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Galectin-3 Is a Target for Proteases Involved in the Virulence of *Staphylococcus aureus*

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ABSTRACT *Staphylococcus aureus* is a major cause of skin and soft tissue infection. The bacterium expresses four major proteases that are emerging as virulence factors: aureolysin (Aur), V8 protease (SspA), staphopain A (ScpA), and staphopain B (SspB). We hypothesized that human galectin-3, a β-galactoside-binding lectin involved in immune regulation and antimicrobial defense, is a target for these proteases and that proteolysis of galectin-3 is a novel immune evasion mechanism. Indeed, supernatants from laboratory strains and clinical isolates of *S. aureus* caused galectin-3 degradation. Similar proteolytic capacities were found in *Staphylococcus epidermidis* isolates but not in *Staphylococcus saprophyticus*. Galectin-3-induced activation of the neutrophil NADPH oxidase was abrogated by bacterium-derived proteolysis of galectin-3, and SspB was identified as the major protease responsible. The impact of galectin-3 and protease expression on *S. aureus* virulence was studied in a murine skin infection model. In galectin-$3^{+/+}$ mice, SspB-expressing *S. aureus* caused larger lesions and resulted in higher bacterial loads than protease-lacking bacteria. No such difference in bacterial load or lesion size was detected in galectin-$3^{-/-}$ mice, which overall showed smaller lesion sizes than the galectin-$3^{+/+}$ animals. In conclusion, the staphylococcal protease SspB inactivates galectin-3, abrogating its stimulation of oxygen radical production in human neutrophils and increasing tissue damage during skin infection.

KEYWORDS galectin-3, *Staphylococcus aureus*, neutrophils, protease, staphopain, skin infection, virulence, virulence regulation, virulence factors

Staphylococcus aureus is a versatile human pathogen that causes infection in a wide range of tissues and is a major cause of both superficial and fatal infections (1, 2). This bacterium modulates immune responses through the expression of an orchestra of virulence factors (2, 3), including the following four major proteases (Table 1): the metalloprotease aureolysin (Aur), the cysteine proteases staphopain A (ScpA) and staphopain B (SspB), and the serine protease V8 protease (SspA) (4, 5). They are encoded under the three operons *aur*, *scp*, and *ssp* as proenzymes that are partly activated by each other or by autocatalysis (see Fig. S1 in the supplemental material) (4, 6–8). Their functions are not fully defined, although many observations suggest important roles in pathogenesis (5, 9).

Galectin-3 is a member of a family of soluble β-galactoside-binding lectins present

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TABLE 1 Description of *S. aureus* proteases

Proteases			Inhibitors		
Acronym	**Name**	**Protease class**	**Acronym**	**Name**	**Activity**
Aur	Aureolysin	Metalloprotease		EDTA	Ion chelator[a]
SspA	Glutamyl endopeptidase or V8 protease	Serine protease		Pefabloc SC	Serine protease inhibitor[a]
SspB	Staphopain B	Cysteine protease	SspC	Staphostatin B	Specific for SspB[b]
ScpA	Staphopain A	Cysteine protease	ScpB	Staphostatin A	Specific for ScpA[b] (and EcpA[c])

[a]Broad-spectrum inhibitors were used against the metallo- and serine proteases.
[b]Specific inhibitors were used against the two cysteine proteases (5, 58).
[c]Close homology to *Staphylococcus epidermidis* also allows inhibition of epidermidis cysteine protease (EcpA) (45).

in many species (10). It exhibits significant regulatory functions both in host defense against infection and in aseptic inflammation (11–13). Several findings suggest that galectin-3 participates in antimicrobial defense, having opsonizing and bacteriostatic properties or inducing infection-reducing effects in the host (14–16), but there are also data showing that galectin-3 can contribute to microbial pathogenesis, e.g., by promoting adhesion to host cells or increasing the tissue-destructive response of inflammatory cells (14, 15, 17, 18). Galectin-3 is produced in epithelia and by several immune cells and is released by monocytes and macrophages in response to bacterial lipopolysaccharide (19, 20). The lectin has been proposed to facilitate the transmigration of neutrophils, the most abundant circulating leukocyte, from blood into tissue during the initial steps of inflammation (16, 21). Galectin-3 also induces activation of the neutrophil NADPH oxidase, producing reactive oxygen species (ROS) (22, 23), and can opsonize apoptotic neutrophils to facilitate their clearance by macrophages (24).

Galectin-3 ligand binding is mediated through the C-terminal carbohydrate recognition domain (CRD), a globular structure that is highly preserved within the galectin family (10). In addition to the CRD, galectin-3 has a collagen-like N-domain that is sensitive to proteolytic cleavage (25). This N-domain is essential for oligomerization of galectin-3 and for many of the galectin-3-induced effects in inflammation (14, 22, 26). There are proteases known to cleave galectin-3; human neutrophil elastase and matrix metalloproteinases-2, -7, and -9 can proteolytically process the lectin (25, 27, 28), a feature shared by proteases from protozoa (29) and bacteria (26, 30, 31). Hence, N-terminal cleavage of galectin-3 could be suggested as a regulatory mechanism in infection and inflammation. Whether such galectin-3 processing is achieved by *S. aureus* proteases has so far not been investigated.

We found that the *S. aureus* protease staphopain B (SspB) had the capacity to cleave galectin-3, thereby inhibiting the neutrophil-activating ability of the lectin. Furthermore, galectin-3-cleaving activity was common among clinical isolates of *S. aureus*. In an *in vivo* murine model of *S. aureus* skin infection, severe tissue damage was associated with SspB expression, but only in galectin-3-expressing mice, suggesting that proteolytic processing of galectin-3 may contribute to bacterial virulence.

