_{max} and K_{cat}.

Results

Mitogenicity profiles, epidermolytic, and esterase activity

Active site mutants. Mutants H72A, D120A, and S195C retained significant mitogenic activity (Fig. 2), and the peak activity (~10^5 cpm) and concentration at which lymphocyte mitogenicity was lost (0.1 ng/well) were similar and comparable to wild-type ETA. In contrast, esterase (Table II) and epidermolytic (Table III) activity were completely abolished. No epidermolysis was seen at concentrations as high as 100 μg per mouse (the highest dose tested), which is about 200 times the minimum dose at which wild-type ETA caused epidermolysis (Table III). These mutants were completely inactive as casein proteases (data not shown), consistent also with the lack of casein protease activity seen for wild-type ETA. These findings support the hypothesis that esterase, and thus likely a specific serine protease activity (with an as yet unidentified substrate), is required for causation of SSSS. Furthermore, the data suggest that lymphocyte mitogenic activity is a distinct and separable property of ETA from esterase and epidermolytic activities.

Active site structure maintenance mutants. Mutant P192A displayed essentially wild-type-like lymphocyte mitogenic activity, but mutant S211A was significantly less mitogenic than wild-type ETA (Fig. 3). S211A was mitogenic only at 10 and 1.0 μg/well, and counts at those concentrations were only about one half of those of wild-type ETA at those concentrations. The S211A mutant possessed wild-type esterase activity (in fact, S211A had slightly higher esterase activity; Table II), but still required a higher dose of toxin (5–10 μg per mouse) to cause epidermolysis (Table III), whereas P192A was esterolytic and epidermolytic at wild-type concentrations. These data suggest a role for Ser^{211} in binding a peptide substrate in vivo that is neither present nor necessary for the protein to act on a specific ester substrate, since the esterase activity remained unaffected despite a mutation at this residue, but the dose required to cause disease was much higher. S211A may also reorient the active site in such a way as to affect pathogenic potential but not esterase activity.

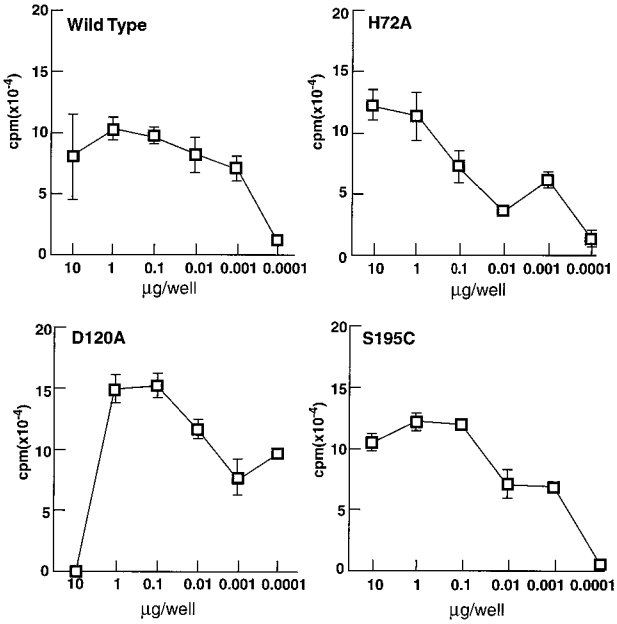

FIGURE 2. Lymphocyte mitogenic activity of active site mutants (H72A, D120A, and S195C). Data are representative of several experiments using different batches of toxin and different rabbits. The mitogenicity profile of each toxin is shown with the profile of wild-type toxin seen during the same assay as the mutant profile. Background cpm = 5567 ± 2056 from wells containing cells only. Wells contained 2×10^5 splenocytes in 200-μl volumes. Wells were labeled with 1.0 μCi of [^{3}H]thymidine 18 h before harvesting DNA.

D-loop mutants. Three mutants made at the D-loop residues (P162A, F163G, and D164A) retained significant (and somewhat

