A Competitive Mechanism for Staphylococcal Toxin SSL7 Inhibiting the Leukocyte IgA Receptor, FcαRI, Is Revealed by SSL7 Binding at the Ca2/Cα3 Interface of IgA*

Leukocyte recruitment and effector functions like phagocytosis and respiratory burst are key elements of immunity to infection. Pathogen survival is dependent upon the ability to overwhelm, evade or inhibit the immune system. Pathogenic group A and group B streptococci are well known to produce virulence factors that block the binding of IgA to the leukocyte IgA receptor, FcαRI, thereby inhibiting IgA-mediated immunity. Recently we found *Staphylococcus aureus* also interferes with IgA-mediated effector functions as the putative virulence factor SSL7 also binds IgA and blocks binding to FcαRI. Herein we report that SSL7 and FcαRI bind many of the same key residues in the Fc region of human IgA. Residues Leu-257 and Leu-258 in domain Cα3 of IgA lie at the Ca2/Cα3 interface and make major contributions to the binding of both the leukocyte receptor FcαRI and SSL7. It is remarkable this *S. aureus* IgA binding factor and unrelated factors from streptococci are functionally convergent, all targeting a number of the same residues in the IgA Fc, which comprise the binding site for the leukocyte IgA receptor, FcαRI.

*Staphylococcus aureus*, a commensal organism of the human skin and nose (1), is also a significant human pathogen responsible for conditions such as Scarlet fever, toxin shock, septicemia, and endocarditis. The *S. aureus* genome contains three clusters of superantigen and superantigen-like genes, designated SaP1n1–2 and -3 (2). SaP2n2 contains the Staphylococcus superantigen-like (ssl) genes, previously designated as staphylococcal enterotoxin-like (SET) genes (3). These genes are highly represented in clinical isolates of *S. aureus* and are inferred to contribute to pathogenicity of these strains (4). Crystallographic studies of SSL5 (SET3) (5) and SSL7 (SET1) (6, 7) proteins have indicated structural similarity to classical superantigens with the SSLs also comprising an (SET3) (5) and SSL7 (SET1) (6, 7) proteins have indicated structural similarity to classical superantigens with the SSLs also comprising an

* MATERIALS AND METHODS*

**Biotinylated SSL7—** Recombinant SSL7 was prepared as described previously (8). Purified SSL7 150 μg/ml (7.5 μM) was reacted in phosphate-buffered saline with 1 mg/ml of EZ-Link sulfo-NHS-LC-biotin at 25 °C, 1 h, and then the remaining biotinylation reagent was reacted by the addition of 100 μl of 0.5 M ethanolamine pH 8.5. Prior to use biotinylated SSL7 was dialyzed extensively against phosphate-buffered saline.

**Transferrin Receptor (TfR)-IgA Fc Construct—** The expression of the transferrin receptor-IgA Fc fusion protein was based on the approach of Stabila et al. (17). In brief, DNA encoding the N terminus and transmembrane region of the human TfR was amplified from cDNA prepared from K562 cells (ATCC, Manassas, VA) using a cDNA synthesis kit (Pharmacia, Melbourne, Australia) and RNAzol (Invitrogen). PCR used the primers oBW07 5′-GGGGAATTCACCACCATGATGGATCAAGCTCAGTACAGCC-3′ and oBW208 5′-CCGGGCCCCTCAGTTTCTGGTTTCTACCC-3′ and the thermostable proofreading polymerase *Pwo* (Roche Diagnostics), and this PCR product was digested with EcoRI and Apal (New England Biolabs, Beverly, MA). A fragment encoding the IgA1 Fc was obtained by NotI and Apal digest of pBAR233. The baculovirus expression vector pBAR233 was a derivative of pFastBac (Invitrogen) containing a FcyRIIa leader sequence, a hexahistidine tag sequence, and a human IgA1-Fc sequence derived by PCR amplification from cDNA prepared from the IgA+ cell line Dakiki (18) using the primers oBW189 5′-GCGGCCCTCAACTCCACCTAC-3′ and oBW190 5′-CTAGTGACGGTGGCGTCCAC-3′. The sequence of this IgA1 Fc cDNA fragment differs from reference sequences (accession: NG_001019, six single nucleotide polymorphisms, NG_001019, six single nucleotide polymorphisms) resulting in two amino acid differences, L271M and R392H (standard IgA1 Bur numbering), consistent with the Dakiki IgA1 being a putative allelic variant. The EcoRI/Apal