RESULTS

Neutrophil activation induced by galectin-3 is abrogated by factors secreted by *S. aureus*. To investigate whether galectin-3 is a target for *S. aureus* proteases, we used galectin-3-induced activation of human neutrophils as a readout (22). Neutrophils responded to activation by galectin-3 with a significant level of production of reactive oxygen species (ROS), in the same range as for the well-known neutrophil agonist phorbol myristate acetate (PMA) (Fig. 1A, inset). Upon preincubation of the lectin with *S. aureus* strain 8325-4 supernatant for 48 h, the ability of galectin-3 to trigger ROS production in neutrophils was abolished (Fig. 1A). Immunoblotting of the supernatant–galectin-3 mixture with an anti-CRD antibody showed that the original 32-kDa galectin-3 had been fully degraded by the supernatant and that the protein was instead present as a 16-kDa fragment. Thus, the N-terminal domain had been proteolytically cleaved, and the fragment consisted solely of the CRD (Fig. 1B). This explains the

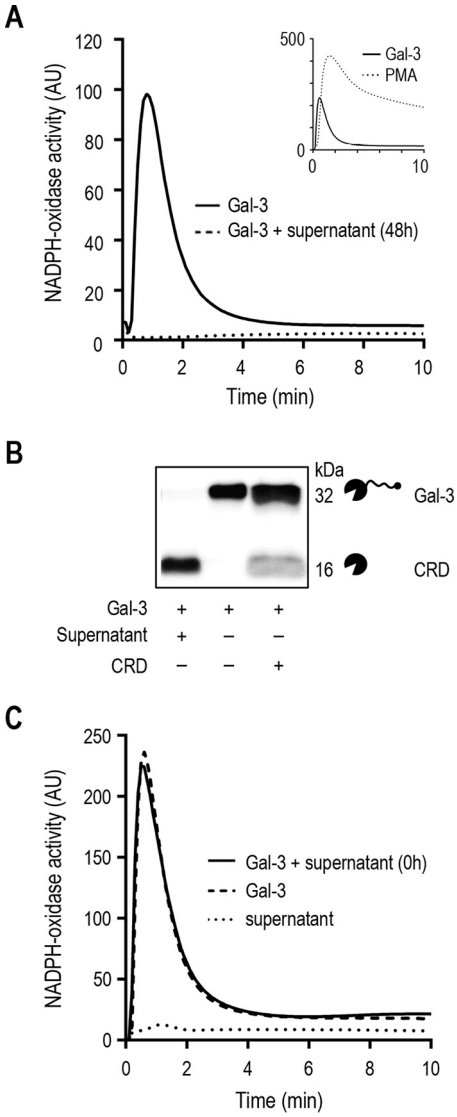

FIG 1 Galectin-3-induced ROS production in neutrophils is prevented by secreted factors from *S. aureus* strain 8325-4. (A) Galectin-3 (400 μg/ml) was incubated in the presence or absence of 2.5% *S. aureus* 8325-4 culture supernatant for 48 h. The preincubated galectin-3 was added to TNF-α-primed neutrophils at a 1:10 dilution. Production of ROS (*y* axis; AU, arbitrary units) was followed over time by isoluminol-amplified chemiluminescence. The results of a representative experiment out of six experiments are shown. The inset shows a comparison of the levels of ROS production upon stimulation with galectin-3 (40 μg/ml) and with PMA (50 nM), a well-defined and potent NADPH oxidase activator. (B) Galectin-3 incubated with or without culture supernatant (see above) was subjected to immunoblotting with an antibody directed toward the CRD. For reference, recombinant galectin-3 (32 kDa) and CRD (16 kDa) are shown. (C) Primed neutrophils were exposed to galectin-3 (40 μg/ml) in the presence or absence of culture supernatant without prior incubation or to culture supernatant only, and ROS production was measured as described above.

abrogation of the neutrophil-activating capacity of galectin-3 by the supernatant, as it is shown that the N-terminal domain is necessary for such activation (22).

To ensure that the culture supernatant did not interfere with the chemiluminescence (CL) detection system *per se*, supernatant was added together with galectin-3 to neutrophils in the CL mixture without preincubation. The supernatant had no effect on the galectin-3-induced ROS production (Fig. 1C). Also, preincubation of horseradish peroxidase (HRP), the included peroxidase, with culture supernatant did not inhibit the CL response (data not shown).

Galectin-3 cleavage by *S. aureus* 8325-4 supernatant is inhibited by staphostatin B. Specific protease inhibitors (Table 1) were employed to identify the galectin-

