Enzymatic and Molecular Characteristics of the Efficiency and Specificity of Exfoliative Toxin Cleavage of Desmoglein 1*

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Exfoliative toxins (ETs) from Staphylococcus aureus blister the superficial epidermis by hydrolyzing a single peptide bond, Gln^106^-Gly^108, located between extracellular domains 3 and 4 of desmoglein 1 (Dsg1). Enzyme activity is dependent on the calcium-stabilized structure of Dsg1. Here we further define the characteristics of this cleavage. Kinetic studies monitoring the cleavage of Dsg1 by ETA, ETB, and ETD demonstrated $k_{cat}/K_m$ values of $2-6 \times 10^4$ M$^{-1}$ s$^{-1}$, suggesting very efficient proteolysis. Proteolysis by ETA was not efficiently inhibited by broad spectrum serine protease inhibitors, suggesting that the enzyme cleavage site may be inaccessible before specific binding to its substrate. Using truncated mutants of human Dsg1 and chimeric molecules between human Dsg1 and either human Dsg3 or canine Dsg1, we show that for cleavage, human-specific amino acids from Dsg1 are necessary in extracellular domain 3 upstream of the scissile bond. If these residues are canine rather than human, ETA binds, but does not cleave, canine Dsg1. These data suggest that the exquisite specificity and efficiency of ETA may depend on the enzyme’s binding upstream of the cleavage site with a very specific fit, like a key in a lock.

Staphylococcus aureus is a frequent bacterial pathogen in human disease. Skin infections are particularly prevalent, with impetigo caused by S. aureus one of the most common infectious diseases in children (1). Approximately 30% of impetigo patients develop bullous impetigo, which is caused by S. aureus strains that produce exfoliative toxins (ETs)†‡ (2, 3). In bullous impetigo, blisters of the superficial epidermis occur at sites of infection. More extensive disease can occur in staphylococcal scalded skin syndrome (SSSS), in which pathogenic autoantibodies bind the desmosomal cell adhesion molecule, desmoglein 1 (Dsg1) (9). This observation led to the hypothesis that the molecular target of ETs is Dsg1. This hypothesis was validated by showing that three different ET isotypes (ETA, ETB, and ETD) specifically cleave mouse and human Dsg1 (hDsg1) but not closely related molecules such as Dsg3 or E-cadherin (9–11). Mutation of the predicted active serine in these ETs caused loss of their proteolytic activity, confirming that they are serine proteases (12).

Dsg1 is in the desmosomal cadherin subfamily of the cadherin supergene family. Desmosomal cadherins, similar to classic cadherins, contain four amino-terminal homologous domains (called extracellular (EC) domains 1–4), of about 100 amino acid each, that are highly conserved among many cadherins (13). A fifth juxtamembrane domain (EC5) is not as well conserved within or among the cadherins. The structure of Dsg1, like other cadherins, is maintained by calcium binding that occurs between EC domains (14–17). This calcium-stabilized structure is thought to be critical in maintaining the adhesive function of classical and, presumably, desmosomal cadherins (18–20).

Recent studies show not only that ETs show a substrate specificity for Dsg1 but also that they hydrolyze only one peptide bond in human and mouse Dsg1 at exactly the same site, after glutamic acid 381 between EC3 and EC4 (12). Furthermore, cleavage of Dsg1 by ETs is dependent on the former’s calcium-stabilized conformation (i.e. denatured or partially denatured Dsg1 cannot be hydrolyzed by ETs) (21). Thus, unlike more typical serine proteases whose specificities are defined by simple amino acid sequences and which can hydrolyze many proteins and model polypeptides, ETs show exquisite specificity for one peptide bond in one correctly folded substrate, Dsg1.

In this study, we show that in addition to being exquisitely specific for a single peptide bond in Dsg1, ETs are efficient in though SSSS is much less common than bullous impetigo, outbreaks that occur in neonatal nurseries have important consequences for health care delivery (2, 4).

