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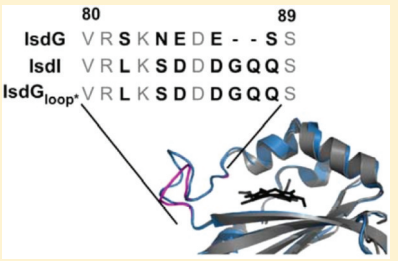
The Flexible Loop of *Staphylococcus aureus* IsdG Is Required for Its Degradation in the Absence of Heme

Michelle L. Reniere,[†] Kathryn P. Haley, and Eric P. Skaar*

Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee 37232, United States

Supporting Information

ABSTRACT: Degradation of specific native proteins allows bacteria to rapidly adapt to changing environments when the activity of those proteins is no longer required. Although these processes are vital to bacterial survival, relatively little is known regarding how bacterial proteins are recognized and targeted for degradation. *Staphylococcus aureus* is an important human pathogen that requires iron for growth and pathogenesis. In the vertebrate host, *S. aureus* fulfills its iron requirement by obtaining heme iron from host hemoproteins via IsdG- and IsdI-mediated heme degradation. IsdG and IsdI are structurally and mechanistically analogous but are differentially regulated by iron and heme availability. Specifically, IsdG is targeted for degradation in the absence of heme. Therefore, we utilized the differential regulation of IsdG and IsdI to investigate the mechanism of regulated proteolysis. In contrast to canonical protease recognition sequences, we show that IsdG is targeted for degradation by internally coded sequences. Specifically, a flexible loop near the heme-binding pocket is required for IsdG degradation in the absence of heme.



The ability of bacterial pathogens to sense and respond to changing environmental conditions is imperative for their survival. Transcriptional and translational mechanisms of regulation have been studied in great detail; however, these pathways require synthesis of new proteins, which is a time-consuming process. The most rapid strategy for altering cellular composition to deal with changing stressors is through protein degradation. Recent studies have suggested that regulated protein degradation is as important to cellular homeostasis as classical transcriptional and translational regulation.[27] To avoid uncontrolled protein degradation, protease substrate specificity is critical. This fact is highlighted by the recent identification of acyldepsipeptides, a novel class of antibiotics that exert toxicity by rendering an intracellular protease constitutively active.[3] However, the mechanisms by which bacteria recognize specific proteins and target them for proteolytic degradation remain ill-defined.

Bacterial pathogens such as *Staphylococcus aureus* must adapt to a variety of host environments to cause infection. For instance, upon entering the host, bacteria encounter an environment that is virtually devoid of available iron. In the vertebrate host, 80% of iron is found as a component of heme, the majority of which is bound to hemoglobin.[5] For *S. aureus* to acquire iron for use as a nutrient source, proteins of the iron-regulated surface determinant (Isd) system bind host hemoglobin, remove the heme cofactor, and transport heme into the cytoplasm.[25] The cytoplasmic components of this system, IsdG and IsdI, are paralogous heme oxygenases that degrade heme to release nutrient iron.[24] IsdG and IsdI are each required for growth on heme as a sole iron source, an environment that is predicted to mimic conditions experienced by the bacterium within the host. Accordingly, IsdG and IsdI are required for staphylococcal pathogenesis in a murine model of systemic infection.[21]

IsdG and IsdI are 64% identical at the amino acid level, and their three-dimensional structures can be superimposed with a root-mean-square deviation of less than 2 Å.[14,24] Moreover, IsdG and IsdI both degrade heme to the chromophore staphylobilin.[22] Although their structures and catabolic mechanisms are nearly identical, IsdG and IsdI are not functionally redundant enzymes. In addition, IsdG and IsdI are differentially regulated by iron and heme availability.[21] Under low-iron conditions, Fur-mediated transcriptional repression is released and *isdG* and *isdI* are transcribed. IsdG is also regulated at the post-transcriptional level such that IsdG is stabilized in the presence of heme. Therefore, IsdI is most abundant under low-iron conditions, while IsdG levels are maximal in iron-depleted environments containing heme.[21] This differential regulation likely allows the bacteria to fine-tune the expression of heme oxygenase activity to adapt to specific environments. However, the mechanism by which IsdG is specifically stabilized by heme has not been elucidated.