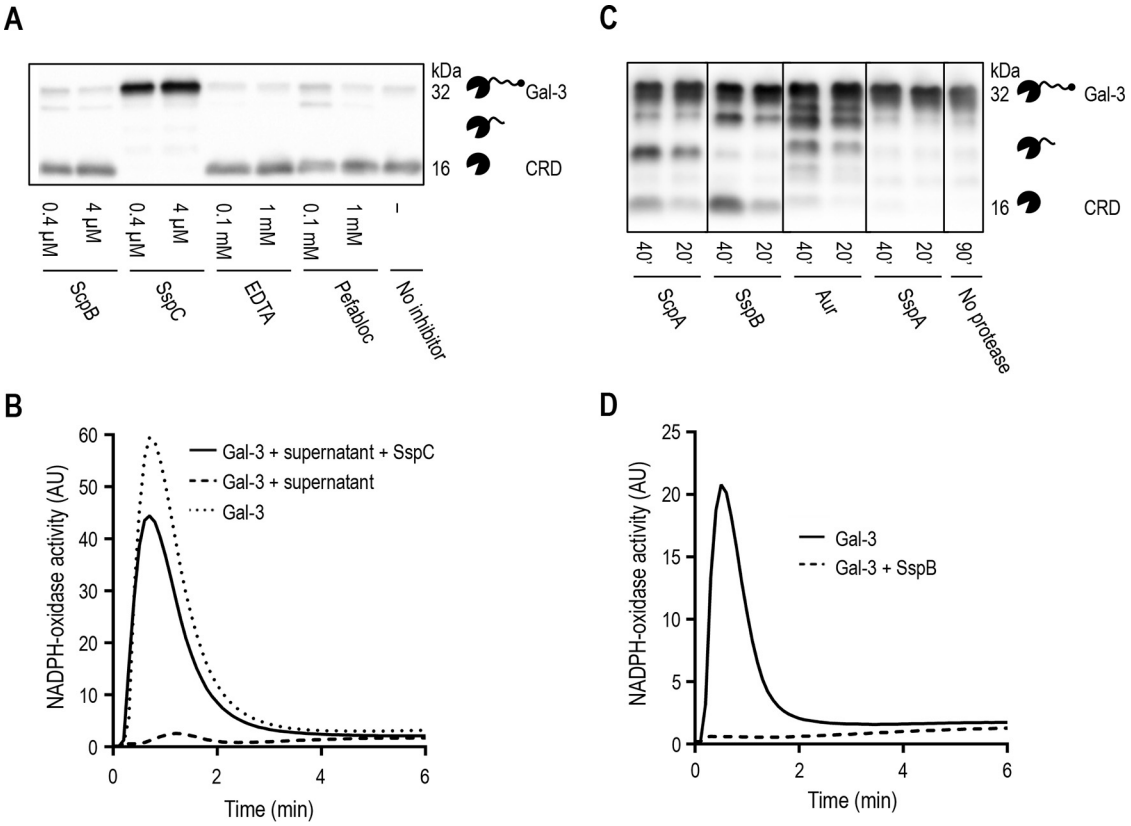

FIG 2 Specific *S. aureus* proteases are responsible for cleavage of galectin-3 into lower-mass fragments. (A) Culture supernatants from *S. aureus* strain 8325-4 were incubated with galectin-3 (100 μg/ml) in the presence or absence of protease inhibitor ScpB, SspC, EDTA, or Pefabloc at the given concentrations for 1 h at 37°C. The samples were then analyzed for content of galectin-3 and fragments thereof by immunoblotting using the anti-CRD antibody. (B) Culture supernatants (diluted 1:40) from strain 8325-4 were incubated with galectin-3 (400 μg/ml) in the presence or absence of SspC (8 μM). The mixture was added to TNF-α-primed neutrophils at a 1:10 dilution, and ROS production (*y* axis; AU, arbitrary units) was followed over time by isoluminol-amplified CL. (C) Galectin-3 (2 μM; 640 μg/ml) was incubated at 37°C with or without 0.2 μM isolated *S. aureus* protease ScpA, SspB, Aur, or SspA in KRG with 1% BSA for the indicated times. Samples were analyzed as described above. All samples were analyzed on the same blot that was then cut and reassembled with the lanes in another order, to increase legibility. (D) Galectin-3 (400 μg/ml) was preincubated with SspB (4 μM) for 24 h at 37°C. The proteolytic activity of SspB was then inactivated by the addition of SspC (8 μM; necessary due to cytotoxic effects of SspB at the given concentrations) before the mixture was added to neutrophils at a 1:10 dilution. ROS production was measured as described above.

3-cleaving agents in the culture supernatant. *S. aureus* 8325-4 is a commonly used laboratory strain that carries a deletion in the *rsbU* gene, which causes an upregulation of proteases (4). The cleavage of galectin-3 by strain 8325-4 supernatants was preserved in the presence of protease inhibitors with one exception: when SspB was inhibited by SspC, no apparent cleavage could be distinguished, indicating that SspB has galectin-3-cleaving properties (Fig. 2A). This was supported by the corresponding experiment in the neutrophil activation system, i.e., the incubation of galectin-3 and culture supernatant in the presence or absence of SspC; the protease inhibitor totally rescued the galectin-3-induced response (Fig. 2B).

Staphopain A, staphopain B, and aureolysin from *S. aureus* cleave galectin-3 into specific fragment patterns. The stand-alone ability of the four proteases to cleave galectin-3 was tested using SspA, SspB, ScpA, and Aur isolated from *S. aureus*. The galectin-3-only sample showed minor but visible fragmentation formed already during the preparation of the lectin. In the presence of ScpA or SspB, additional midsize and CRD-only fragments of galectin-3 were produced (Fig. 2C). Aur cleaved galectin-3 into mostly higher-molecular-mass fragments, suggesting that it primarily digested the more distant parts of the N-terminal collagen-like domain. Thus, ScpA and Aur, aside from SspB, apparently have galectin-3-processing capacity, despite the fact that their

TABLE 2 Protease expression of *S. aureus* strain 8325-4 and mutant strains

S. aureus strain	Protease expression[a]			
	Aur	SspA	SspB	ScpA
8325-4	++	++	++	++
Δ*aur* mutant[b]	−	+	+	++
Δ*sspA* mutant	++	−	−	++
Δ*sspB* mutant	++	++	−	++
Δ*scpA* mutant	++	++	++	−

[a]Activity or expression of proteases is denoted as active (++), moderately active (+), or inactive/lacking (−).
[b]The Δ*aur* mutant has reduced SspA and SspB activities due to incomplete processing of SspA in the absence of Aur (8).

corresponding inhibitors did not block galectin-3 cleavage in culture supernatants (Fig. 2A). SspA showed little or no degradation of galectin-3.

