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Mechanisms of Blister Formation by Staphylococcal Toxins

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Many children suffer from the bacterial skin diseases bullous impetigo and staphylococcal scalded skin syndrome (SSSS). *Staphylococcus aureus*, which produces exfoliative toxins (ETs), causes these diseases. Recently, it was proven that ETs cleave the cell adhesion molecule desmoglein (Dsg) 1, which plays an important role in maintaining the proper structure and barrier function of the epidermis. Surprisingly, Dsg1 is also the antibody target in the autoimmune disease pemphigus foliaceus. Skin biopsies from pemphigus foliaceus patients show the same pathology as those from bullous impetigo and SSSS patients. The crystal structure of ET suggests that it is a serine protease with an inactive catalytic site, which may become activated when ET binds a specific receptor. This receptor binding is thought to cause a change in conformation that exposes the catalytic site. It has recently been shown that Dsg1 specifically binds and activates ET, which in turn cleaves the bound Dsg1 at only one peptide bond. This process is absolutely dependent on the calcium-dependent conformation of Dsg1. These data suggest that ETs have a very high specificity for human Dsg1, and that *S. aureus* uses ETs to disrupt the barrier of the human epidermis in order to survive and proliferate on the human body.

Key words: desmoglein, exfoliative toxin, pemphigus foliaceus, serine protease, staphylococcal scalded skin syndrome.

Overview

Almost 30 years ago, exfoliative toxins (ETs) were identified as causing the blisters characteristic of bullous impetigo and staphylococcal scalded skin syndrome (SSSS). Since then many researchers have tried to find the target for ETs. The crystal structure as well as amino acid sequence indicated that ETs may be serine proteases. However synthetic peptides that were well cleaved by other serine proteases were shown not to be substrates for ETs. In addition, the crystal structure of ETs indicated that they may be folded into an inactivate form that might be activated by binding to a specific receptor, which could then be cleaved and released. However, the identity of such a putative receptor remained a mystery until recently when it was shown that ETs cleave desmoglein (Dsg) 1, which belongs to the cadherin supergene family and plays an important role in cell-cell adhesion in epidermal keratinocytes. Further analysis indicated that ETs are actually serine proteases that bind specifically to Dsg1 and cleave only one site between extracellular domain (EC) 3 and EC4, and then release it. ETs only recognize Dsg1 if it is in its native calcium-dependent conformation. In addition, amino acids found 110 residues upstream of the cleavage site in Dsg1 are necessary for its cleavage by ETs. These data suggest that ETs may fit into Dsg1, as a key in a lock, which may account for the exquisite specificity of ETs for Dsg1. The cleavage of Dsg1 results in a blister due to the loss of keratinocyte cell adhesion just below the stratum corneum. Such specific targeting of Dsg1 by ETs allows *Staphylococcus aureus* to proliferate and spread beneath the barrier of the skin.

Introduction: ETs and Dsg1

Impetigo, usually caused by *S. aureus*, is the most common bacterial skin disease in children, and may account for up to 10% of all childhood skin problems (*1*). Approximately 25–30% of these patients have bullous impetigo (*2*). Staphylococcal scalded skin syndrome (SSSS) is a generalized form of bullous impetigo that often occurs in neonates, but also may occur in young children or adults with renal failure or who are immunocompromised (*3*). In both diseases, blisters are formed due to the loss of keratinocyte adhesion in the granular layer (*i.e.* the most superficial living layer) of the epidermis. Thus, the blister forms just under the stratum corneum, which normally constitutes the skin barrier to infection. *Staphylococcus* can be cultured from the bullae of bullous impetigo but not usually from those of SSSS.

The bullae in these diseases are caused by toxins released by the staphylococcus. In bullous impetigo the toxin is released locally where infection occurs in the skin, however, in SSSS the toxin circulates systemically from a remote site of injection (*e.g.* the umbilicus or nasopharynx). Even though the toxin circulates throughout the body, it has exquisite specificity in causing a blister only in the epidermis, for example, it does not cause a blister in the closely related stratified squamous epithelia of mucous membranes. Passive transfer studies of ETs into neonatal mice that then develop clinically and histologically typical SSSS have proven that the toxins are pathogenic (*3–6*).

