Analysis of a Novel Prophage-encoded Group A *Streptococcus* Extracellular Phospholipase A$_2^{*}$

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Group A *Streptococcus* (GAS), a Gram-positive bacterial pathogen that causes many types of infections, including pharyngitis and severe invasive diseases. We recently sequenced the genome of a serotype M3 strain and identified a prophage-encoded secreted phospholipase A$_2$ designated SlaA. To study SlaA structure-activity relationships, 20 site-specific mutants were constructed by alanine-replacement mutagenesis of amino acid residues previously described as crucial in the catalytic mechanism of secreted phospholipase A$_2$. Similarly, substitution of five residues in an inferred Ca$^{2+}$-binding loop and three residues in the inferred active site region resulted in loss of activity of 76.5% or greater relative to the wild-type enzyme. Analysis of enzyme substrate specificity confirmed SlaA as a phospholipase A$_2$, with activity against multiple phospholipid head groups and acyl chains located at the sn-2 position. PCR analysis of 1,189 GAS strains representing 48 M protein serotypes commonly causing human infections identified the slaA gene in 129 strains of nine serotypes (M1, M2, M3, M4, M6, M22, M28, M75, and st3757). Expression of SlaA by strains of these serotypes was confirmed by Western immunoblot. SlaA production increased rapidly and substantially on co-culture with Detroit 562 human pharyngeal epithelial cells. Together, these data provide new information about a novel extracellular enzyme that participates in GAS-human interactions.

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1 The abbreviations used are: GAS, Group A *Streptococcus*; PLA$_2$, phospholipase A$_2$; sPLA$_2$, secreted phospholipase A$_2$; MGAS, Musser Group A *Streptococcus* strain; SlaA, Group A streptococcal phospholipase A$_2$; rSlaA, recombinant SlaA; PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; FA, fatty acid; LPC, lysophosphatidylcholine; LPS, lysophosphatidylserine; PI3P, phosphatidylinositol 3-phosphate; PL, phospholipid.
| MGAS no. | emm type | Locality     | Year | Disease | slaA allele | SlaA expression | M. E. | L. E. | Stationary | Co-culture |
|----------|-----------|--------------|------|---------|-------------|-----------------|-------|-------|------------|------------|
| 1556     | 1         | Germany      | 1989 | Invasive | slaA1       |                 |       |       |            |            |
| 9461     | 1         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 7564     | 2         | Illinois     | 1998 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 7635     | 2         | Illinois     | 1998 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 8467     | 2         | Texas        | 1999 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9173     | 2         | Illinois     | 1998 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9421     | 2         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9422     | 2         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9465     | 2         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9652     | 2         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9728     | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9729     | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9762     | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 10062    | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 10270    | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 12507    | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 12508    | 2         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 159      | 3         | Utah         | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 259      | 3         | California   | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 274      | 3         | Colorado     | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 315      | 3         | Texas        | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 322      | 3         | Oregon       | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 1558     | 3         | Germany      | 1989 | Invasive | slaA1 |                 |       |       |            |            |
| 1559     | 3         | Germany      | 1989 | Invasive | slaA1 |                 |       |       |            |            |
| 1666     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1562     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1563     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1564     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1565     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1566     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1567     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1568     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1569     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1570     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1571     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1572     | 3         | Germany      | 1990 | Invasive | slaA1 |                 |       |       |            |            |
| 1610     | 3         | Minnesota    | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 1611     | 3         | Minnesota    | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 1612     | 3         | Minnesota    | 1987 | Invasive | slaA1 |                 |       |       |            |            |
| 1638     | 3         | Minnesota    | 1986 | Invasive | slaA1 |                 |       |       |            |            |
| 3297     | 3         | Minnesota    | 1995 | Invasive | slaA1 |                 |       |       |            |            |
| 3396     | 3         | Minnesota    | 1995 | Invasive | slaA1 |                 |       |       |            |            |
| 3398     | 3         | Ontario      | 1995 | Invasive | slaA1 |                 |       |       |            |            |
| 3384     | 3         | Ontario      | 1994 | Invasive | slaA1 |                 |       |       |            |            |
| 3388     | 3         | Ontario      | 1995 | Invasive | slaA1 |                 |       |       |            |            |
| 3389     | 3         | Ontario      | 1995 | Invasive | slaA1 |                 |       |       |            |            |
| 6129     | 3         | Texas        | 1998 | Invasive | slaA1 |                 |       |       |            |            |
| 6132     | 3         | Texas        | 1998 | Invasive | slaA1 |                 |       |       |            |            |
| 6134     | 3         | Texas        | 1998 | Invasive | slaA1 |                 |       |       |            |            |
| 6147     | 3         | Texas        | 1998 | Invasive | slaA1 |                 |       |       |            |            |
| 6165     | 3         | Texas        | 1998 | Invasive | slaA1 |                 |       |       |            |            |
| 6740     | 3         | Texas        | 1998 | Invasive | slaA1 |                 |       |       |            |            |
| 7555     | 3         | Illinois     | 1998 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 7630     | 3         | Illinois     | 1998 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9497     | 3         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9507     | 3         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9611     | 3         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9246     | 3.1       | Illinois     | 1995 | Invasive | slaA1 |                 |       |       |            |            |
| 9254     | 3.2       | Ontario      | 2000 | Invasive | slaA1 |                 |       |       |            |            |
| 9487     | 4         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 12509    | 4         | Illinois     | 2000 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9591     | 6         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9593     | 6         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9616     | 6         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9645     | 6         | Texas        | 2001 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9705     | 6         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
| 9761     | 6         | Texas        | 2002 | Pharyngitis | slaA1 |                 |       |       |            |            |
**Streptococcal-secreted Phospholipase A₂**