The ability of galectin-3 to activate neutrophils was totally abrogated after incubation of the lectin with purified SspB (Fig. 2D), in line with the effect seen for galectin-3 incubated with bacterial supernatant (Fig. 1A). For this experiment, galectin-3 was incubated with SspB for 24 h, since the galectin-3 incubated with SspB for 40 min still contained substantial amounts of full-length lectin (32 kDa) (Fig. 2B).

Galectin-3 is cleaved by *S. aureus* 8325-4 strains expressing staphopain B. We verified the above-described findings using culture supernatants of *S. aureus* 8325-4 and 8325-4 Δ*aur*, Δ*sspA*, Δ*sspB*, and Δ*scpA* mutant strains that each lacked one of the proteases (Table 2). The Δ*sspB* and Δ*sspA* strains lost all apparent galectin-3-cleaving activity, while the other mutants were still able to process the lectin (Fig. 3). SspB is downstream from SspA both in transcription and in activation (see Fig. S1 in the supplemental material) (4, 8). The other proteases are partly or fully autoactivated (4, 6–8), which supports the idea that SspB is the protease that cleaves galectin-3 in strain 8325-4. The Δ*aur* strain supernatant still had a reduced level of cleavage of galectin-3, explained by inefficient SspB activation, as full activity of its activator, SspA, requires processing by Aur (8).

In summary, although isolated ScpA, SspB, and Aur all have proteolytic specificity toward galectin-3, the above-described data identify SspB as the galectin-3-cleaving protease of *S. aureus* 8325-4 and, thereby, also the inhibitor of galectin-3-mediated ROS production.

Clinical isolates of *S. aureus* from skin infections and invasive infections show various levels of galectin-3 cleavage. In culture supernatants from clinical isolates of *S. aureus* from invasive infections and superficial skin infections, digestion of galectin-3 was seen in several samples from both groups, with various degrees of efficiency (Fig. 4A). This indicates that the galectin-3 processing may be part of a virulence-determining system *in vivo*. In fact, a closer analysis of the galectin-3 processing by

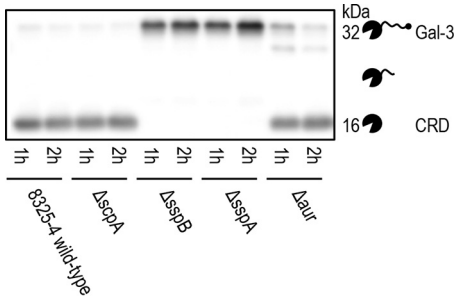

FIG 3 Galectin-3 is cleaved by *S. aureus* strains expressing SspB. Culture supernatants from strain 8325-4 and Δ*scpA*, Δ*sspB*, Δ*sspAB*, and Δ*aur* mutants were incubated with galectin-3 (100 μg/ml) for 1 or 2 h, and the content of galectin-3 and fragments thereof in each was analyzed by immunoblotting with anti-CRD antibody.

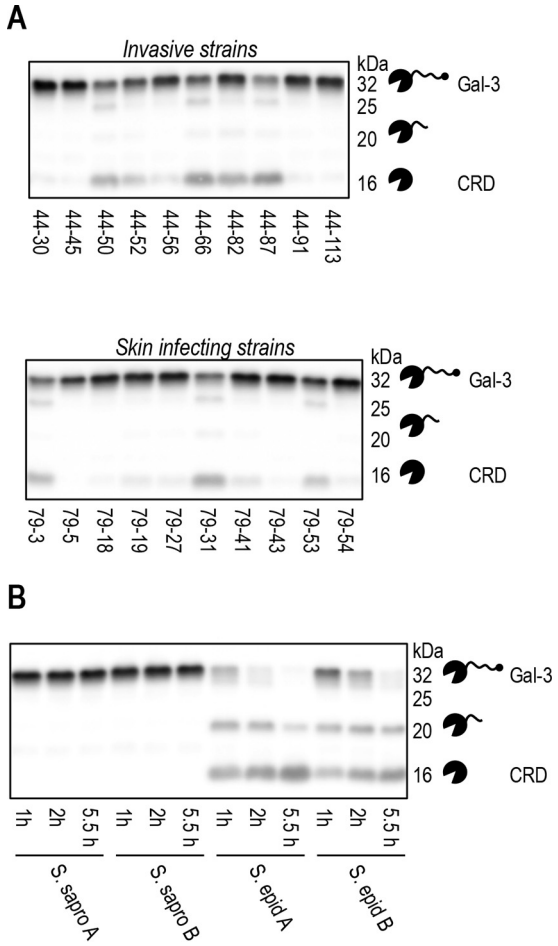

FIG 4 Clinical isolates of *S. aureus* and *S. epidermidis* but not *S. saprophyticus* cleave galectin-3. Culture supernatants of 10 strains of *S. aureus* isolated from invasive infections and 10 strains from superficial skin infections (A) or of isolates of two strains each of *S. saprophyticus* (S. sapro A and S. sapro B) and *S. epidermidis* (S. epid A and S. epid B) (B) were incubated with galectin-3 (100 μg/ml) for 16 h (A) or 1, 2, and 5.5 h (B). Galectin-3 fragmentation was analyzed by immunoblotting using the anti-CRD antibody.

clinical *S. aureus* strains suggests that ScpA and SspB, and to some extent Aur, also may cleave galectin-3 when acting cooperatively (Fig. S3).

In the presence of culture supernatants of two coagulase-negative staphylococci, *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*, a clear distinction in galectin-3 cleavage capacities could be observed (Fig. 4B). *S. saprophyticus* showed no tendency to cleave galectin-3, while the more virulent *S. epidermidis* digested a large portion of the full-length galectin-3 into CRD and at least one other midsize fragment (Fig. 4B).