We sought to utilize the differential heme-dependent regulation of IsdG and IsdI to investigate the mechanism by which IsdG is specifically targeted for degradation. Here we show that IsdG catalytic activity is not required for heme-dependent IsdG stability. Furthermore, IsdG degradation is ATP-dependent, suggesting that an as-yet unidentified ATP-dependent protease may be responsible for IsdG degradation in the absence of heme. We also show that IsdG is targeted for degradation by an amino acid motif located within the primary

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sequence. This is a unique in vivo demonstration of a bacterial protein that is specifically targeted for degradation by a sequence not located within the N- or C-terminus. Finally, we identify the flexible loop of IsdG as a critical determinant of IsdG degradation. Combined, these results begin to elucidate the mechanism of heme-dependent IsdG stability. Moreover, these studies will lead to an improved understanding of targeted protein degradation in general, as the IsdG recognition sequence may be widely utilized in protein turnover in Gram-positive pathogens.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *S. aureus* clinical isolate Newman was used in all experiments.[9] Isogenic mutants lacking *isdG* and/or *isdI* have been described previously.[17,21] Bacteria were grown in tryptic soy broth (TSB) at 37 °C with shaking at 180 rotations per minute unless otherwise stated. Chloramphenicol (10 μg/mL) was included in the medium for growth of all strains harboring *pOS1*-derived vectors. Luria broth (LB) was used for the growth of *Escherichia coli* with ampicillin (100 μg/mL) or chloramphenicol (34 μg/mL) when necessary for plasmid selection.

Genetic Manipulation. The Δ*ftsH* and Δ*hslUV* isogenic mutants were made by deleting the genes using the p*KOR1* vector, as previously described.[2] The Δ*clpP* isogenic mutant was a generous gift from H. Ingmer (University of Copenhagen, Copenhagen, Denmark). The *pisdG* complementation vector encodes a full-length copy of *isdG* under the control of the *S. aureus* lipoprotein diacylglycerol transferase (*lgt*) constitutive promoter in the *pOS1*-derived vector.[4,23]

Pfu mutagenesis was used to create point mutations in *pET15b.isdG*.[28] The $IsdG_{loop*}$ mutant was made by successive individual point mutants in the IsdG loop region via inverse polymerase chain reaction (iPCR). All mutants were confirmed by sequencing (Vanderbilt University DNA sequencing facility). Plasmids were then transformed into *E. coli* BL21-(DE3) *pREL* for expression and purification, as described previously.[24] IsdG mutant coding sequences were excised using NdeI and BamHI and were inserted into *pOS1plgt*.[4] Plasmids were then transformed into restriction negative *S. aureus* strain RN4220 and subsequently transformed into strain Newman Δ*isdGI*.

Construction of Chimeric Proteins. To create IsdI–IsdG chimeras, a *pET15b.isdI_isdG* plasmid was first constructed. The *isdG* coding sequence was amplified from genomic DNA with phosphorylated primers and then purified via column purification, according to the manufacturer's specifications (Qiagen). The *pET15b.isdI* vector was linearized with BamHI and Klenow-treated to produce blunt ends. The *isdG* coding sequence was then ligated into *pET15b.isdI*, following treatment of the linearized vector with shrimp alkaline phosphatase. Directionality was confirmed by PCR. This created the *pET15b.isdI_isdG* vector in which the coding sequence of *isdG* directly follows that of *isdI*. Chimeras 1–3 were then created by iPCR using the *pET15b.isdI_isdG* vector as a template (see Table S1 of the Supporting Information for primers).

Chimera 4 was created by amplifying the coding sequence for residues 12–89 of IsdG from genomic DNA with phosphorylated primers. Following iPCR of *pET15b.isdI* and gel purification of the linear vector, the amplified *isdG* fragment was ligated in to create *pET15b.chimera4*. Chimera 5 was created by iPCR of *pET15b.chimera4* and PCR amplification of a 76 bp fragment of *isdI* with phosphorylated primers (see Table S1 of the Supporting Information for primers). All chimeric proteins were confirmed by sequencing. The chimera coding sequences were excised using NdeI and BamHI and were inserted into *pOS1plgt*.[4] Plasmids were then transformed into restriction negative *S. aureus* strain RN4220 and subsequently into strain Newman Δ*isdGI*.

Pulse–Chase Analysis and Immunoprecipitation. Pulse–chase analyses were performed as described previously.[21] Briefly, midlog cultures of *S. aureus* Δ*isdG pisdG* were washed extensively in methionine-free medium and resuspended in methionine-free medium supplemented with iron sulfate (10 μM) or heme (10 μM) when stated. After pulsing the mixture with [^{35}S]methionine for 2 min, we added the chase solution and removed samples at various time points. Following lysostaphin treatment, the protoplasts were washed and lysed. Cell lysates were incubated with IsdG antisera, and subsequently, protein-A Sepharose beads were added. After being extensively washed, the antibody–protein complexes were eluted in reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and were separated by electrophoresis. Dried gels were analyzed using a PhosphorImager, and bands were quantified using Multi Gauge software version 3.0 (Fuji Film).

Immunoblot. *S. aureus* protoplasts were analyzed for IsdG and IsdI expression as described previously.[21] Briefly, overnight cultures of *S. aureus* were sedimented and treated with lysostaphin to digest the cell wall. After centrifugation, the protoplasts were resuspended in SoluLyse Bacterial Protein Extraction Reagent (Genlantis). Samples were then sonicated to homogeneity and normalized by total protein concentration, as measured by BCA analysis. Following separation via 15% SDS–PAGE, gels were analyzed by immunoblotting with polyclonal antisera for IsdG or IsdI. Before being blotted, the nitrocellulose membranes were stained with the Novex Reversible Membrane Protein Stain (Invitrogen) to confirm equal loading.