Table II. *Esterolytic properties of wild-type ETA and mutants*

Type	Protein[a]	K_m (mM)	V_{max} (mM min^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}mM^{-1}$)
	ETA	7.10 ± 0.89	0.76 ± 0.05	32.8 ± 2.10	4.64 ± 0.65
	V8 protease	0.10 ± 0.01	0.71 ± 0.03	376 ± 14.9	3600 ± 332
Active site mutants	H72A	Inactive	Inactive	Inactive	Inactive
	D120A	Inactive	Inactive	Inactive	Inactive
	S195C	Inactive	Inactive	Inactive	Inactive
Active site structure maintenance mutants	P192A	6.10 ± 1.4	0.72 ± 0.07	27.0 ± 2.60	4.50 ± 1.10
	S211A	5.20 ± 1.30	0.81 ± 0.10	35.1 ± 4.10	6.80 ± 1.90
D-loop mutants	P162A	ND[b]			
	F163G	7.20 ± 1.06	0.67 ± 0.05	28.7 ± 2.20	4.00 ± 0.67
	D164A	0.82 ± 0.05	0.80 ± 0.02	34.6 ± 1.00	42.0 ± 2.80
	D164G	2.46 ± 0.17	0.32 ± 0.01	7.28 ± 0.23	2.96 ± 0.22
Substrate specificity mutants	K213A	Inactive	Inactive	Inactive	Inactive
	K213E	Inactive	Inactive	Inactive	Inactive
	K213T	Inactive	Inactive	Inactive	Inactive
Other mutants	E12A	ND[b]			
	I62V	ND[b]			
	R87G	6.45 ± 0.26	0.69 ± 0.02	29.9 ± 0.82	4.64 ± 0.23

[a] All proteins were tested for esterolytic activity with the use of *N*-t-Boc-L-Glutamic acid α-phenyl ester as a substrate. Esterase activity was measured spectrophotometrically at 270 nm. V8 protease, a known serine protease, served as a positive control.
[b] ND, not yet done.

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Table III. *Epidermolytic dose (ED) of toxin required for activity*

	Protein Tested	Minimum ED (μg/mouse)[a]
	Wild-type	0.5 μg
Active site residues	H72A	Inactive[b]
	D120A	Inactive[b]
	S195C	Inactive[b]
Active site maintenance residues	P192A	0.5 μg
	S211A	5–10 μg
D-loop residues	P162A	0.5 μg
	F163G	0.5 μg
	D164A	0.5 μg
	D164G	0.5 μg
Substrate specificity mutants	K213A	Inactive[b]
	K213E	Inactive[b]
	K213T	Inactive[b]
Randomly mutagenized residues	I62V	0.5 μg
	R87G	1.0 μg

[a] Wild-type ETA or mutants were suspended in PBS and injected intradermally (in 50-μl volumes) into the necks of 2- to 3-day-old BALB/c mice. At least 2 mice were used per protein per dose tested. Animals were monitored for positive Nikolsky signs (i.e., separation of epidermal layers of skin) for 3 h after injection.

[b] Inactive mutants were found to be nonepidermolytic at concentrations as high as 100 μg/mouse (the highest dose tested).

enhanced) lymphocyte mitogenic activity compared with wild-type ETA, whereas the D164G mutant was completely inactive (Fig. 4). However, all of these mutants were active as esterases (Table II) and as epidermolytic agents (Table III) at concentrations comparable to wild type with one exception; D164A displayed a significant increase in esterase activity (~ 9-fold higher than wild-type ETA), but had only wild-type epidermolytic activity. D164A bound substrate better than wild type (as indicated by K_m), and

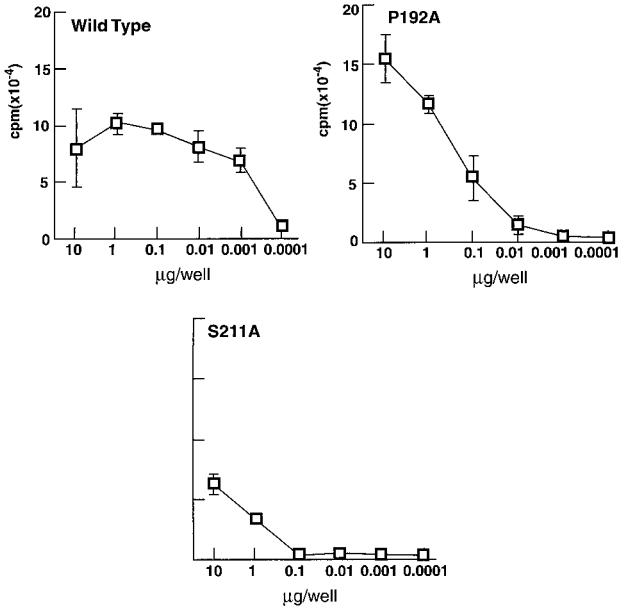

FIGURE 3. Lymphocyte mitogenic activity of active site structure maintenance mutants (P192A, and S211A). Data are representative of several experiments using different batches of toxin and different rabbits. The mitogenicity profile of each toxin is shown with the profile of wild-type toxin seen during the same assay as the mutant profile. Background cpm = 5567 ± 2056 from wells containing cells only. Wells contained 2×10^5 splenocytes in 200-μl volumes. Wells were labeled with 1.0 μCi of [^{3}H]thymidine 18 h before harvesting DNA.