### Table 1—continued

| MGAS no. | emm type | Localitiy | Year | Disease | slaA allele | M. E. | L. E. | Stationary | Co-culture |
|----------|----------|-----------|------|---------|-------------|-------|-------|-------------|------------|
| 9837     | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | pos. | pos. | pos. |
| 9868     | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | neg. | pos. | neg. |
| 9875     | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | neg. | neg. | neg. |
| 10169    | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | pos. | neg. | pos. |
| 10276    | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | neg. | neg. | neg. |
| 10282    | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | neg. | pos. | neg. |
| 10286    | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | neg. | neg. | neg. |
| 10287    | 6        | Texas     | 2002 | Pharyngitis | slaA1 | neg. | pos. | pos. | pos. |
| 10387    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 10388    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 10390    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 10391    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 10392    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 10393    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 10394    | 6        | Pennsylvania | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 12510    | 6        | Arkansas   | 2000 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 12511    | 6        | Arkansas   | 2000 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 6269     | 22       | Texas      | 1998 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 7556     | 22       | Illinois   | 1998 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 9619     | 22       | Texas      | 2001 | Pharyngitis | slaA1 | neg. | neg. | neg. | neg. |
| 9866     | 22       | Texas      | 2001 | Pharyngitis | slaA1 | neg. | neg. | neg. | neg. |
| 9857     | 22       | Texas      | 2002 | Pharyngitis | slaA1 | neg. | neg. | neg. | neg. |
| 582      | 28       | Canada     | 1991 | Invasive  | slaA1 | neg. | neg. | neg. | neg. |
| 594      | 28       | Canada     | 1991 | Invasive  | slaA1 | neg. | neg. | neg. | neg. |
| 6180     | 28       | Texas      | 1998 | Invasive  | slaA1 | neg. | neg. | neg. | neg. |
| 6274     | 28       | Texas      | 1998 | Invasive  | slaA1 | neg. | neg. | neg. | neg. |
| 7348     | 28       | Texas      | 1999 | Invasive  | slaA1 | neg. | pos. | neg. | pos. |
| 7359     | 28       | Texas      | 1999 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 7500     | 28       | Montana    | 1999 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 7577     | 28       | Illinois   | 1998 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 7585     | 28       | Illinois   | 1998 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 7596     | 28       | Illinois   | 1998 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 7598     | 28       | Illinois   | 1998 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 7615     | 28       | Illinois   | 1998 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 7925     | 28       | Illinois   | 1998 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 7934     | 28       | Illinois   | 1998 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 7944     | 28       | Ontario    | 1996 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 8004     | 28       | Ontario    | 1999 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 8342     | 28       | Finland    | 1995 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 8349     | 28       | Finland    | 1995 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 8354     | 28       | Finland    | 1995 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 8359     | 28       | Finland    | 1995 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 8364     | 28       | Finland    | 1995 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 9233     | 28       | Ontario    | 1992 | Invasive  | slaA2h | neg. | neg. | pos. | pos. |
| 9249     | 28       | Ontario    | 1996 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 9458     | 28       | Texas      | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 9468     | 28       | Texas      | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 9594     | 28       | Texas      | 2001 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 9771     | 28       | Texas      | 2002 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 9796     | 28       | Texas      | 2002 | Pharyngitis | slaA1 | neg. | neg. | pos. | pos. |
| 9250     | 75       | Ontario    | 1996 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |
| 9231     | 3757     | Ontario    | 1992 | Invasive  | slaA1 | neg. | neg. | pos. | pos. |