RESULTS

IsdG Catalytic Activity Is Dispensable for Its Heme-Dependent Stability. IsdG protein levels are stabilized when *S. aureus* is grown in medium supplemented with heme.[21] In the presence of an electron donor, IsdG degrades heme to release iron and staphylobilin. Staphylobilin is a novel chromophore that was recently identified, and its function in the cell has not yet been determined.[22] In vitro staphylobilin remains bound in the active site of IsdG; however, it is not known if this is representative of IsdG function in vivo. Therefore, it is possible that IsdG is stabilized by staphylobilin rather than heme. In an effort to parse the catalytic activity and the heme-dependent stability of IsdG, we performed pulse–chase and immunoprecipitation analysis to monitor the stability of a catalytically inactive variant of IsdG. The asparagine residue at position 7 within IsdG is critical for heme degradation, and therefore, an N7A mutant is catalytically inactive.[14,24] This is despite the fact that IsdG N7A binds heme in a manner equivalent to that of the wild type. If staphylobilin is required in the active site for IsdG stability, the N7A variant should not be stabilized in the presence of heme, as this mutant is unable to synthesize staphylobilin.

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Following a 2 min pulse with [^{35}S]methionine, excess unlabeled methionine was added to the medium and levels of labeled IsdG were followed over time by immunoprecipitation. These experiments were performed in *S. aureus* Δ*isdG* that express *isdG* constitutively from a plasmid to analyze protein stability independently of transcriptional regulation.[21] We found that the abundance of IsdG N7A and IsdG wild type is increased in the presence of heme to a similar extent (Figure 1). Moreover, the heme-dependent stability of IsdG in the N7A mutant is not due to altered expression of IsdI or the heme uptake machinery, which could have an indirect effect on intracellular heme abundance (Figure S1 of the Supporting Information). These results demonstrate that the catalytic activity of IsdG is dispensable for its stability. Therefore, the heme-dependent stability of IsdG is not dependent upon the formation of staphylobilin. Interestingly, we observed a statistically insignificant increase in IsdG N7A stability in the absence of heme, as compared to that of wild-type IsdG (Figure 1B). We hypothesize that this slight increase in IsdG N7A stability may be due to the binding of endogenously synthesized heme. The decrease in heme oxygenase activity may result in the accumulation of endogenous heme, which could bind and stabilize the inactive protein. This hypothesis remains to be tested, but it does introduce the possibility that endogenously synthesized heme may be recycled by heme oxygenases.

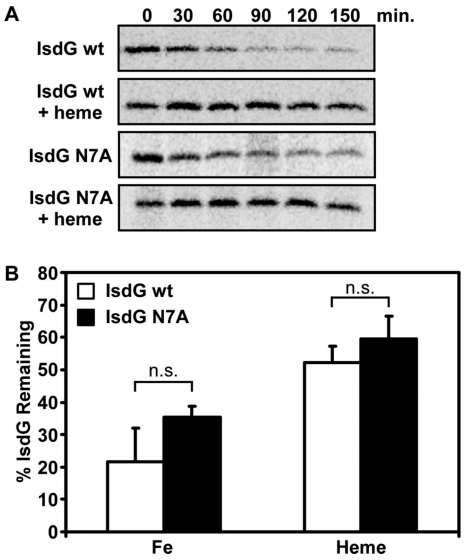


Figure 1. The catalytic activity of IsdG is not required for its heme-dependent stability. Pulse–chase analyses of an IsdG N7A variant expressed in *S. aureus* Δ*isdG pisdG*. IsdG N7A binds heme but is catalytically inactive. (A) Representative phosphorimages of immunoprecipitation over time in the presence and absence of heme. (B) Percent IsdG remaining 150 min after addition of the chase solution. Error bars represent the standard deviation of at least three independent experiments. There is no statistical difference (n.s.) between IsdG wild type and N7A, as measured by a Student's *t* test ($p > 0.14$).

IsdG Stability Is Increased by Inhibiting ATPases. We next sought to elucidate the mechanism by which IsdG is degraded in the absence of heme. We hypothesize that IsdG is not required by the cell when exogenous heme is scarce and is therefore targeted for degradation by intracellular proteases. To determine if IsdG degradation in the absence of heme is mediated by an ATP-dependent protease, we analyzed the stability of IsdG in the presence of the respiratory poison sodium arsenate. Sodium arsenate structurally mimics inorganic phosphate, thereby inhibiting all cellular ATPases. A significant increase in IsdG stability was observed upon addition of sodium arsenate to the medium, an effect that is independent of heme (Figure 2 and Figure S2 of the Supporting Information). These results indicate that IsdG degradation is an ATP-dependent process, supporting the hypothesis that IsdG is specifically degraded by an ATP-dependent protease.