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The TIR fragment and the Apal/NotI IgA Fc fragment were simultaneously ligated into the EcoRI and NotI restriction sites of the gateway vector pENTR1A (Invitrogen) yielding pBAR355. The LR clonase reaction was used with gateway reading frame-A cassette adapted pCR3 (Invitrogen) to create the construct pBAR357 expressing the N-terminal transmembrane region of the transferrin receptor fused, at the hinge region, to the IgA Fc. The IgA1-Fc mutants were constructed by PCR of pBAR357 using Turbo Pfu (Stratagene) followed by phosphorylation and ligation of the linear PCR product as previously described (19). The LL257,258MI mutation reaction used the primer pair oBW308 GTT-TCAGAAAGCAACCTTACG and oBW269 GATCATCAGGTCC-CTCGAGGGCCCG, the A442R mutation used the primer pair oBW266 ACACAGAAGACCATCGACCG and oBW202 GAAGGCAGGCG-GAGGGCCCTCG, and the PLAF(440–443)HNHY mutation used oBW-270 GCCCTGCAACAACACTACACACAGAGACCACATCGACCCG and oBW271 CTCGTGCCCCACCATGCAG, and the N263T mutation used the primer pair oBW268 GGTTCAGAAGCGACCCTCAC and oBW309 TAAGAGCAGGTCTCTCGAGTGCGG.

FcαRI-Fcγ2b Construct—The Fc region of mouse IgG2b was amplified from cDNA prepared from Balb/c splenocytes using the primers oBW137 GGATCCGAGCCACGCGGGCCATTTTC and oBW172 GCCCGGTCTATTTAACCGGAGACCGGGA as described above. Splice overlap PCR was used to add the sequence GGGCCGCTCTGCA-GAACTGTTCCTGGTGGATCC onto the 3'-end of the cDNA encoding the FcαRI ectodomain (pBAR152) (20) immediately following the codon for Ile-208 (i.e. corresponding amino acid sequence I208-GPAEL1PRGS, thrombin cleavage site in italics). These two DNAs were ligated at their BamHI sites, and the chimeric DNA was subcloned into the Stul site of pFastBac (Life Tech) making pBAR213. The chimeric DNA was then further subcloned into the EcoRI/XbaI sites of pcDNA3 (Invitrogen). CHO-K1 cells were transfected with this construct, pBAR225, using Lipofectamine 2000 (Invitrogen) and resistant colonies selected with 1 mg/ml G418. The cell line 225CHO expressing the WT and mutant IgA fusion proteins was determined to be pH10.5 1 h on ice with 0.2 µg (10 pmol) of biotinylated SSL7. Unbound SSL7 was removed, and the cells were incubated in 1/400 dilution of 0.5 mg/ml phycocerythrin-conjugated streptavidin (Pharmingen) for 1 h on ice. FcαRI binding activity of the mutants of IgA Fc was measured by incubating cells (105)3 with 225CHO cell supernatant containing FcαRI-Fcγ2b fusion protein (~0.5 µg, 5 pmol). Unbound receptor was removed, and the cells were incubated in a 1/200 dilution of FITC-conjugated sheep Fab’2 anti-mouse Ig (Silenus/Chemicon) for 1 h on ice.

RESULTS

Recombinant SSL7 Binding to IgA—S. aureus colonizes mucosal sites and this prompted us to characterize the interaction of SSL7 with IgA, the major subclass of IgA at the mucosa. Here we report rSSL7 binding to a human chimeric IgA2 anti-NP antibody. The antibody was reacted with a NP-derivatized biosensor surface with ~700 response units of antibody binding and a variation between cycles of ±10 response units (Fig. 1A). The binding to this immobilized IgA2 of SSL7 at different concentrations was recorded (Fig. 1B) after 3000 s when equilibrium was being approached. Plotting the equilibrium binding responses fitted best to a single binding site with an apparent affinity of 5.0 ± 1.5 nM (n = 5, Fig. 1C, one representative experiment KD = 6.8 ± 0.8 nM).

SSL7 Inhibits FcαRI-Fcγ2b Binding to IgA Fc—The Fc region of human IgA1 derived from Dakiki cells was fused to the N-terminal region of the transferrin receptor, a type II integral membrane protein. FACS analyses of transfections of CHOP cells with the WT and with the A422R IgA Fc transferrin receptor fusion constructs is shown in Fig. 2. The surface expression of the WT and mutant IgA fusion proteins was determined...
The expression of WT and A442R mutant TfR-IgA Fc fusion proteins were determined by FACS analysis of transiently transfected CHO cells. The nonspecific staining of mock-transfected cells is shown in the solid gray histograms. A, surface expression of WT (bold solid line) and A442R (thin solid line) proteins was determined using FITC-labeled anti-human IgA polyclonal antiserum. B, the SSL7 binding activities of the WT (bold solid line) and A442R (thin solid line) TfR-IgA Fc proteins were determined using biotin-labeled rSSL7 and streptavidin-labeled PE. C, the FcRI binding activities of the WT (bold solid line) and A442R (thin solid line) IgA proteins was determined using FcRI-Ig fusion protein and FITC-labeled anti-mouse Ig. D, FcRI binding to TfR-IgA Fc is inhibited by rSSL7. WT TfR-IgA Fc fusion protein was expressed in CHO cells and incubated for 10 min in the absence or presence of 2 μg (40 μg/ml) rSSL7. Cells were then incubated with FcRI-Ig and FcRI-Ig binding was detected with sheep FITC-labeled polyclonal IgG anti-mouse IgG.