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Strikingly, an autoantibody-mediated disease, pemphigus foliaceus (PF), has an identical histopathology to that seen in bullous impetigo and SSSS (*7*). In PF, as in these staphylococcal toxin-mediated diseases, blisters occur in the superficial epidermis from loss of adhesion of keratinocytes in the granular layer. PF is caused by autoantibodies that bind Dsg1, a transmembrane glycoprotein of desmosomes. It is thought that these anti-Dsg1 antibodies cause adhesive dysfunction of Dsg1 that results in the blister (*8*). Like anti-Dsg1 antibodies in PF, ET causes blisters only in the superficial epidermis, and not in mucous membranes. Furthermore, the histology of ET- and anti-Dsg1–induced blisters is identical. Because in man and neonatal mice ET causes blisters that are similar or identical to those caused by PF anti-Dsg1 IgG, we hypothesized that it specifically inactivates Dsg1. This hypothesis was confirmed when it was shown that three different isotypes of ETs (ETA, ETB, ETD) cleave Dsg1 (*7*, *9–11*).

Dsg1 is a member of the cadherin supergene family, and is, therefore, termed a desmosomal cadherin (*12–14*). These cadherins characteristically contain extracellular repeating domains of about 100 amino acids in length that are highly conserved. Most cadherins are involved in calcium-dependent cell-cell adhesion, and calcium is thought to be important in maintaining their structure and function (*15–17*). The amino acid sequence of desmosomal and classical cadherins is highly conserved at the calcium-binding sites. Therefore, as in the case of classical cadherins, calcium is presumed to maintain the structure and function of desmosomal cadherins.

ET is a bacterial protein that exhibits the hallmark structural and amino acid sequence features of a serine protease (*e.g.* a characteristic pocket with the Ser-His-Asp triad responsible for mediating catalysis) (*18*, *19*). Although bacterial, ET demonstrates structural homology more similar to the mammalian proteases of the chymotrypsin family than to the bacterial subtilisin family. Specific features resemble those in the subclass of chymotrypsin family proteases found in pronase, a mixture of hydrolytic enzymes produced by the fungus *Streptomyces griseus*. Based on the crystal structure of the primary substrate specificity pocket (S1 subsite), ET is believed to cleave at a Glu-X peptide bond (*18*, *19*). The basic His213 and Lys216 residues in this pocket are thought to stabilize the acidic glutamic acid in the substrate. Despite these predictions based on structural and sequence homologies, the ET peptidase activity of typical model peptide and protein substrates had not been reliably demonstrated until recently. Only low activities for an ester substrate and α-melanocyte stimulating hormone, a substrate of unclear significance, have been reported (*20*).

The absence of hydrolytic activity for model substrates is believed to be related to the improper formation of the oxyanion hole by ETA (and perhaps ETB), as it is normally folded in the absence of its specific substrate. In an active serine protease, the substrate to be cleaved fits in a cleavage pocket in such a way that the oxyanion hole stabilizes the covalent transition state that is produced by nucleophilic attack of the Ser in the catalytic triad on the carbonyl bond of the substrate peptide. This nucleophilic attack produces a transition molecule with a negative charge on the attacked carbonyl oxygen. In serine proteases of the chymotrypsin family, amide nitrogens from conserved residues Ser195 (numbered according to chymotrypsin) and Gly193 form the oxyanion hole. However, the oxyanion hole is not properly configured in ETA (and perhaps ETB). The bond between Pro192 and Gly193 is turned 180° compared to typical serine proteases, thereby placing the carbonyl oxygen of Pro 192 instead of the amide nitrogen of Gly193 in the oxyanion hole. This unusual orientation has led to the hypothesis that ETA and ETB are inactive when secreted from the bacteria and must rely on interaction with another component to be converted to the active form. This interaction would presumably produce a conformational change in ETA and ETB to properly align Gly193 in the active site. Such an "induced-fit" activation mechanism (*21*) would furnish the protease with a high degree of substrate specificity in that cleavage would be dependent on the recognition of both the proper amino acid residues by the S1 pocket and interaction with a binding site capable of affecting the structure of the oxyanion hole. ETA does contain a highly charged long α-helical N-terminus, unique among the chymotrypsin family, that may function as this postulated binding site, although it is not clear how an interaction at this region would affect the conformation of Gly193.