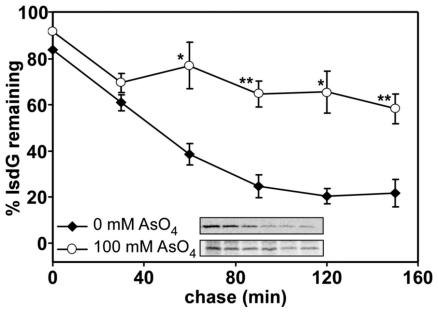
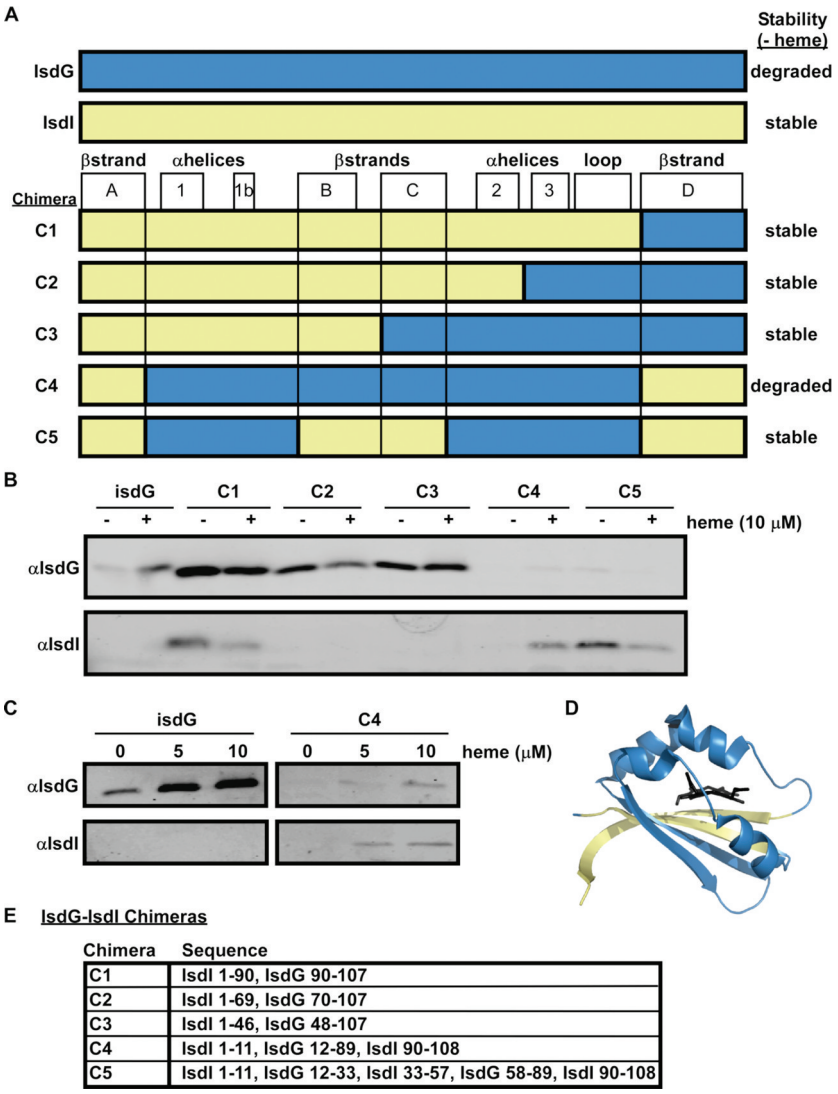


Figure 2. IsdG degradation is ATP-dependent. Pulse–chase analysis of IsdG in *S. aureus* Δ*isdG pisdG* exposed to the respiratory poison AsO_4. Insets are representative phosphorimages of immunoprecipitation over time. Error bars represent the standard deviation of at least three independent experiments. Asterisks indicate statistically significant differences, as measured by a Student's *t* test (*$p < 0.025$; **$p < 0.009$).

Sodium arsenate is a general ATPase inhibitor that likely has many effects on the cell in addition to inhibiting proteolysis. Therefore, to more directly test the role of ATP-dependent proteases in IsdG degradation, we analyzed IsdG stability in *S. aureus* strains deficient in known intracellular proteases. To date, only three proteins have been identified as intracellular ATP-dependent proteases in *S. aureus*. These proteases are ClpP, FtsH, and HslV. ClpP is the primary intracellular protease in *S. aureus* and has been shown to be important for stress response, metal homeostasis, autolysis, degradation of antitoxins, and regulation of virulence factors.[8,18] In contrast, FtsH and HslV (also called ClpQ) appear to play only minor roles in stress survival and virulence.[10,16]

Surprisingly, we did not observe an increase in IsdG protein levels in any of the protease mutants (Figure S2 of the Supporting Information). It is conceivable that more than one protease can degrade IsdG, and this redundancy may mask the effect of inactivating any single protease mutant. However, previous work has demonstrated that a Δ*clpP*Δ*hslV* double mutant is not viable, making it difficult to test this possibility.[10] ATP-independent cellular peptidases may also play a role in protein turnover, although these have not been characterized in *S. aureus*. Taken together, these results suggest that IsdG is not degraded by ClpP, FtsH, or HslV, and that an as-yet-unidentified protease may degrade IsdG in the absence of heme.