In the 1970s Melish et al. (5–7) showed that ETs produced by S. aureus injected into neonatal mice caused blister formation in skin similar to that seen in patients with bullous impetigo and SSSS. It remained unclear exactly how ETs cause this blister until it was discovered that they have structural and sequence homologies to serine proteases, at which time it was suggested that they act as proteolytic enzymes that were predicted to cleave after either a glutamic or aspartic acid (8). However, they could not be shown to cleave model polypeptide substrates. Their normal pathophysiologic substrate, if any, remained a mystery until it was realized that the blisters in bullous impetigo and SSSS show clinical and histological similarity to those of pemphigus foliaceus, an autoimmune disease in which pathogenic autoantibodies bind the desmosomal cell adhesion molecule, desmoglein 1 (Dsg1) (9). The discovery of the molecular target of ETs is Dsg1. This hypothesis was validated by showing that three different ET isotypes (ETA, ETB, and ETD) specifically cleave mouse and human Dsg1 (hDsg1) but not closely related molecules such as Dsg3 or E-cadherin (9–11). Mutation of the predicted active serine in these ETs caused loss of their proteolytic activity, confirming that they are serine proteases (12).

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hydrolyzing that peptide bond. We show that the catalytic site of ETA is protected from typical serine proteases inhibitors and that domains in Dsg1 amino-terminal, but not carboxyl-terminal, to the cleavage site are necessary for its cleavage by ETA.

Finally, we show that although ETA binds to canine Dsg1 (cDsg1) it does not cleave it, but it will do so if 5 amino acids that are upstream of the cleavage site are mutated to hDsg1 residues. The results of these studies suggest that the exquisite...
specificity and efficiency of ETA in cleavage of Dsg1 depend on the enzyme's binding upstream of the cleavage site with the proper fit, like a key in a lock, to properly align, and perhaps activate, its catalytic site.

MATERIALS AND METHODS

Recombinant ETs—DNA encoding the ETD mature sequence was amplified by PCR using pQE-ETD-His (provided by Dr. Motoyuki Tateyama, University of Miyazaki) as a template (11). PCR was also used to add nucleotides encoding the ETA signal peptide to the 5'-end of the ETD sequence and to add nucleotides encoding the V5 and His tags to the 3'-end of the ETD sequence. ETA was subcloned into the shuttle vector, pCE104 (12). pCE104 with ETD-His was electroporated into EN4220 S. aureus electocompetent cells (12). We also used recombinant wild-type ETA, ETB, and ETAS195A (enzymatically active serine, at position 195 as defined by classical chymotrypsin serine protease numbering, mutated to alanine) with V5 and His tags as previously described (12). Recombinant ETs were purified on Ni²⁺-nitrilotriacetic acid columns (Qiagen, Valencia, CA) according to the manufacturer's protocol and then dialyzed against phosphate-buffered saline. Protein concentrations of ETs were estimated with a Protein Assay Kit (Bio-Rad) and on Simply Blue (a modified Coomassie G-250 stain) on SDS-PAGE gels compared with standards and each other.

Production and ET Cleavage of Human Desmoglein 1—The entire EC domain of human recombinant Dsg1 with a His and E tag (hDsg1E) on the carboxyl terminus was produced as a secreted protein by baculovirus as previously described (22). For kinetic analysis, hDsg1E was purified from insect cell culture supernatant on an anti-E tag column (Amersham Biosciences) and each other.

For protease inhibitor studies, 1 mM DFP, 100 nM DCI, and 50 nM ETA were detected with chemiluminescence (ECL or ECL Plus; Amersham Biosciences) or anti-ETA sheep polyclonal antibody conjugated with horseradish peroxidase (Toxin Technology, Sarasota, FL), diluted in blocking buffer of 5% fat-free milk powder in phosphate-buffered saline. The sheets were incubated for 1 h, washed with 0.1% Tween 20 in phosphate-buffered saline, and then used directly for immunoprecipitation. E tagged proteins were precipitated with Anti-E tag- Sepharose (Amersham Biosciences) at 4 °C for 1 h. Immunoprecipitates were washed 10 times with 1% Triton X-100 TBS plus Ca²⁺ and then eluted with Laemmli sample buffer at 100 °C.

Western Blotting—Proteins in Laemmli sample buffer were separated by 4–20% SDS-PAGE (Bio-Rad) and then transferred to nitrocel-lulose sheets (Trans blot; Bio-Rad). The sheets were incubated for 1 h in blocking buffer of 5% fat-free milk powder in phosphate-buffered saline. The E tag antibody conjugated with horseradish peroxidase (Amersham Biosciences) or anti-ETA sheep polyclonal antibody conjugated with horseradish peroxidase (Toxin Technology, Sarasota, FL), diluted in blocking buffer, was applied for 1 h at room temperature. After four washes with 0.1% Tween 20 in phosphate-buffered saline, the signals were detected with chemiluminescence (ECL or ECL Plus; Amersham Biosciences).