*a* MGAS, Musser GAS strain number.  
*b* Based on DNA sequence analysis of the *emm* gene.  
*c* Expression studied by Western immunoblot analysis.  
*d* M.E., mid-exponential phase of growth.  
*e* L.E., late-exponential phase of growth.  
*f* Stationary, stationary phase of growth.  
*g* Co-culture, GAS was co-cultured with D562 human epithelial cells.  
*h* The *slaA2* allele differed from *slaA1* by one synonymous (silent) nucleotide change.

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**Mature** protein was purified from *E. coli* strains grown at 37 °C in 4 liters of Luria-Bertani broth supplemented with 100 μg of ampicillin per liter. Expression of recombinant protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside at OD600 nm of 0.5, and growth was continued until early stationary phase. The cells were harvested by centrifugation, suspended in 40 ml of 10 mM Tris-HCl buffer, pH 8.0, and sonicated for 15 min. Cell debris was removed by centrifugation, and the supernatant was loaded onto a DEAE Sepharose Fast Flow column (Amersham Biosciences) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. Proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in 10 mM Tris-HCl buffer, pH 8.0. Fractions were analyzed for the presence of rSlaA by SDS-PAGE, and peak fractions were pooled. rSlaA was precipitated by adding (NH₄)₂SO₄ (70% saturation at 25 °C). The precipitate was collected by centrifugation, suspended in 10 ml of 10 mM Tris-HCl, pH 8.0,
and loaded onto a Phenyl-Sepharose 6 Fast Flow (high sub) column (Amersham Biosciences) equilibrated with 0.8 mM \((\text{NH}_4)_2\text{SO}_4\) in 10 mM Tris-HCl buffer, pH 8.0. Protein was eluted with a step gradient of \((\text{NH}_4)_2\text{SO}_4\) (0.16, 0.08, 0.016, and 0.04 mM) in 10 mM Tris-HCl buffer, pH 8.0. Fractions containing rSlaA were pooled, \((\text{NH}_4)_2\text{SO}_4\) was added to 1.0M, and the fractions were analyzed by SDS-PAGE, to determine the final concentration of rSlaA. The purity of the protein was determined by Coomassie Blue staining and visualized using a Personal FX Phosphorimager and ImageMaster software (Amersham Pharmacia BioTech).

**Analysis of Enzyme Substrate Specificity—**Phospholipase A\(_2\) activity was detected using two fluorometric or radiolabeled substrates in mixed micelle form and radiolabeled dipalmitoyl-[1,2-palmitoyl-1,3-C\(_{31}\)H\(_{53}\)](phosphatidylcholine (PC) in vesicle form) (26). For radiolabeled assays, 8 \(\mu\)g of SlaA was mixed with 0.454 mM radiolabeled substrate and 58 \(\mu\)M cold (unlabeled) dipalmitoyl PC in 50 mM Tris-HCl, pH 7.5, 2 mM CaCl\(_2\), and 0.1 mM PANT-labeled-X-100. The radiolabeled substrates used included dipalmitoyl-[1,2-palmitoyl-1,3-C\(_{31}\)H\(_{53}\)](phosphatidylcholine (PC)) (26). The test substrate was sonicated three times for 30 s and heated to 60 °C under nitrogen before addition of the enzyme. The reactions were incubated at 37 °C for 2 h, and stopped by addition of chloroform-methanol-hydrochloric acid (50:50:0.3, v/v/v). For one-dimensional thin-layer chromatography, the lipids were analyzed with Silica Gel 60 plates, in chloroform-methanol-25% aqueous amonia (80:20:1, v/v/v). The lipid spots were visualized with iodine vapor, and the radioactive lipids were visualized using autoradiography in conjunction with a phosphorimager.