The mechanisms by which bacterial proteins are recognized and targeted for degradation are not well understood. Because of their significant similarity, the differentially regulated heme oxygenases IsdG and IsdI are ideal proteins for investigating the structural features that are recognized by the proteolytic



Chimera	Sequence
C1	IsdI 1-90, IsdG 90-107
C2	IsdI 1-69, IsdG 70-107
C3	IsdI 1-46, IsdG 48-107
C4	IsdI 1-11, IsdG 12-89, IsdI 90-108
C5	IsdI 1-11, IsdG 12-33, IsdI 33-57, IsdG 58-89, IsdI 90-108

Figure 3. The N- and C-termini are not required for IsdG degradation. (A) Schematic of IsdG (blue), IsdI (yellow), and chimeric proteins. Regions of secondary structure are indicated above chimeras 1−5 (C1−C5, respectively). (B) Immunoblot analyses of chimeric proteins in the presence (+) and absence (−) of heme (10 μM). (C) Immunoblot analyses of IsdG wild type and chimera 4 in increasing concentrations of heme. To better detect chimera 4, twice the total amount of protein was loaded in each lane, as compared to IsdG. (D) Structure of the IsdG−heme complex (Protein Data Bank entry 2ZDO) color-coded to show the sequence contribution of IsdG and IsdI to chimera 4 (C4). The color scheme is the same as in panel A, with heme colored black. (E) Table describing the amino acid sequences contained within each chimera.

machinery of *S. aureus*. To determine the minimal region necessary for IsdG degradation in the absence of heme, we constructed a panel of chimeric IsdI−IsdG proteins and assessed their stability in the presence and absence of heme (Figure 3A). All chimeric proteins were recombinantly expressed in *E. coli* and were found to bind and degrade heme like the wild type, indicating that they are properly folded and catalytically active (Figures S3 and S4 of the Supporting Information). The chimeras were then constitutively expressed in *S. aureus* Δ*isdGI* grown in the presence or absence of heme, and stability was analyzed by immunoblotting. As these proteins contain regions from both IsdG and IsdI, we utilized polyclonal antibodies raised against each protein for detection.[24] The relative immunoreactivity of each chimera was analyzed with both IsdG and IsdI antisera to ensure that each chimeric protein was detectable (Figure S5 of the Supporting Information). Notably, expression of the heme uptake machinery is not altered in bacteria expressing chimeric heme oxygenases (Figure S8 of the Supporting Information).

Chimeras 1, 2, and 3 (C1−C3, respectively) contain the N-terminus of IsdI and an increasing number of amino acids from the C-terminus of IsdG. Each of these chimeras is stable in the presence and absence of heme, indicating that the C-terminus of IsdG is not sufficient to induce its targeted degradation (Figure 3B). Conversely, chimera 4 (C4) contains IsdI sequence at both the N- and C-termini (C4) and is detected only in the presence of heme, as seen with wild-type IsdG (Figure 3C,D). This is particularly intriguing as protein targeting sequences within bacteria are typically located at either terminus of the substrate protein. Recently, it was shown that a degradation tag normally at the terminus of an *E. coli* protease substrate can be recognized when engineered to be in the interior of the primary sequence. This result establishes that bacterial proteases are capable of degrading substrates with internal recognition sequences in vitro.[12] However, the results