Galectin-3 aggravates skin lesions in an *in vivo* skin abscess model with *S. aureus* strain 8325-4. In order to investigate the influence of galectin-3 and *S. aureus* SspB expression *in vivo*, we used a murine infection model. As galectin-3 is highly prevalent in epithelium (19), we chose to study subcutaneous skin infection rather than invasive infection. In the chosen murine model of *S. aureus*-induced skin infection (32, 33), wild-type (Gal-3$^{+/+}$) and galectin-3 knockout (Gal-3$^{-/-}$) mice were challenged with the wild-type strain 8325-4 and the Δ*sspB* mutant, as illustrated in Fig. S2A.

Immunohistochemical analysis of skin sections collected on the third day of infection from Gal-3$^{+/+}$ mice was performed for healthy and infected skin regions. In healthy skin (Fig. 5A), galectin-3 staining was strongly positive in the adnexa or squamous epithelial cells. Dermal macrophages and stromal fibroblasts were positively stained, while adipocytes, myocytes, and vascular epithelium were negative for

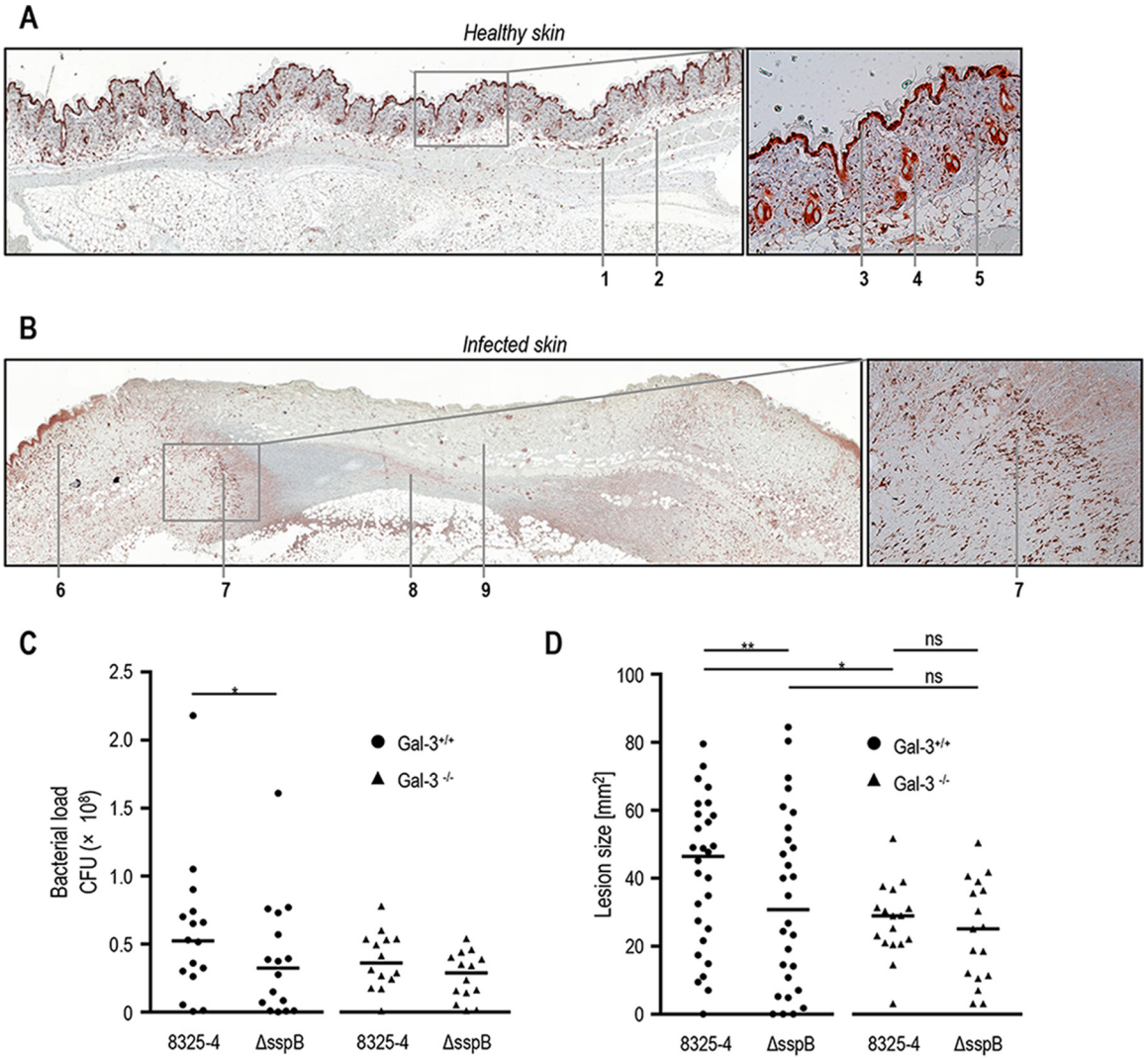

FIG 5 SspB enhances *S. aureus* virulence in a skin infection model in the presence of galectin-3. Gal-3$^{+/+}$ or Gal-3$^{-/-}$ mice were injected with 1×10^7 CFU of *S. aureus* 8325-4 or the Δ*sspB* mutant on either flank and observed for 3 days, after which they were sacrificed. (A and B) Skin biopsy specimens of healthy skin (A) and *S. aureus* 8325-4-infected lesions (B). Histological biopsy specimens were immunohistochemically analyzed for galectin-3 using an anti-galectin-3 antibody. (A) 1, muscle tissue; 2, fat tissue; 3, squamous epithelial cells; 4, adnexa epithelial cells; 5, dermal macrophages or dendritic cells. (B) 6, normal tissue; 7, infiltrating macrophages; 8, *S. aureus* cells; 9, necrotic tissue. (C) Bacterial loads in skin biopsy specimens collected at day 3 were determined by viable count. (D) Lesion sizes caused by strain 8325-4 and the Δ*sspB* mutant, respectively, were recorded, and the sizes at day 3 are shown. Horizontal bars designate median values. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test to compare the two strains inoculated on the same mouse and the Mann-Whitney test to compare the strains between the two groups of mice. Data from the two experiments are pooled. *, $P < 0.05$; **, $P < 0.01$; ns, no significant difference.

galectin-3 staining. The patterns are consistent with previous studies of galectin-3 in human epidermal tissue (19).