ETs hydrolyze one peptide bond between the EC3 and EC4 domains in Dsg1

After treatment with ET, the 80-kDa recombinant extracellular domain of human Dsg1 made by baculovirus (hDsg1E) is cleaved into two major peptides of 50 and 30 kDa, suggesting ET hydrolyzes only one peptide bond. Western blotting with an anti–E-tag, which recognizes the carboxy-terminus of hDsg1E, identified the 30 kDa band, defining the 30 kDa product as the carboxy-terminal cleavage product. We performed N-terminal amino acid sequence analysis of this product, which revealed that ET cleaves human Dsg1 after glutamic acid residue #381 between the EC3 and EC4 domains. The cleavage site in mouse Dsg1 is the same. ETA, ETB and ETD all cleave at this same site (*22*). None of these ETs cleaves closely related cadherins such as Dsg3 or E-cadherin. These data suggest that ETs cleave Dsg1 very specifically.

ETA is a serine protease

Previous crystal structure data for ETs indicate that they have the structure of a serine protease. To prove that assumption, we mutated the putative active serine residue #195 of ETA, ETB and ETD into alanine (S195A). Incubation of these mutant ETs with hDsg1E failed to show the characteristic 30-kDa cleavage product (or any cleavage at all) by Western blotting with anti–E-tag (*22*). These data suggest that ETs are serine proteases. To show that the substitution of serine by alanine did not result in a loss of activity due to a major conformational change, we replaced the active serine with cysteine, which also resulted in a marked decrease in the hydrolysis of hDsg1E.

Binding of ETs to Dsg1

Crystal structural studies of ETA have suggested that it may be an inactive enzyme due to an inappropriate alignment of certain residues forming the active site. As a consequence, it has been postulated that ETA may need to bind to its specific substrate or receptor in order to become catalytic. After incubation of wildtype ETA or the ETA S195A mutant with mouse Dsg1F (full length Dsg1 with a FLAG tag on its C-teminus) or mouse Dsg3F (full length Dsg3 with a FLAG tag on its C-teminus), immunoprecipitation was performed using anti-FLAG conjugated agarose beads. The immunoprecipitated proteins were analysed by SDS-PAGE followed by immunoblotting with anti-FLAG or anti-ETA. Dsg1F and ETA S195A co-precipitated but wildtype ETA with Dsg1F did not, suggesting that ETA binds to Dsg1, but that after cleavage, ETA is released. Neither wildypte ETA nor ETA S195A showed binding to Dsg3F, demonstrating that the binding of ETA with Dsg1 is specific (*22*).

Ca^{2+}-depleted Dsg1 is not cleaved by ETs, and the sequential addition of Ca^{2+} does not reverse resistance to proteolysis

Typical serine proteases, such as trypsin or V8 protease often cleave large protein substrates into small pieces because they cleave after specific amino acid sequences that are usually repeated many times in a protein (*i.e.* they are sequence-specific proteases). In contrast, ETs cleave only one site in one molecule, Dsg1, which is a highly structured protein whose conformation is stabilized by calcium. These observations led us to hypothesize that the unique specificity of ETs for Dsg1 may require a specific interaction with its Ca^{2+}-dependent conformation. To determine whether the ability of ETs to cleave Dsg1 is dependent on the conformation of the latter, Dsg1 was preheated or pretreated at high or low pH before incubation with ETA. ETA did not cleave such denatured Dsg1. To determine if the proper conformation for cleavage by ETA is maintained by calcium, we incubated hDsg1E with EDTA. As controls for ETA we used trypsin and staphylococcus V8 protease, which are in the same protease family (chymotrypsin) as ETA. hDsg1E treated with EDTA was not cleaved by ETA, even if the calcium was subsequently added back suggesting that the change in conformation by calcium depletion is not readily reversible. In contrast to the results with ETA, EDTA did not block hDsg1E digestion by trypsin and V8 protease. These results suggest that the cleavage of Dsg1 by ETs is dependent on a Ca^{2+}-stabilized conformation of Dsg1 (*23*).

ETs are efficient enzymes for the cleavage of Dsg 1

Time course studies were performed with purified hDsg1E incubated with various concentrations of ETs. Densitometry analysis of the disappearance of substrate or appearance of cleavage products with time indicated values of k_{cat}/K_M of 62,000 M^{-1} s^{-1}, 26,000 M^{-1} s^{-1}, and 19,000 M^{-1} s^{-1} for ETA, ETB, and ETD, respectively (*24*). These values indicate the efficient cleavage of a large protein substrate (*25–27*).