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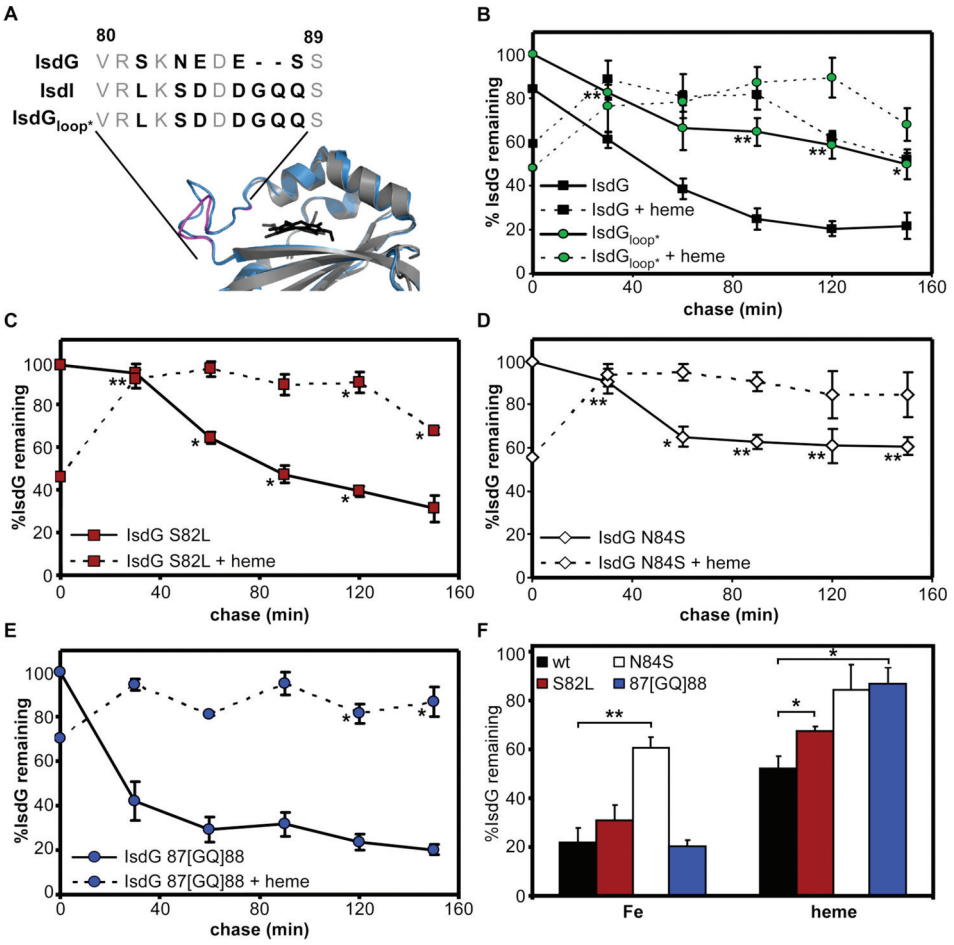

Figure 4. The flexible loop is required for IsdG degradation in the absence of heme. (A) Alignment of the flexible loop regions of IsdG and IsdI (Protein Data Bank entry 2ZDP) analyzed with PyMol. IsdG$_{loop*}$ is an IsdG mutant with residues 82−88 mutated to the corresponding amino acid sequence of IsdI. The crystal structure of IsdI (blue) is superimposed with that of IsdG (gray), and the IsdG flexible loop is highlighted (magenta). (B−E) Quantification of pulse−chase analyses of IsdG loop mutants expressed in *S. aureus* Δ*isdG*. (F) Percent IsdG remaining 150 min after the addition of the chase solution. Error bars represent the standard error of at least three independent experiments. Asterisks indicate statistically significant differences as compared to the wild-type IsdG value in the same growth medium, as measured by a Student's *t* test (*$p < 0.03$; **$p < 0.01$).

reported here provide a unique in vivo demonstration of a native protein that is targeted for degradation by a sequence not at the N- or C-terminus of the protein.

Upon discovering that IsdG degradation does not require sequence from either terminus, we sought to further refine the minimum region necessary to target a protein for degradation. A recent proteome-wide analysis of limited proteolysis concluded that proteolytic cleavage is most often observed in α-helices and disordered regions, and only very rarely in β-strands.[26] Therefore, we constructed C5, a chimera in which all the α-helices and disordered regions are sequences from IsdG and only the β-strands are encoded by IsdI sequence (Figure 3A). Contrary to our hypothesis, C5 is not degraded in the absence of heme and, in fact, appears to be more stable in the absence of heme. These results indicate that the central β-strands play a role in IsdG stability (Figure 3B).

The Flexible Loop Is Required for IsdG Degradation.

To identify specific motifs responsible for the targeting of IsdG, we focused on regions that undergo a conformational change upon heme binding. In silico analysis of IsdG and IsdI sequences indicated that amino acids 82−88 of IsdG comprise the region of highest divergence between the two proteins and the region exhibiting the greatest surface exposure (Figure 4A). The apoprotein crystal structures revealed this region to be a disordered loop that becomes ordered and more rigid upon porphyrin binding.[14,29] Thus, amino acids 82−88 comprise the section of highest sequence divergence between IsdG and IsdI, is the region with the most dramatic conformational change upon heme binding, and lacks secondary structure in the absence of heme. On the basis of these observations, we hypothesized that this region may play a role in the differential stability of IsdG and IsdI. This hypothesis is supported by a recent report demonstrating that the presence of a flexible loop is the critical component of an optimum protease cleavage site.[26]

To test the role of the flexible loop in IsdG stability, we constructed a chimeric IsdG protein that has the IsdI loop amino acid sequence substituted for the IsdG loop (IsdG$_{loop*}$) and tested its stability (Figure 4A). Thermal denaturation analysis reveals that this mutation does not negatively impact the folding properties of IsdG (Figure S7 of the Supporting Information). Pulse−chase analysis and immunoprecipitation were used for these analyses for increased sensitivity to detect slight changes in stability. These experiments revealed that

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$IsdG_{loop*}$ is significantly more stable than wild-type IsdG in the absence of heme (Figure 4B). More than 50% of the labeled $IsdG_{loop*}$ protein remained after 150 min in comparison to ~20% of the wild type. In fact, $IsdG_{loop*}$ is as stable in the absence of heme as wild-type IsdG in the presence of heme. The $IsdG_{loop*}$ mutant in the presence of heme shows a trend toward stability greater than that of the wild type; however, this difference is not significant (Figure 4B). These results demonstrate that the IsdI loop sequence confers heme-independent stability upon IsdG.