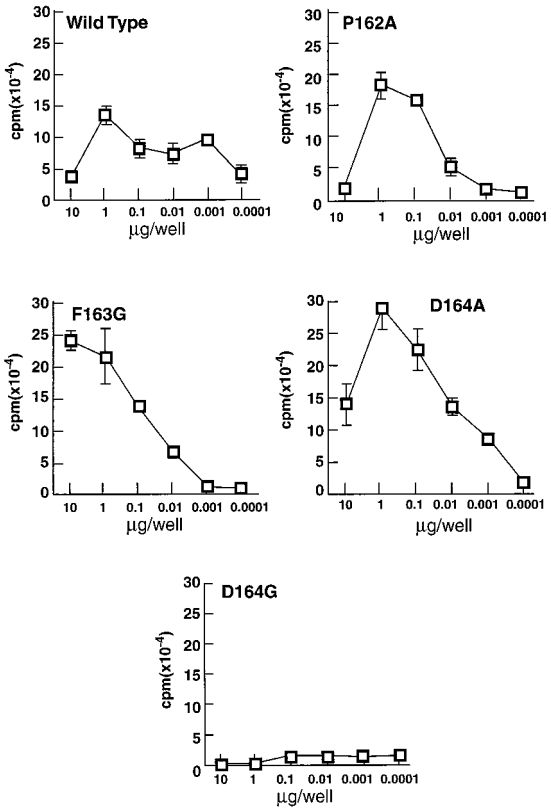

FIGURE 4. Mitogenic activity of D-loop mutants (P162A, F163G, D164A, and D164G). Data are representative of several experiments using different batches of toxin and different rabbits. The mitogenicity profile of each toxin is shown with the profile of wild-type toxin seen during the same assay as the mutant profile. Background cpm = 14998 ± 1331 from wells containing cells only. Wells contained 2×10^5 splenocytes in 200-μl volumes. Wells were labeled with 1.0 μCi of [^{3}H]thymidine 18 h before harvesting DNA.

D164G was intermediate between D164A and wild type, likely because the active site of D164G was more open than wild type but less so than for D164A. This must have resulted from differences in the flexibility of the D-loops. In addition, D164G had a lower K_{cat} (turnover rate) than both D164A and wild type, again likely because of differences in the flexibility of the D-loops. The above data also demonstrate that ETA has intrinsic lymphocyte mitogenic activity in that the D164G mutant lost activity compared with wild type, and that mitogenicity and epidermolytic activity are separable.

Substrate specificity mutants. Mutants at Lys^{213} (K213A, K213E, and K213T) retained partial lymphocyte mitogenic activity (Fig. 5). These mutants were not esterolytic (Table II), also consistent with the lack of activity seen in active site mutants. These data agree with the hypothesis that a mutation at Lys^{213} would be rendered esterolytically inactive, since a glutamic acid ester was used as a substrate. It was also interesting to note that these mutants were also unable to cause epidermolysis (Table III), which may lend support to the idea of a very specific peptide substrate in vivo.

N-terminal α-helix mutants. The mutation at Glu^{12} (E12A) possessed wild-type ETA activity in lymphocyte mitogenicity (Fig. 6), esterase activity (Table II), and epidermolytic activity (Table III). Interestingly, an N-terminal deletion mutant (Δ5–22) appeared to be degraded as it was made in *E. coli*, and was either not expressed or not transformed in *S. aureus* after repeated attempts. Other studies have shown that N-terminal α-helix deletion mutants of ETA

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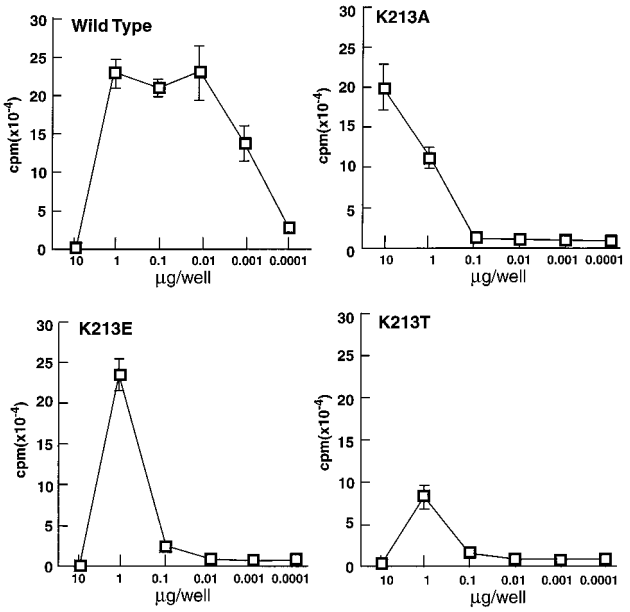