**Enzyme Activity Assay—**Rat liver microsomes were used as a source of SlaA. The reaction mixture contained 50 mM Tris-HCl, 10 mM CaCl\(_2\), 100 \(\mu\)M SlaA, and 1 mM 1-palmitoyl, 2-arachidonyl \([-1\text{,2-C\(_{31}\}^14\text{C}\}_3\text{H}_{53}\}\)]PC. The reaction was initiated by addition of the enzyme, and the reaction was stopped after 3 min by addition of 200 mM Na\(_2\)CO\(_3\) and 10% trichloroacetic acid. The radioactivity was determined using a liquid scintillation counter.

**Assessment of Transferase Activity—**The transferase activity of SlaA was assessed using a radiometric assay. The reaction mixture contained 50 mM Tris-HCl, 10 mM CaCl\(_2\), 100 \(\mu\)M SlaA, and 1 mM 1-palmitoyl, 2-arachidonyl \([-1\text{,2-C\(_{31}\}^14\text{C}\}_3\text{H}_{53}\}\)]PC. The reaction was initiated by addition of the enzyme, and the reaction was stopped after 3 min by addition of 200 mM Na\(_2\)CO\(_3\) and 10% trichloroacetic acid. The radioactivity was determined using a liquid scintillation counter.

**DNA Sequence Analysis of the slaA Gene among GAS Strains—**The slaA gene was sequenced using PCR-amplified gene fragments and internal primers. All sequence data were collected with an Applied Biosystems model 3700 instrument.
cultures of GAS test strains were inoculated into 25 ml of fresh THY medium and cultured for 3 h to an OD_{600} of 0.2–0.3. The bacteria were collected by centrifugation, washed once in phosphate-buffered saline, and suspended in 1 ml of phosphate-buffered saline. Bacteria (100 μl) were added to the D562 cells and incubated for 3 h at 37 °C. To test the time course of induction, one well was harvested immediately to serve as a 0 h control and then at 1, 2, and 3 h, respectively. The medium was removed and centrifuged at 4,000 × g to remove bacteria and host-cell debris. The supernatant was concentrated to 0.06 ml with a Nanosep 10,000 MWCO device (Pall Corporation, East Hills, NY). Each sample was mixed with 15 μl of 5× SDS sample buffer containing 1% 2-mercaptoethanol, boiled for 5 min, and an aliquot (10 μl) was resolved on a Ready Gel 15% Tris-HCl gel (Bio-Rad). Western immunoblot analysis was performed as described above with α-SlaA antibody diluted 1:100,000.

RESULTS

slaA Gene Mutagenesis and Protein Purification—To identify amino acid residues in SlaA potentially important for structure-activity relationships, SlaA, textilotoxin, Naja naja atra venom sPLA₂, bovine pancreatic sPLA₂, and the four subunits of textilotoxin were aligned with Clustal W using default parameters. Amino acid residues that match the consensus sequence are shaded in dark gray, and similar residues are shaded in light gray. The Ca²⁺-loop consensus sequence region is highlighted in blue, and the active site consensus sequence region is highlighted in red. Amino acid residues chosen for replacement are underlined. Conserved cysteine residues are indicated by green arrows. SlaC, phospholipase A₂ from Group C Streptococcus.

FIG. 1. Amino acid alignment of SlaA and related sPLA₂. C-terminal region amino acid residues of SlaA, N. n. atra venom sPLA₂, bovine pancreatic sPLA₂, and the four subunits of textilotoxin were aligned with Clustal W using default parameters. Amino acid residues that match the consensus sequence are shaded in dark gray, and similar residues are shaded in light gray. The Ca²⁺-loop consensus sequence region is highlighted in blue, and the active site consensus sequence region is highlighted in red. Amino acid residues chosen for replacement are underlined. Conserved cysteine residues are indicated by green arrows. SlaC, phospholipase A₂ from Group C Streptococcus.
Amino acid residues located in the putative active site and Ca\(^{2+}\)-binding loop regions of SlaA were well conserved in these sPLA\(_2\) enzymes (Fig. 1). Conserved amino acid residues located in and around the putative active site and Ca\(^{2+}\)-binding loop regions were replaced with alanine residues by site-specific mutagenesis. Additional conserved amino acids were replaced because of their putative structural importance based on known structure-activity relationships in Groups I and II sPLA\(_2\) enzymes (31). The twenty mutant proteins were overexpressed in E. coli and purified to apparent homogeneity (Fig. 2). Western immunoblot analysis using specific rabbit and mouse monoclonal antibody confirmed that the correct protein had been purified (Fig. 2).