In the infected skin (Fig. 5B), galectin-3 staining was lost within the necrotic region. A strong intensity of galectin-3 staining was instead seen lining the outer region of the lesion, accompanying the presence of inflammatory cells, as well as colocalizing with the bacteria in the central part of the lesion. Similar patterns of galectin-3 staining were seen when strain 8325-4 was exchanged for the Δ*sspB* mutant, although the lesions were significantly smaller (see below). Furthermore, staining for CRD (detecting both full-length galectin-3 and CRD) gave the same patterns as for full-length galectin-3 (data not shown).

The weight loss of the mice was minimal, as expected for such a mild and localized infection; it did not differ between the Gal-3$^{+/+}$ and Gal$^{-/-}$ mice (Fig. S2B). The bacterial-load assessment at day 3 showed significantly higher CFU counts for strain

8325-4 than for the Δ*sspB* mutant (Fig. 5C). The difference, however, was significant only among the Gal-3$^{+/+}$ mice. In Gal-3$^{+/+}$ mice, the lesion sizes were significantly larger than the lesions in the mice lacking galectin-3 (Fig. 5D). Furthermore, the lesions caused by strain 8325-4 were clearly larger than those induced by the Δ*sspB* strain, but again, the difference was only significant in the presence of galectin-3 (Fig. 5D). Altogether, the data suggest that SspB is in fact a virulence factor and that its function as such is dependent on the presence of galectin-3.

DISCUSSION

Galectin-3 is emerging as a regulator of inflammation that also has a direct impact on infection (11–13). We have previously shown that galectin-3 induces ROS production in tissue neutrophils (22), an effect that may contribute not only to bacterial killing but also to collateral tissue destruction, and there are numerous examples of galectin-3 regulating inflammatory processes and infections *in vivo* (14–18, 21, 29). The function of galectin-3 is dependent on its collagen-like N-terminal domain, and cleavage of this domain by human or pathogen proteases may attenuate galectin-3 function and thereby influence inflammatory regulation and bacterial virulence (25–28, 30, 31). This study was undertaken to investigate whether *S. aureus* proteases suggested to be associated with virulence can modulate the function of galectin-3 and, if so, to characterize any role that this phenomenon plays in *S. aureus* pathogenicity.

The four major proteases of *S. aureus* are well preserved among clinical strains (34) and are emerging as determinants of *S. aureus* virulence (5, 9, 35). The proteases have described targets, but there is no consensus in the literature as to their biological importance. There are, however, some clues as to their function; in line with cleaving extracellular matrix and clot-forming proteins, they facilitate tissue destruction and modulate secreted and surface-associated virulence factors (36, 37). These activities promote detachment from colonized tissues, dissipation of biofilms, and hiding from detection by immune cells (5, 37, 38). Many immunomodulating mechanisms of the proteases have been identified (3), including effects on phagocyte chemotaxis and phagocytosis of *S. aureus* (37, 39–42). Furthermore, the proteases have been shown to inactivate human protease inhibitors (e.g., α_1-antitrypsin), to cleave phagocyte "do-not-eat-me" surface receptors (CD31), to inhibit complement activation (9), and to induce phagocyte cell death (42, 43). We now add the processing of galectin-3 as a possible staphylococcal immunomodulating function.

In our first experiments, we used the laboratory strain 8325-4, which carries a deletion in the control gene *rsbU* upstream from *scp*, *ssp*, and *aur*, to ensure high expression of all four proteases (4, 44). In bacterial supernatants, the main galectin-3-cleaving protease was SspB, shown by using specific protease inhibitors and purified proteases, as well as mutant *S. aureus* strains. The ScpA and Aur proteases also had galectin-3-processing capacity in isolation, but this activity appeared minor within culture supernatants compared to that of SspB, as neither mutants nor inhibitors had any obvious impact on the cleavage.

The proteolytic patterns of galectin-3 produced by the clinical isolates indicate that the *in vivo* situation is more complex. The degree of cleavage varied between strains, and it appears that more than one protease may participate in galectin-3 processing *in vivo*, as discussed briefly in the legend to Fig. S3 in the supplemental material. Whether this influences the outcome *in vivo* can presently only be speculated upon. Our data indicate that the invasive strains, when grown *in vitro*, are more likely to cleave galectin-3 than are noninvasive strains. The clinical strains also appear to produce additional, larger fragments apart from the 16-kDa CRD fragment. Whether these intermediate fragments have biological function will be investigated in future studies.

We also investigated the coagulase-negative staphylococci *S. epidermidis* and *S. saprophyticus* for galectin-3-degrading capacity. *S. epidermidis*, expressing proteases homologous to *S. aureus* Aur, SspA, and ScpA (5, 45), exhibited galectin-3-cleaving capacity that could be inhibited by ScpB (Fig. S3), indicating a close relationship with ScpA. Thus, the ability to cleave galectin-3 appears to be a preserved function in

adherent staphylococcal species; both *S. epidermidis* and *S. aureus* form biofilms on medical devices (2, 5), and it may be hypothesized that galectin-3 processing is associated not only with superficial skin infection but also with graft-related infection. *S. saprophyticus*, commonly causing urinary tract infection, lacked galectin-3-processing capacity even though galectin-3 is present throughout the urinary tract (46), in line with the lack of any known exoprotease expression in this species (47).