FIGURE 5. Mitogenic activity of substrate specificity mutants (K213A, K213E, and K213T). Data are representative of several experiments using different batches of toxin and different rabbits. The mitogenicity profile of each toxin is shown with the profile of wild-type toxin seen during the same assay as the mutant profile. Background cpm = 6501 ± 672 from wells containing cells only. Wells contained 2×10^5 splenocytes in 200-μl volumes. Wells were labeled with 1.0 μCi of [^{3}H]thymidine 18 h before harvesting DNA.

are stable and biologically active (12). About 1.5 mg of toxin was demonstrated immediately after ethanol precipitation of DH5α *E. coli* containing the deletion of residues 5–22 in 2.4 liters of beef heart medium, and about 400 μg remained after isoelectric focusing (IEF). After dialysis to remove ampholytes, none of the mutant

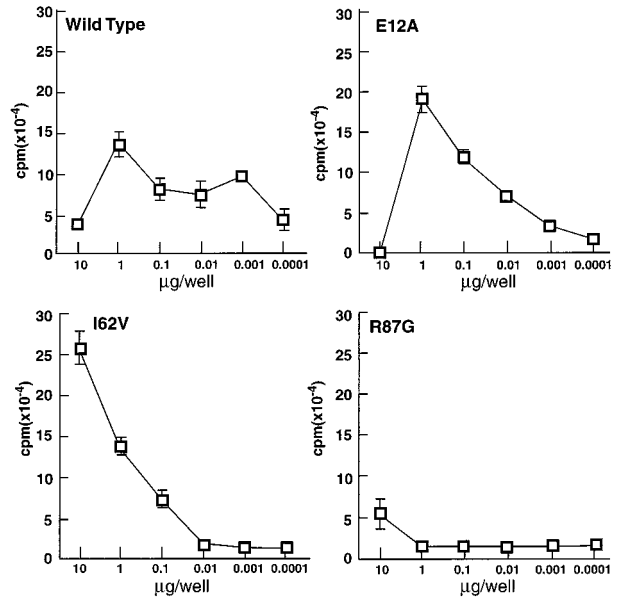

FIGURE 6. Mitogenic activity of mutants E12A, I62V, and R87G. Data are representative of several experiments using different batches of toxin and different rabbits. The mitogenicity profile of each toxin is shown with the profile of wild-type toxin seen during the same assay as the mutant profile. Background cpm = 14998 ± 1331 from wells containing cells only. Wells contained 2×10^5 splenocytes in 200-μl volumes. Wells were labeled with 1.0 μCi of [^{3}H]thymidine 18 h before harvesting DNA.

could be detected. It is thought that the N-terminal α-helix in some way might protect ETA such that it is not subject to degradation, either from outside sources (e.g., *E. coli* DH5α proteases) or via autodegradation.

Other mutants. Two other mutants made via random mutagenesis were evaluated for activity. It was shown that the conservative mutation (I62V) retained lymphocyte mitogenic activity (Fig. 6), esterase activity (Table II), and epidermolytic activity (Table III). This is consistent with what one would expect to find with a fairly conservative amino acid change in a biologically "unimportant," internal region of the molecule. However, whereas the R87G mutant was esterolytically comparable to wild-type ETA (Table III), the epidermolytic activity (Table II) was slightly lower than wild-type ETA, and the lymphocyte mitogenic activity was significantly lower than wild-type ETA (Fig. 6). Like S211A, R87G had a higher epidermolytic dose (ED) than did wild-type ETA.

Discussion

S. aureus makes a large number of secreted virulence factors that allow it to cause serious diseases. Among these are the enterotoxins (23–25), toxic shock syndrome toxin-1 (26–28), and the exfoliative toxins (3). All of these toxins have major effects on the host immune system, primarily acting as superantigens. As such, all of these molecules stimulate T cell proliferation dependent on the composition of the variable region of the β-chain of the TCR, unlike other antigenic peptides (29). For example, ETA has been shown to stimulate human T cells bearing V_β 3, 12, 13.2, 14, 15, 17, and 20 (14). The consequence of the subsequent high level of T cell proliferation is massive cytokine release resulting in non-Ag-specific host damage.