RESULTS

Kinetics of ET Cleavage of Dsg1—To investigate the efficiency of hydrolysis of Dsg1 by ETs, we measured hDsg1 cleavage by ETs as a function of time. Purified recombinant ETA, ETB, and ETD used in these experiments showed a single band by Simply Blue staining of SDS-PAGE (Fig. 1A).

1 μM hDsg1E was incubated with 12.5, 25, and 50 nM ETA or ETB and 25 and 50 nM of ETB and ETD. Both hDsg1E disappearance and product appearance were quantified over time from bands resolved by SDS-PAGE.

SDS-PAGE of a representative cleavage time course with 25 nM ETA is shown in Fig. 1B. Reactions were stopped by the addition of SDS denaturing buffer and subsequent heating to 100 °C, resolved on a 4–20% gradient polyacrylamide gel,
and stained with Simply Blue. The substrate (upper band, arrow) is cleaved with time to the products (two lower bands, arrowhead and open arrow).

Stained bands were quantified by densitometry, and the data were plotted as a function of time for analysis (Fig. 1C). Substrate consumption and product formation were fit to exponen-
tial curves defined by Equations 1 and 2 to obtain $k_{obs}$, the observed rate constant for substrate consumption or product formation as follows,

$$[S] = [S]_0 e^{-k_{cat} t}$$  \hspace{1cm} (Eq. 1)

$$[P] = [S]_0 [S]_0 e^{-k_{cat} t}$$  \hspace{1cm} (Eq. 2)

where [S] represents the substrate concentration (as determined by densitometry) and [P] represents concentration of the products.

Observed rate constants obtained from disappearance and formation data were in good agreement, as expected. For a given ET concentration, curves defined by first order kinetics (i.e. Equations 1 and 2) fit the data points well. This is best shown by the linear fit in Fig. 1D, in which the natural log of [S] is plotted against time. Measurements were made at three (for ETA) or two (for ETB and ETD) ET concentrations. Values of $k_{obs}$ were directly proportional to ET concentrations, also consistent with pseudo-first-order reactions. The values for $k_{cat}/K_m$, which is a measure of enzyme efficiency, were obtained from the slopes of plots of $k_{obs}$ versus ET concentrations, according to Equation 3,

$$k_{cat} = k_{obs} [E]$$  \hspace{1cm} (Eq. 3)

Values of 62,000, 26,000, and 19,000 M$^{-1}$ s$^{-1}$ were obtained for ETA, ETB, and ETD, respectively. These values indicate efficient cleavage of a large protein substrate.

The Broad Spectrum Serine Protease Inhibitors DFP, DCI, and $\alpha_2$-Macroglobulin Do Not Effectively Inhibit the Enzymatic Activity of ETA—We tested the ability of small (DFP and DCI) and large ($\alpha_2$-macroglobulin) broad spectrum serine protease inhibitors to inhibit the enzymatic activity of ETA. There was minimal, if any, inhibition at the concentration tested, whereas both protease inhibitors did efficiently inhibit V8 protease at concentrations equivalent to those of ETA (Fig. 2), although an ~10-fold larger excess of DCI did show inhibition (data not shown). These data suggest that the catalytic site of ETA may be difficult to access in the proper alignment by some serine protease inhibitors or is relatively inactive in the absence of its proper substrate.

hDsg1 EC Domains That Are Upstream, but Not Downstream, of the Cleavage Site Are Critical for Its Hydrolysis by ETA—Previously, we have shown that the ability of ETs to cleave Dsg1 is not simply dependent on the amino acid sequence of the cleavage site but is dependent on the calcium-stabilized conformation of Dsg1 (21). These results suggest that domains distant from the site of hydrolysis might influence the enzymatic efficiency of ETA. Because desmogleins have five well defined EC domains whose relationship to each other is stabilized by calcium, we first determined whether all of these domains were necessary for cleavage by testing truncated molecules of hDsg1E for their susceptibility to hydrolysis by ETA (Fig. 3, A and B). These data indicated that EC5 is not necessary for cleavage. Loss of EC1 decreases the efficiency of cleavage, yet the truncated molecule is still cleaved. Loss of EC1–EC2 prevents cleavage (Fig. 3B). After incubation with ETA, the EC1–EC3 domain can no longer be detected with anti-E tag by Western blotting (Fig. 3B), presumably because of cleavage of the E tag, which is too small to detect on these gels. To confirm the cleavage of EC1–EC3, a GST tag was added after the E tag on the carboxyl-terminal end. Incubation of this new
substrate, EC1–3EGST, with ETA clearly showed cleavage with the product now detectable (Fig. 3C). In this construct, the combination E and GST tag was added to hDsg1 only 4 amino acids after the cleavage site. Further analysis indicates that if these 4 amino acids were truncated, the construct was no longer susceptible to cleavage. These data show that cleavage by ETA is not dependent on intact domains EC4 and EC5.