Expression of Fusion Proteins of Transferrin Receptor with Mutant IgA Fc Regions—Because SSL7 binds to both IgA1 and IgA2 with nanomolar affinity and SSL7 inhibits FcRI binding to IgA, SSL7 may bind directly to the FcRI binding site, which is conserved in IgA1 and IgA2.

Molecular analysis of the SSL7 binding site was performed by mutating residues at the Ca2/Ca3 interface of IgA Fc comprising the FcRI binding site. The MFIs from FACS profiles showed the level of expression of the WT IgA fusion protein varied between experiments from a MFI = 38.6 ± 2) was inhibited 90% in the presence of 2 μg/ml (40 μg/ml) rSSL7 (MFI = 8 ± 2, background staining MFI = 5 ± 1, Fig. 2D). Hence in this experimental system FcRI-Ig binding to the TfR-IgA Fc fusion protein is inhibited by rSSL7 just as rSSL7 inhibits serum IgA binding to isolated leukocytes (8).

Identification of the SSL7 Binding Site in the IgA Fc Region—The SSL7 and FcRI binding activities of the various TR-IgA Fc proteins expressed on the surface of CHO transfectants were evaluated. The binding of biotinylated rSSL7 to WT IgA fusion protein was readily detectable (Fig. 4B, MFI = 350 ± 40, n = 4). In contrast, the level of binding of SSL7 to the A442R mutant IgA Fc fusion protein (MFI = 4.5 ± 0.2) was indistinguishable from the binding to the mock-transfected transiently expressing WT and mutant fusion proteins as indicated. WT expressing cells were first reacted (1 h, on ice) either in the presence or absence of 5 μg (100 μg/ml) rSSL7 and then stained with FITC-labeled anti-IgA. The MFIs for the WT and A442R proteins alone were derived from the FACS profiles shown in Fig. 2; the other FACS profiles are not shown.

FIGURE 2. FACS of anti-IgA, rSSL7, and FcRI-Ig binding to WT and A442R mutant TfR-IgA Fc fusion proteins. The expression of WT and A442R mutant TfR-IgA Fc fusion proteins and their functional activities were determined by FACS analysis of transiently transfected CHO cells. The nonspecific staining of mock-transfected cells is shown in the solid gray histograms. A, surface expression of WT (bold solid line) and A442R (thin solid line) proteins was determined using FITC-labeled anti-human IgA polyclonal antiserum. B, the SSL7 binding activities of the WT (bold solid line) and A442R (thin solid line) TfR-IgA Fc proteins were determined using biotin-labeled rSSL7 and streptavidin-labeled PE. C, the FcRI binding activities of the WT (bold solid line) and A442R (thin solid line) IgA proteins was determined using FcRI-Ig fusion protein and FITC-labeled anti-mouse Ig. D, FcRI binding to TfR-IgA Fc is inhibited by rSSL7. WT TfR-IgA Fc fusion protein was expressed in CHO cells and incubated for 10 min in the absence or presence of 2 μg (40 μg/ml) rSSL7. Cells were then incubated with FcRI-Ig and FcRI-Ig binding was detected with sheep FITC-labeled polyclonal IgG anti-mouse IgG.

FIGURE 3. The A442R mutant IgA Fc has lower apparent binding to anti-IgA, whereas the PLAF440-443NHHY mutant is equivalent to WT. CHO cells were transfected for transient expression of WT and mutant fusion proteins as indicated. WT expressing cells were first reacted (1 h, on ice) either in the presence or absence of 5 μg (100 μg/ml) rSSL7 and then stained with FITC-labeled anti-IgA. The MFIs for the WT and A442R proteins alone were derived from the FACS profiles shown in Fig. 2; the other FACS profiles are not shown.