SSSS is a disease primarily seen in neonates and is associated with production of one or both of the two exfoliative toxins (ETA and ETB). SSSS is characterized by generalized exfoliation of outer epidermal layers at the level of the desmosomes. As well, neonates often display edema and erythematous skin rash as might be expected due to cytokine release.

There have been two major theories to explain the exfoliation seen in SSSS. First, it was hypothesized that the ETs cause epidermolysis as a consequence of direct action at the desmosomes. This is supported by our recent observations that ETs have structures similar to serine protease enzymes (11, 30). Furthermore, it has been shown that ETs have esterase activity (Ref. 13) and this study). However, protease activity has yet to be demonstrated. The second hypothesis to explain the epidermolysis seen in SSSS is that the ETs stimulate cytokine release with subsequent edema, which physically forces layers of skin apart at the desmosomes (4). In support of this hypothesis are data showing that ETs are superantigenic (14, 31) and that edema and reddening of skin occur in SSSS.

The studies presented here were conducted to evaluate the contributions of both direct activity and superantigenic activity on the causation of skin peeling in SSSS. Lymphocyte mitogenic activity remained in most mutants, irrespective of esterase or epidermolytic activity, which indicates that mitogenicity and esterase/epidermolytic activity are distinct and separable properties of ETA. However, some mutants did appear to have markedly reduced mitogenic activity (R87G, D164G, S211A, and the K213 mutants, especially K213T). Moreover, the two mutants with higher than normal (yet still epidermolytically active) epidermolytic doses, namely, R87G (1.0 μg/mouse, 2 times a normal ED) and S211A (5–10 μg/mouse, 10–20 times a normal ED) were significantly less mitogenic than wild type and most mutant forms of ETA. Collectively, these data suggest that superantigenicity is not required for the skin peeling seen in SSSS. However, superantigenicity may lead to edema and skin reddening; neither of these latter two effects were seen in the 1- to 3-h time required to cause skin

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peeling in mice, but are seen in SSSS. Interestingly, residues whose mutants were deficient in mitogenicity are all clustered about the active site, but are not considered to be part of the serine protease catalytic triad itself.

Most significantly, esterase and epidermolytic activity were found to correlate with one another. This lends support to the idea that some sort of serine protease activity (or at least, serine esterase activity) either directly causes or contributes to the causation of SSSS. All mutants that retained esterase activity also retained epidermolytic activity. Conversely, mutants that lost esterase activity also lost epidermolytic activity (i.e., still epidermolytically inactive at 100 μg/mouse, 500 times a normal ED). Various other serine proteases were tested for epidermolysis, such as trypsin, subtilisin, and staphylococcal V8 protease. None of these proteins were capable of causing epidermolysis, which indicates that ETA possesses special properties that endow it with its signature biological activity.

Neither wild-type ETA, nor any mutant form of ETA showed proteolytic activity for azocasein or any other peptide substrate studied to date. An ester substrate, such as used in this study, may be more accessible to the putative active site by virtue of its size and increased flexibility (ester bonds are typically more flexible than rigid, planar, peptide bonds).

The unusual properties displayed by the Asp^{164} mutants indicate that this may be a particularly "sensitive" residue in terms of biological activity. The fact that mitogenic activity was abolished in D164G may be due to replacing Asp^{164} with a residue that allows much more main-chain flexibility in an important area of the protein in terms of binding immune system cells. A D164A mutation may reorient the D-loop in such a way as to make the active site more accessible, at least to an ester substrate, without losing main chain rigidity.

In conclusion, while much is still unknown about how ETA causes SSSS, many of the structural, immunological, and biological properties of the toxin have been studied in detail, and several conclusions can be drawn from available information. Data gathered to date have shown that ETA does act as a superantigen in the absence of any potential contaminating factors. Structural analyses and biological activity (i.e., esterase activity) studies have shown that ETA is likely acting as a serine protease, possibly cleaving a protein or proteins at the stratum granulosum. The correlation between esterase activity and epidermolysis indicates that an epidermal site-specific protease activity is likely the mechanism by which ETA causes exfoliation. Superantigenicity may also play a supplementary role in the causation of SSSS such as production of rash and edema, but is not required for the progression of epidermolysis.

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