The importance of *S. aureus* proteases in promoting infection *in vivo* is supported by the fact that Δ*sspB* or Δ*sspA* Δ*sspB* mutants show reduced virulence, while augmented SspB expression results in increased virulence in murine models of systemic or local infection (4, 48, 49). Our results showing that an Δ*sspB* strain displays attenuated virulence during skin infection are in total accordance with this; the lesions were smaller and the bacterial counts lower for the SspB-lacking strain than for the SspB-expressing 8325-4. Interestingly, the difference in virulence between SspB-expressing and SspB-deficient *S. aureus* strains was dependent on the presence of galectin-3 in the host; in the Gal-$3^{+/+}$ mice, the protease-expressing bacteria generated larger lesions and higher bacterial burdens than the Δ*sspB* bacteria, while both strains caused similar, and minor, tissue destruction in the Gal-$3^{-/-}$ mice. Thus, galectin-3 does not appear to increase the inflammatory response to infection *per se*, since the lesion size was independent of the presence of galectin-3 when SspB was not present. Hence, galectin-3 expression has to be combined with protease activity to result in the observed increase in lesion size/inflammation.

The contribution of galectin-3/CRD to the bacterially induced tissue damage in the skin infection model probably involves several different mechanisms, on which we can only speculate at this point. The epithelial damage by the bacteria *per se*, enhanced by protease expression, induces an augmented inflammatory activation and recruitment of neutrophils, the most important cell type in fighting *S. aureus* skin infection (3). Neutrophil migration into infected tissue has previously been shown to be affected by galectin-3 (16, 50), which is produced in increased amounts during infectious/inflammatory processes (14, 16). Neutrophil activation by galectin-3, enhanced after extravasation of the cells into tissue, may contribute to tissue destruction by the production of toxic oxygen radicals and cytokines and the release of neutrophil proteases (15, 22, 25). Another mechanism could involve the cleavage of galectin-3 by SspB (as shown here) and by neutrophil-released metalloproteases and serine proteases (25, 27, 28), impairing the production of immunomodulatory ROS, as well as galectin-3-dependent opsonophagocytic processes, possibly resulting in impaired infection control and aggravated tissue destruction (14, 15, 24). Furthermore, protease-cleaved galectin-3 (CRD) may possibly have proinflammatory effects *per se*, contributing to inflammatory tissue damage, as seen in other disease models (51–54). These suggested mechanisms can appear to work in opposite directions, but opposing functions are well-known phenomena in the galectin field. For example, systemic *Neisseria* infection is dampened in Gal-3 knockout mice, while streptococcal pneumonia is instead significantly enhanced (14, 15). Furthermore, galectin-3 may have several different functions in one and the same model (15), and it is not always clear how the combination of different mechanisms ends up giving the result achieved (11).

Whether SspB-processed galectin-3, i.e., CRD, is present in the skin of the infected mice cannot be stated with certainty. When probing healthy skin with the antibody that detects full-length galectin-3, intense staining was seen in the epithelium, the initial point of attack for the bacteria. Galectin-3 was also present at the lesion site, both around the infecting *S. aureus* bacteria and in the surrounding tissue lining the necrotic area, meaning that galectin-3 is available for interaction with secreted bacterial products during all phases of infection. To determine the presence of processed galectin-3 (CRD), a CRD-recognizing antibody was used, detecting both full-length galectin-3 and processed CRD. This staining was largely similar to that of galectin-3 only (by the N terminus-directed antibody M3/38). Since the antibodies cannot be compared with regard to staining intensity, the conclusion that all staining is due to galectin-3 should not be drawn, but rather, it should be concluded that all processed galectin-3 (CRD), if

present in the tissue, colocalizes with the full-length galectin-3. The relative levels of full-length galectin-3 and CRD, however, cannot be determined.

In conclusion, our data imply that galectin-3 processing is an active feature of *S. aureus* virulence. The *S. aureus* protease SspB can cleave the immunomodulatory protein galectin-3, thereby not only causing inactivation of galectin-3-dependent biological functions but also resulting in the appearance of a novel protein, the CRD, which so far has no determined biological effects and is an exciting subject for future studies.

MATERIALS AND METHODS

Chemicals and reagents. Isolated proteases (SspA, SspB, ScpA, and Aur) and recombinant inhibitors (SspC and ScpB) were from Preparitis (Krakow, Poland). Pefabloc SC was from Boehringer Mannheim GMBH (Mannheim, Germany). Tumor necrosis factor alpha (TNF-α), EDTA, sodium dodecyl sulfate (SDS), glycerol, and isoluminol were from Sigma-Aldrich (St. Louis, MO, USA). Bromophenol blue was from LKB-Produkter AB (Stockholm, Sweden). Formaldehyde, paraffin, eosin, and hematoxylin were from Histolab Products AB (Gothenburg, Sweden), and H_2O_2 from Acros Organics (Geel, Belgium). Bovine serum albumin (BSA) was from Roche Diagnostic (Mannheim, Germany), Ficoll-Paque was from Fisher Scientific GTF AB (Gothenburg, Sweden), and dextran from Pharmacosmos (Holbaek, Denmark). Affinity-purified polyclonal chicken anti-CRD antibody was produced by Capra Science (Ängelholm, Sweden); the antibody was produced in chickens because of the low immunogenicity between mammal CRDs (10). Horseradish peroxidase (HRP)-conjugated donkey anti-chicken antibody was from Abcam (Cambridge, United Kingdom), while HRP-conjugated rabbit anti-rat antibody was from Dako (Stockholm, Sweden). Mini-Protean TGX polyacrylamide precast gels and the Clarity Western ECL kit were from Bio-Rad Laboratories (Sundbyberg, Sweden).