Because truncations may result in a generally unstable overall structure, we used domain swapping between hDsg1E and hDsg3 to further test which domains in Dsg1 are critical (Fig. 4). These data suggest that sequences in hDsg1 between 213 and 450 are probably sufficient for cleavage. This conclusion was confirmed by insertion of just these sequences from Dsg1 into Dsg3 (Fig. 5). In fact, finer analysis indicated that only amino acids 214–398 from the EC2-EC3 domain of Dsg1, inserted into Dsg3, were sufficient for cleavage (data not shown). Although sufficient in the overall structure of Dsg3, these amino acids must be in the proper overall conformation of a desmoglein, because when inserted into E-cadherin, they could no longer be hydrolyzed by ETA (Fig. 5B).

5 Amino Acids 110 Residues Upstream of the Cleavage Site in Dsg1 Are Necessary for Cleavage—To further define which particular amino acids in the EC2-EC3 region of hDsg1 are critical for cleavage, we used cDsg1 because it is highly homologous to human and mouse Dsg1 and shares identical amino acids around the cleavage site (Fig. 6A) yet is not hydrolyzed by ETA (Fig. 6, C and D). Swapping sequences of hDsg1 into cDsg1 near the EC2-EC3 junction allowed its cleavage (Fig. 6, C and E). We then determined which amino acids in this junction of cDsg1 were different from those in hDsg1 and mouse Dsg1, which, like hDsg1, is cleaved by ETs (12) (Fig. 6B). Because cDsg1 is highly homologous to the human and mouse Dsg1, there were only a limited number of candidate amino acids that might be critical to allow cleavage of cDsg1. Substitution of these amino acids from hDsg1 to cDsg1 allowed us to find 5 amino acids in hDsg1 that are critical to allow hydrolysis of cDsg1 by ETA (Fig. 7, A and B). These amino acids are about 110 residues upstream of the cleavage site (Fig. 7C).

ETA Binding and Ability to Cleave Are Independent in Canine Dsg1—We speculated that the inability of ETA to hydrolyze cDsg1 could be due to its inability to bind that substrate, as with hDsg3 (12), and substitution of amino acids from the hDsg1 sequence might restore binding and, therefore, cleavage. However, co-immunoprecipitation of an ETA with the active serine mutated to alanine with cDsg1 and hDsg1 showed that both could bind the enzyme. (A mutant must be used to prevent
hydrolysis of the hDsg1-positive control (12) (Fig. 8). These data suggest that cleavage of Dsg1 by ETA depends not only on binding but also on proper alignment and/or activation of the enzyme active site.

DISCUSSION

Exfoliative Toxins Are Efficient Enzymes—The calculated values of $k_{cat}/K_m$ of $2-6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ show efficient enzymes. These $k_{cat}/K_m$ values are similar to those for the best tetrapeptide synthetic substrates for the acidic amino acid-specific endopeptidases *S. aureus* V8 protease and glutamic acid-specific *Streptomyces griseus* proteinase, which are $6 \times 10^3$ and $9 \times 10^2 \text{ M}^{-1} \text{s}^{-1}$, respectively (28). These $k_{cat}/K_m$ values are also greater than those obtained for the hydrolysis of the esterolytic substrate ($N$-$t$-Boc-$\text{L}$-Glu-$\text{L}$-phenyl ester) by ETA and ETB, which were reported to range from $3$ to $6 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ (29). It also
Fig. 7. Mutation of 5 amino acids in cDsg1 into human sequences restores its susceptibility to cleavage by ETA. A, amino acid sequences of point-mutated cDsg1 with residues mutated to human sequences underlined. B, mutation of 5 amino acids in cDsg1, shown in construct c271–7, restores susceptibility for cleavage by ETA. C, mutated sequences (in black, thin arrows) in c271–7 superimposed on the c-cadherin crystal structure. Note that the mutated sequences are on a loop proximal to the cleavage site (in black, thick arrow).
should be considered that the values we calculated for $k_{cat}/K_m$ for the hydrolysis of a large asymmetric protein, Dsg1. The interaction between two macromolecules is naturally going to be less efficient than that of an enzyme with a small substrate due to fact that only a relatively small area of each large protein is interactive (30–32). For example, $k_{cat}/K_m$ values for enzyme-small model peptide substrate reactions can be as high as $10^8$ to $10^9$ M$^{-1}$ s$^{-1}$ (33). The basal rate constant for productive collisions (i.e. encounters with the enzyme and substrate in the proper orientation for hydrolysis) between two large proteins in the absence of electrostatic effects is estimated at $-10^5$ M$^{-1}$ s$^{-1}$ (30). The $k_{cat}/K_m$ values for ET hydrolysis of Dsg1 approach this value. From these data, it can be appreciated that ETs are efficient enzymes. Our data also suggest that ETA is more efficient than ETB and ETD, although this observation may not reflect significant biological differences, since all of the enzymes are extremely efficient and pathological.