ET cleavage of Dsg 1 is only weakly inhibited by typical serine protease inhibitors

We measured the inhibition of ET hydrolysis of Dsg 1 by small [di-isopropylphosphofluoridate (DFP) and 3,4-dichloroisocoumarin (DCI)] and large (α2 macroglobulin) serine protease inhibitors. At concentrations that almost totally inhibit V8 protease, all are very poor inhibitors of ET activity. These results suggest that the catalytic site of ETs is difficult to access or that it is inactive when incubated with the inhibitors (*18*, *19*, *28*).

hDsg1 EC domains that are upstream, but not downstream, of the cleavage site are critical for its hydrolysis by ETA

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. These results suggest that domains distant from the site of hydrolysis might influence the enzymatic efficiency of ET. Because desmogleins have five well-defined EC domains whose relationship to each other is stabilized by calcium, we first determined if all these domains were necessary for cleavage by testing truncated molecules of hDsg1E for their susceptibility to hydrolysis by ETA. The results indicated that EC5 is not necessary for cleavage. A loss of EC1 decreases the efficiency of cleavage, yet the truncated molecule is still cleaved (*24*). A loss of EC1-2 prevents cleavage. After incubation with ETA, the EC1-3 domain with a carboxy-terminal E-tag can no longer be detected with anti–E-tag by Western blotting, presumably because of the cleavage of the E-tag, which is too small to detect on these gels. To confirm the cleavage of EC1-3, a GST-tag was added after the E-tag on the carboxy-terminal end. Incubation of this new substrate, EC1-3EGST, with ETA clearly showed cleavage with a now detectable product. In this construct, the combination E- GST-tag was added to hDsg1 only 4 amino acids after the cleavage site. Further analysis indicated that if these 4 amino acids are truncated, the construct is no longer susceptible to cleavage. These data show that cleavage by ETA is not dependent on intact domains EC 4 and 5.

Because truncations may result in a generally unstable overall structure, we used domain swapping between hDsg1E and hDsg3 to test further which domains in Dsg1 are critical. These data suggest that sequences in hDsg1 between 213 and 450 (which are in EC 2 and EC4 domains) are probably sufficient for cleavage. This conclusion was confirmed by insertion of just these sequences from Dsg1 into Dsg3. In fact, finer analysis indicated that only amino acids 214–398 from the EC 2-3 domain of Dsg1, when inserted into Dsg3, are sufficient for cleavage. 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, the chimeric molecule could not be hydrolyzed by ETA.

Five amino acids 110 residues upstream of the cleavage site in Dsg1 are necessary for cleavage

To define further which particular amino acids in the EC 2-3 region of hDsg1 are critical for cleavage, we used canine Dsg1 (cDsg1) (*29*) because it is highly homologous

to human and mouse Dsg1 and shares identical amino acids around the cleavage site, yet is not hydrolyzed by ETA. Swapping sequences of hDsg1 into cDsg1 near the EC23 junction allowed its cleavage. We then determined which amino acids in this junction of cDsg1 are different from those in hDsg1 and mouse Dsg1 which, like hDsg1, is cleaved by ETs. Because cDsg1 is highly homologous to the hDsg1 and mouse Dsg1, there are only a limited number of candidate amino acids that might be critical to the cleavage of cDsg1. Substitution of these amino acids from hDsg1 to cDsg1 allowed us to identify 5 amino acids in hDsg1 that are critical for the hydrolysis of cDsg1 by ETA. These amino acids are located about 110 residues upstream of the cleavage site (*24*). The location of these amino acids, as determined on the homologous C-cadherin crystal structure (*30*) is on a loop proximal to the cleavage site, suggesting that this loop may be important for the properly alignment of the enzyme.

Conclusion

ETs, serine proteases, specifically bind and cleave Dsg1 at only one peptide bond between the EC3 and EC4 domains. The cleavage of Dsg1 by ETs requires that the proper conformation of Dsg1 be maintained by calcium, otherwise the enzyme is inactive. Five amino acids located 110 amino acids upstream of the cleavage site are critical for Dsg1 hydrolysis. These data suggest that ETs may fit into Dsg1 as a key in a lock in order to align, and possibly activate, the catalytic site. This highly specific and efficient cleavage of Dsg1 allows the bacteria to form a blister under the normal barrier of the skin in order to survive and proliferate.

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