Bacterial strains and growth conditions. The *S. aureus* laboratory strains that were used in this study were strain 8325-4 and Δ*scpA*, Δ*sspA*, Δ*sspB*, and Δ*aur* mutants thereof (Table 2) (4). Two clinical isolates each of *S. saprophyticus* and *S. epidermidis* were kindly provided by the Department of Clinical Bacteriology, Sahlgrenska University Hospital, Gothenburg, Sweden. Ten clinical isolates each of *S. aureus* from invasive infections and superficial skin infections were a kind gift from Gunnar Jacobsson (32, 55), Department of Infectious Diseases, Skaraborg Hospital, Skövde, Sweden. All strains were kept in 15% glycerol at −80°C for long-term storage.

Bacteria grown overnight in 1.5 ml tryptic soy broth (TSB) were diluted 1:1,000 in 15 ml fresh TSB and incubated overnight. To obtain bacterium-free supernatants, the cultures were centrifuged at 4,000 × *g* for 10 min at 4°C and supernatants were filtered through 0.2-μm double sterile filters (Acrodisc syringe filter; VWR, Sweden), aliquoted, and stored at −80°C.

To prepare bacteria for the murine skin infection model, *S. aureus* 8325-4 and 8325-4 Δ*sspB* overnight cultures were diluted 1:100 in fresh TSB and incubated for another 16 h. The bacteria were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS; pH 7.2) with 5% BSA and 10% dimethyl sulfoxide (DMSO), and stored at −80°C. The concentration of bacteria was determined by viable counts.

Production of recombinant galectin-3 and CRD. Recombinant galectin-3 and CRD were produced in *Escherichia coli* and purified as previously described (22, 26).

Isolation of neutrophils. Neutrophils from healthy blood donors were separated from buffy coats, obtained from the Sahlgrenska University Hospital Blood Centre, Gothenburg, as described by Bøyum et al. (56), and diluted to a concentration of 1×10^7 cells/ml in Krebs-Ringer phosphate buffer (KRG; pH 7.3) with 1 mM Ca^{2+}.

Neutrophil production of ROS. Neutrophil NADPH oxidase-derived reactive oxygen species (ROS) were measured using an isoluminol-amplified chemiluminescence system as previously described (57). Neutrophils (5×10^6 cells/ml) primed with TNF-α (10 ng/ml at 37°C for 20 min) in KRG with 1 mM Ca^{2+} were stimulated with galectin-3, and ROS release was recorded over time; the results are given in arbitrary units (AU).

Proteolytic digestion of galectin-3. Galectin-3 was incubated for different times with various dilutions of isolated *S. aureus* proteases (SspA, SspB, ScpA, and Aur) or culture supernatants, as stated in each figure legend. Where indicated, culture supernatants were premixed with protease inhibitors (Pefabloc, SspC, ScpB, or EDTA) (5, 58) prior to the addition of galectin-3. For experiments where the readout was activation of neutrophils, the lectin was incubated for a longer time (48 h) with diluted (0.25%) supernatant in order to avoid cytotoxic effects of undiluted supernatants (data not shown).

SDS-PAGE and Western blotting. Galectin-3 and fragments thereof were analyzed by SDS-PAGE and immunoblotting, using a chicken anti-human polyclonal CRD antibody (2.2 μg/ml) and a donkey anti-chicken IgG HRP-labeled secondary antibody (described in detail in the supplemental material).

***In vivo* skin abscess model.** Galectin-3 knockout mice on the 129/Sv background (59) were backcrossed for four generations with C57BL/6 mice to generate 97.75% C57BL/6 mice (60). Wild-type (Gal-$3^{+/+}$) and galectin-3 deficient (Gal-$3^{-/-}$) mice were bred as heterozygotes, controlled by continuous genotyping (60). To obtain animals for the present study, homozygote animals were bred for three generations. The animals were maintained in the animal facility at the Department of Rheumatology and Inflammation Research, University of Gothenburg. The study was approved by the Gothenburg Ethical Committee for Animal Research.

A skin infection model modified from previous studies (32, 33) was performed in Gal-$3^{+/+}$ and Gal-$3^{-/-}$ mice in parallel, using *S. aureus* strains 8325-4 and 8325-4 Δ*sspB* from the premade batches.

Mice at the age of 9 to 15 weeks and of both genders were used for experiments. Age and sex were matched between wild-type and knockout animals.

The bacteria were washed in PBS, and the concentration was adjusted to 2×10^8 CFU/ml. The backs and both flanks of the mice were shaved, and 50-μl amounts of strains 8325-4 and 8325-4 Δ*sspB* were injected subcutaneously on either flank on the same mouse (see Fig. S2A in the supplemental material). Two days after injection, the mice were weighed, and the areas of the lesions formed were measured. In the first experiment, nearly half of the mice from each group were sacrificed at day 3. Skin biopsy specimens of the infected areas were collected and homogenized, and the bacterial loads were assessed by viable count. The remaining mice were studied for another 7 days to record the recovery after infection. No differences in lesion sizes or weight changes were detected between the two mouse strains during healing up until day 10 (data not shown). In the second experiment, all mice were sacrificed at day 3. Biopsy specimens from two Gal-$3^{+/+}$ mice were used for histological analysis (described in detail in the supplemental material), while the remaining biopsy specimens were analyzed for bacterial loads.

Statistical analysis. Differences in lesion sizes and bacterial loads were analyzed using the Wilcoxon matched-pairs signed-rank test for comparisons of paired samples and the Mann-Whitney test for comparisons between groups. Statistical analyses were performed in Prism software (version 6.05; GraphPad, La Jolla, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00177-17.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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We confirm that we have no conflicts of interest.

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