To more precisely define the sequence of IsdG required for its targeted degradation, a series of single-point mutations in the flexible loop of IsdG were constructed. Pulse−chase analyses showed that IsdG S82L is more stable than the wild type in the absence of heme 30, 60, and 120 min following addition of the chase solution (Figure 4C). However, like the wild type, only ~20% of the labeled S82L mutant protein remains after 150 min. This suggests that the S82L mutation slows but does not completely inhibit the degradation of IsdG. In contrast, IsdG N84S is significantly more stable than the wild type in the absence of heme at all time points after 60 min (Figure 4D). Moreover, IsdG N84S stability is not significantly altered upon addition of heme, revealing that mutation of a single asparagine abolishes the heme-dependent stability of IsdG. This difference in stability is not due to altered heme binding or degradation, as all IsdG mutants tested are able to bind and degrade heme like the wild type (Figure S6 of the Supporting Information). IsdG 87[GQ]88, in which the flexible loop is elongated by two amino acids, is not altered in terms of its overall stability (Figure 4E). This was unexpected given that longer flexible loops that extend the cleavage site away from the surface of the protein are favorable for protease-mediated degradation.[26] Thus, it is possible that the flexible loop is involved in protease recognition but is not the site of proteolytic cleavage.

Combined, these results demonstrate that asparagine 84 is the critical component of the flexible loop required to target IsdG for degradation (Figure 4F). Pulse−chase analyses revealed that an IsdI protein encoding the loop sequence of IsdG ($IsdI_{loop*}$) is stable in the presence and absence of heme (Figure S9 of the Supporting Information). This result is consistent with the heme-independent stability of chimeras 2, 3, and 5 (Figure 3), indicating that the IsdG flexible loop is not sufficient for targeting a protein for degradation. Proteases are not solely influenced by the amino acid sequence of the cleavage site. Rather, sequences surrounding the cleavage site are also known to play a role in the specificity of protease recognition. Secondary site interactions at surfaces distinct from the cleavage site may also be important for effective protease cleavage.[26] Therefore, we conclude that the flexible loop is required for IsdG degradation in the absence of heme, although it is not a sufficient targeting sequence for proteolysis.

DISCUSSION

In the iron-depleted environment of the host, *S. aureus* expresses proteins of the Isd system to obtain heme iron during infection.[25] This system includes cell wall-anchored hemoprotein receptors, membrane heme transport proteins, and the cytoplasmic heme oxygenases IsdG and IsdI. We predict that upon encountering an iron-depleted environment devoid of heme *S. aureus* adapts by decreasing heme oxygenase activity in the cell. This is accomplished through the specific proteolysis of IsdG. Precedence for substrate-dependent stability comes from the quorum-sensing regulator TraR in *Agrobacterium tumefaciens*. TraR is resistant to proteolysis only when synthesized in the presence of its signal molecule, an autoinducing peptide (AAI).[30] In the absence of substrate, TraR is targeted for rapid proteolysis by a sequence in the amino terminus of the protein.[6] It is postulated that the hydrophobic core of TraR is exposed to solvent in the absence of AAI, resulting in aggregation and proteolysis. However, in this system, TraR synthesized in the absence of substrate is rapidly degraded with a half-life of 2 min. The half-life of IsdG in the absence of substrate is significantly greater (~60 min). This suggests that IsdG is properly folded in the absence of heme but is targeted for degradation at a step subsequent to translation and protein folding. This supposition is further supported by the observation that recombinant IsdG purified from *E. coli* is obtained at yields equal to that of IsdI. Recombinant IsdG is also able to bind and degrade heme with kinetics similar to those of IsdI, indicating that it is properly folded.[24]

Post-translational regulation mediated by proteolysis must be a highly specific process to prevent degradation of native proteins. The best characterized methods for substrate specificity in bacteria are the N-end rule pathway and the SsrA tag. The N-end rule describes a universal system across kingdoms in which the stability of a protein is dependent on its NH_2-terminal residue.[1] The SsrA tag is an 11-amino acid peptide added to the C-terminus of incomplete polypeptides upon ribosome stalling. Approximately 0.5% of *E. coli* proteins are SsrA-tagged during normal translation;[15] however, the process of SsrA tagging has not been investigated in *S. aureus*. The rate of IsdG degradation makes it an unlikely substrate for the N-end rule or the SsrA-tagging system, as both targeting mechanisms result in rapid degradation of substrates, whereas IsdG degradation occurs over ~60 min.[15,19,21] Moreover, we have shown that the first 11 amino acids and the last 18 amino acids of IsdG are not required for degradation in the absence of heme. *E. coli* ClpAP and ClpXP are capable of degrading proteins with internal recognition tags in vitro.[12] More specifically, Hoskins et al. engineered a protein with a ClpA recognition motif moved from the amino terminus to an interior amino acid sequence. The engineered protein was degraded in vitro, although with kinetics slower than those of the native protein. The experiments described herein identify IsdG as a native protein targeted for degradation in vivo by a sequence outside the N- or C-terminus.