**ETA Is Not Inhibited by Broad Spectrum Serine Protease Inhibitors**—The broad spectrum serine protease inhibitors, DFP, DCI, and c$_s$-macroglobulin, do not efficiently inhibit ETA. These data suggest that somehow the active site of the enzyme is either inaccessible or not in the proper conformation to interact with these inhibitors and is consistent with the idea that only by binding to its physiologic substrate does the catalytic site of ETA become properly activated and/or aligned with the peptide bond in Dsg1 that it hydrolyzes. Consistent with this idea, some crystal structure analyses of ETA and ETB have suggested that their oxyanion hole is properly formed (35). However, one structural analysis of ETB suggests that this loop may be important in properly aligning the enzyme.

**Key in Lock Model for Specificity and Efficiency of ETA**—Our present data, together with previous data showing the importance of calcium-stabilized conformation of Dsg1 for its cleavage by ETA (21), suggest that the enzyme binds the substrate upstream of the cleavage site, which may align the catalytic site with the scissile bond. Furthermore, our data on resistance of ETA to serine protease inhibitors is consistent with an inactive or inaccessible catalytic site that we hypothesize is activated and/or made accessible to the scissile bond when the proper binding to the substrate occurs. Activation of ETA must require a very specific type of binding interaction, because although ETA binds to Dsg1 it does not cleave it. However, changing 5 amino acids in a loop just upstream of the cleavage site in Dsg1 allows its cleavage by ETA. The structure of this loop might allow a very specific "fit" that leads to enzyme activation and can, in this context, be thought of as similar to a key (ETA) in a lock (Dsg1).

Finally, our kinetic data indicate that either an efficient enzyme once aligned properly or, alternatively, electrostatic interactions between ETA and Dsg1 enhance the alignment beyond simple diffusion (30).

The key in lock model that explains the specificity of ETs is not unique. For example, it has also been applied to the specificity of coagulation factors. One example is the specificity of thrombin for fibrinogen, another system involving the interaction between two large proteins (37). Thrombin exhibits a number of unique structural features that provide specificity. The deep cleft bordering the active site called the 60-insertion loop limits the accessibility of the active site. An exosite defined as a highly positively charged region away from the active site is complementary to a negatively charged region in fibrinogen. An argy binding site requires apolar residues to occupy certain positions of substrates. Thus, the substrate must interact with all of these regions, like a key in a lock, to be hydrolyzed efficiently. Although our studies are not as detailed as those with thrombin, certain parallels can be made. Recognition of Dsg1 as a substrate is conformation-specific rather than sequence-specific and probably involves a specific exosite interaction between ETA and a region of extracellular domain 3 of Dsg1. Although the value of $k_{cat}/K_m$ for hydrolysis of Dsg1 by ETA was not as high as that observed for thrombin hydrolysis of fibrinogen (38), Dsg1 is concentrated in vivo in desmosomes on cell surfaces. In these structures, hydrolytic rates may be much faster due to the effective concentration of the substrate.

In conclusion, ETs have evolved to efficiently and specifically hydrolyze one bond in one substrate through specific interactions with that substrate, depending on both the substrate’s amino acid sequence and conformation at and distal to the cleavage site. The enzyme is thus able to efficiently and specifically target the exact molecule that allows blister formation in dog skin (2, 36). Substitution of these 5 amino acids from hDsg1 allows its cleavage. The location of these amino acids, as determined on the homologous C-cadherin crystal structure (15), is on a loop proximal to the cleavage site (Fig. 7C), suggesting that this loop may be important in properly aligning the enzyme.

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