Assessment of Mutant and Wild-type SlaA Enzymatic Activity—Mutant proteins with amino acid replacements located in the putative active site and Ca\(^{2+}\)-binding loop regions had the greatest loss of enzymatic activity (Fig. 3). For example, replacement of the putative catalytic histidine residue (His-137) and aspartate residues (Asp-128, Asp-131, and Asp-138) in the putative active site region abolished activity (Fig. 3). In addition, replacement of amino acid residues located in the putative Ca\(^{2+}\)-binding loop resulted in loss of activity ranging from 76.5% (G116A) to 100% (Y112A) (Fig. 3). In contrast, replacement of amino acid residues Val-126, Val-129, and Cys-151 resulted in mutant proteins with increased enzymatic activity (Fig. 3).

Native-PAGE analysis was used to gain insight into the structural integrity of the mutant proteins. Three of the four mutant proteins with a cysteine residue replacement (C113A, C134A, and C151A) had substantially slower electrophoretic mobility than the wild-type enzyme, presumably due to altered protein conformation (Fig. 4). The electrophoretic mobility of the C140A mutant protein was not altered substantially, consistent with the hypothesis that C140 is located in the enzyme active site region and not involved in disulfide bond formation. The other mutant proteins lacked or had only modestly altered electrophoretic migration, suggesting that the observed changes in enzyme activity were not caused by gross changes in protein conformation.

Assessment of SlaA Substrate Specificity—Use of radiolabeled substrates and 1D-TLC showed that SlaA had activity against phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine. SlaA was not active against phosphatidylinositol 3P, as assessed with the fluorescent-labeled (BODIPY) 1D-TLC assay. With respect to the specificity of enzymatic activity against fatty acyl groups, SlaA-cleaved palmitic, oleic, and arachidonic acid present in the sn-2 position of the substrate. SlaA cleavage products were more readily released from substrates present in a mixed micelle assay than a PC vesicle assay, similar to other sPLA\(_2\)'s (26).

Incubation of SlaA with dipalmitoyl-[2-palmitoyl-1-\(^{14}\)C]PC produced only palmitic acid as assessed by 1D-TLC. That is, no
lysophosphatidylcholine (LPC) was detected, indicating that SlaA cleaved only at the sn-2 position. These results confirmed that SlaA has PLA2, rather than PLA1, activity. Moreover, incubation of SlaA and dipalmitoyl-[1,2-palmitoyl-1-14C]PC produced detectable palmitic acid and LPC, as analyzed by 1D-TLC. This result confirmed that SlaA has PLA, rather than phospholipase B (able to cleave at sn-1 and sn-2 positions at similar rates), activity.

**Distribution of the slaA Gene among GAS Strains and Analysis of slaA Allelic Variation**—The slaA gene was discovered as a consequence of sequencing the genome of a serotype M3 GAS strain. We showed previously that the slaA gene and the prophage encoding it were not present in serotype M3 strains until the mid-1980s, whereas the vast majority of serotype M3 strains recovered since the mid-1980s have the slaA gene and prophage (7). However, it is not known if the slaA gene is present in strains other than serotype M3 GAS. To determine if slaA was widely distributed in natural populations of GAS, 1,189 strains representing 48 emm types were studied by PCR. The slaA gene was present in 129 of the 1,189 strains, including emm1, emm2, emm3, emm4, emm6, emm22, emm28, emm75, and st3757 (Table I).

To test the hypothesis that allelic variation existed in the slaA gene present in these emm types, slaA was sequenced in the 129 GAS strains (Table I). 128 of 129 strains had the same slaA allele, designated slaA1. Only one additional allelic variant was identified (designated slaA2), and this variant was found in only one emm28 strain (MGAS9233). The slaA2 allele differed from slaA1 by one synonymous (silent) nucleotide change.

**Analysis of in Vitro Expression of SlaA**—Previous study of serotype M3 strain MGAS315 showed that SlaA was actively secreted into the culture supernatant of this organism (18). However, in vitro production by other GAS strains has not been studied. To test the hypothesis that organisms with the slaA gene expressed SlaA in vitro, 51 strains of diverse emm types were studied. As assessed using culture supernatants obtained at three phases of growth, relatively few (n = 17) strains produced immunoreactive SlaA (Table I). This result was unexpected given that we previously found that patients infected with serotype M3 strains seroconverted to SlaA (7). In addition, the slaA gene sequencing data did not reveal nucleotide variation that would account for lack of in vitro expression of immunoreactive SlaA.