Substrate recognition by bacterial proteases is multifaceted and requires precise structural presentation, cleavage site sequence, subsite specificity, and appropriate secondary site interactions.[26] Studies of the structural features of proteolytic substrates found that loop size and target sequence play critical roles in defining the susceptibility of protein substrates.[7,26] Variations in the recognition sequence, such as conformational changes that occur upon binding substrate, can also affect proteolytic sensitivity.[11] Herein, we have shown that the flexible loop is necessary but not sufficient for IsdG degradation. These results indicate that other factors, which may include subsite sequence specificity and secondary site recognition, play a role in IsdG stability. Ongoing experiments are aimed at elucidating the mechanism of IsdG degradation, particularly to identify the minimal region sufficient to target a staphylococcal protein for degradation.

We have also demonstrated that the intracellular ATP-dependent proteases, ClpP, FtsH, and HslV, are not required for IsdG degradation in the absence of heme (Figure S2 of the Supporting Information). These proteases were originally identified on the basis of homology to *E. coli* proteases. *E. coli* also encodes the cytoplasmic serine protease Lon, which is highly conserved across kingdoms. However, Lon is not encoded within the genomes of some pathogenic Firmicutes, including *S. aureus*.[13] In addition to the cytoplasmic proteases, *S. aureus* encodes many secreted proteases that are required for pathogenesis. However, these are not active in the cytoplasm so the contribution of secreted proteases to IsdG stability was not analyzed in this study. It is possible that *S. aureus* expresses novel as-yet-unidentified proteases. This is supported by the observation that in the absence of exogenous heme, the yield of recombinant apo-IsdG purified from *E. coli* is greater than that of IsdI. This observation suggests that the protease responsible for IsdG degradation is not expressed in *E. coli* (data not shown). Alternatively, this could be due to a lack of recognition factors or protease cofactors that are necessary for IsdG degradation but are absent from *E. coli*. Future experiments will focus on identifying the factor(s) required for IsdG degradation.

IsdG and IsdI are each required for growth of *S. aureus* on heme as a sole iron source, an environment likely encountered during infection of vertebrates. Accordingly, IsdG and IsdI are both essential for staphylococcal pathogenesis. Furthermore, a *S. aureus* mutant lacking *isdG* is significantly more impaired than an *isdI* mutant in colonization of both the hearts and kidneys of infected animals.[21] This organ-specific phenotype is not yet understood; however, we hypothesize that the variability in iron and heme concentrations in each organ may dictate the differential requirement for IsdG and IsdI during infection. Mutants lacking the hemoglobin receptor IsdB are also severely attenuated in the hearts of infected animals, demonstrating that heme acquisition during pathogenesis is particularly critical in colonization of the heart.[20] Therefore, the increased stability of IsdG in the presence of heme may provide an advantage to *S. aureus* during infection of the heart, allowing it to efficiently utilize heme iron from host hemoglobin. Moreover, inhibiting protease function and/or specificity is a viable therapeutic option, as demonstrated by the novel class of antibiotics called acyldepsipeptides, which exert toxicity through the constitutive activation of bacterial proteases.[3] Combined, these facts suggest that elucidating the mechanism of IsdG degradation may lead to the identification of novel therapeutic targets. As *S. aureus* is becoming increasingly resistant to all available antibacterial agents, identifying novel therapeutic targets is imperative to combat this important pathogen.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Immunoblot analyses of IsdE and IsdI in *S. aureus* Δ*isdG pisdG* N7A (Figure S1), analysis of IsdG stability in protease-deficient strains and cells treated with arsenate (Figure S2), degradation of heme by IsdI−IsdG chimeric proteins (Figure S3), thermal denaturation profiles of chimeras 4 and 5 compared to IsdG (Figure S4), immunoreactivity of chimeric IsdG−IsdI proteins (Figure S5), degradation of heme by IsdG and IsdI loop mutants (Figure S6), thermal denaturation profiles of the $IsdG_{loop*}$ mutant compared to IsdG wild type (Figure S7), immunoblot analyses of IsdE and IsdI in *S. aureus* Δ*isdGI* expressing IsdI−IsdG chimeras (Figure S8), pulse−chase analyses of $IsdI_{loop*}$ stability (Figure S9), and primers used in this study (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Vanderbilt University Medical Center, 1161 21st Ave., South, A-5102 MCN, Nashville, TN 37232-2363. Phone: (615) 343-0002. Fax: (615) 343-7392. E-mail: eric.skaar@vanderbilt.edu.

Present Address

[†]Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200.

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