FIGURE 4. The SSL7 and FcRI binding activities of the WT and Ca2/Ca3 mutant IgA proteins. WT, A442R, PLAF440-443NHHY, LL257,258MI, and N263T mutant IgA fusion proteins were expressed transiently and analyzed for apparent surface expression with FITC label anti-IgA (A), biotinylated rSSL7 binding (B), and FcRI-Ig binding (C).
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FIGURE 5. The Fc region of IgA depicting the FcγRI and SSL7 binding sites at the Ca2/Ca3 interface. The IgA Fc structure (Herr et al. (16) PDB accession 1OW0) was displayed as a transparent surface and α carbon trace. The residues mutated in this study, (Leu-257 and Leu-258 in Ca2 and residues 440–443, PLAF, in Ca3) are displayed as thick lines. These residues comprise the major contacts for FcγRI and are also essential to SSL7 binding. The carbohydrate linked to Asn-263 (N-263) which, although reaching the Ca2/Ca3 interface, does not interact with SSL7 or FcγRI, is also depicted in thick lines.

Discussion

SSL7, a putative staphylococcal virulence factor, binds serum and secretory IgA from multiple species and prevents human IgA interaction with the leukocyte IgA receptor, FcγRI (8). S. aureus infection of the bloodstream can lead to septicemia, and asymptomatic carriage of S. aureus can occur in the nose while life-threatening infections of the respiratory tract also occur (12). We previously had characterized SSL7 binding to serum IgA, of which the major form is IgA1 (8). The importance of S. aureus adaptation to mucosal environments prompted us to characterize the interaction of SSL7 with IgA2, the major mucosal subclass of IgA. Here we report that IgA2 also bound SSL7 with nanomolar affinity. This high affinity interaction marks SSL7 as a potential factor in the survival of S. aureus not only in the blood, but also at mucosal sites. This study has further shown that the SSL7 binding site and that of FcγRI utilize common residues in the Fc region of human IgA. Residues Leu-257 and Leu-258 in the AB loop/helix of domain Ca2 of IgA and residues 440–443 PLAF in the FG loop of domain Ca3 of IgA have been found in domain swap, mutagenesis (23–25), and x-ray crystallographic studies (16) to make major contributions to the binding of the leukocyte receptor FcγRI (Fig. 5). Mutation of these residues resulted in an expected decrease in soluble FcγRI-Ig binding consistent with the structural data (16) and, in addition, completely abrogated SSL7 binding indicating the binding sites of SSL7 and FcγRI comprise many of the same residues. In the crystal structure of IgA bound to FcγRI, the N-linked carbohydrate of IgA extends down the external face of the Ca2 domain to the Ca2/Ca3 interface (16) (Fig. 5) and thus may potentially be involved in interaction with molecules binding to this region. The role of the carbohydrate in SSL7 binding was examined using the N-linked glycosylation site point mutant N263T. The N263T IgA Fc mutant was expressed at levels equivalent to WT and bound similar levels of SSL7 indicating that the carbohydrate did not contribute to SSL7 binding. Thus despite the fact that other bacterial proteins containing an OB-fold domain have been shown to bind oligosaccharides (26), we conclude that this is not the case for the SSL7/FcγRI interaction. Interestingly the IgA Asn-263-linked glycan is close to (8 Å), but not contacting, FcγRI in the crystal complex (16), and in this study only a modest effect on the binding of FcγRI-Ig to the glycan deficient N263T mutant was observed.

Leukocyte recruitment, phagocytosis, and respiratory burst are critical aspects of protective immunity to Gram-positive bacteria. Interfering with FcγRI-mediated recognition of IgA opsonized bacteria by blocking the receptor binding site on IgA is a strategy utilized by pathogenic group A (27, 28) and group B streptococci (29, 30) to frustrate IgA-mediated protective immunity. Our report that SSL7 binds IgA with high affinity and inhibits interaction with FcγRI (8) clearly demonstrates that this evasion strategy is utilized by staphylococci as well as streptococci. It is notable that these two organisms also share superantigens, which are structurally similar to the SSL structure. It is striking these unrelated IgA binding factors from group A and group B streptococci (29) and from S. aureus are functionally convergent, all targeting the same residues at the Ca2/Ca3 interface of IgA (Fig. 5), the binding site for FcγRI. A second noteworthy parallel is that the different streptococcal proteins in addition to IgA binding also bind complement proteins, with the M proteins (Sir22/Arp4) also binding the classical complement regulator C4bp (and IgG) (31) and the β-protein also binding the complement regulator factor H (32). An unrelated S. aureus protein, extracellular fibrinogen binding protein (Efb) also inhibits the complement pathway, in this case by binding C3 (33). Taken together these observations, including the binding of C5 and the inhibition of complement subsequent to C5 by SSL7 (8), suggest strong selection of pathogenic group A and group B streptococci (27–30) and pathogenic S. aureus to evade both host complement and IgA/Fc receptor effector systems.

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