Banks et al. (18) recently reported that serotype M3 strain MGAS315 significantly up-regulated SlaA production when grown in vitro in the presence of D562 human pharyngeal epithelial cells. Hence, we tested the hypothesis that analogous up-regulation of SlaA production would occur in the 51 GAS strains tested. Consistent with the hypothesis, a strikingly
increased amount of immunoreactive SlaA was detected in the tissue culture medium (Fig. 5). Moreover, virtually all GAS strains produced detectable immunoreactive SlaA in this assay (Table I).

To further probe the characteristics of the interaction between GAS and D562 pharyngeal cells, we conducted a time course experiment to assess the rapidity with which increased immunoreactive SlaA occurred on initiation of co-culture. Genetically representative strains selected from four M-serotypes causing abundant episodes of pharyngitis and invasive disease were used, including M2 strain MGAS12508, M3 strain MGAS1563, M6 strain MGAS10394, and M28 strain MGAS6180. The bacteria were co-cultured with D562 cells for a maximum of 3 h. Western immunoblot analysis (Fig. 6) showed that immunoreactive SlaA was absent in the culture supernatant at the zero hour time point for all strains. Immunoreactive SlaA increased rapidly over the 3 h of co-culture for all strains analyzed. The greatest increase in immunoreactive SlaA occurred between 1 and 2 h, indicating rapid induction of SlaA.

**DISCUSSION**

The protein we characterized in this study was the first secreted PLA₂ enzyme to be isolated from a pathogenic bacterium, making SlaA an important addition to the PLA₂ superfamily. Although the biologic function of SlaA has not been elucidated, it is reasonable to hypothesize that it participates in host-pathogen interaction. Secreted PLA₂ enzymes can have a wide range of functions, including cellular toxicity, anticoagulant activity, proinflammatory effects, and bactericidal activity (30). The presence of anti-SlaA antibodies in convalescent sera obtained from patients with infections caused by serotype M3 GAS strains and the substantial up-regulation of SlaA production in response to interaction with human epithelial cells support the idea that SlaA participates in GAS pathogenesis (7, 18).

**Predictions of Structural Features Derived from the Amino Acid Sequence**—Historically, sPLA₂-s that have been characterized have been obtained mainly from mammalian tissue, blood, and vertebrate and insect venoms (30). Secreted PLA₂-s have a histidine residue as their catalytic amino acid residue and, with a few exceptions, a highly conserved active site region (DXCCXXHDXCY) and Ca²⁺-binding loop (XYCGXXGXX) (31, 32, 33). Comparison of SlaA with other sPLA₂-s revealed some notable differences. As expected, SlaA has diverged considerably from sPLA₂-s made by eukaryotic organisms, but the active site and Ca²⁺-binding loop regions are well conserved. One striking difference between SlaA and eukaryotic sPLA₂-s is the small number of cysteine residues in SlaA relative to the other sPLA₂-s. sPLA₂-s are generally cysteine-rich and contain 5–8 disulfide bonds that contribute to structural integrity (30). Among PLA₂-s assigned to group I, II, V, or X, six disulfide bonds are absolutely conserved. In contrast, SlaA has only six cysteine residues, and only one putative disulfide pair (Cys-113–Cys-134) is conserved relative to the other sPLA₂-s. sPLA₂-s are generally cysteine-rich and contain 5–8 disulfide bonds that contribute to structural integrity (30). Among PLA₂-s assigned to group I, II, V, or X, six disulfide bonds are absolutely conserved. In contrast, SlaA has only six cysteine residues, and only one putative disulfide pair (Cys-113–Cys-134) is conserved relative to the enzymes in the groups listed above.

Recently a new group (XIV) of PLA₂-s was described that includes enzymes made by the soil bacterium *Streptomyces violaceoruber* and *Tuber borchii*, a symbiotic fungus (34, 35). These discoveries showed that sPLA₂-s are not produced exclusively by higher eukaryotes. These two enzymes have high amino acid sequence homology to one another, especially in their active site and Ca²⁺-binding regions. Compared with

![Figure 5](http://www.jbc.org/)

**Figure 5.** Western immunoblot analysis showing up-regulation of *in vitro* expression of SlaA occurring on co-culture of GAS with D562 human epithelial cells. Serotype M3 strain MGAS1563 was used as a representative organism. M.E., mid-exponential phase of growth; L.E., late-exponential phase of growth; Stationary, stationary phase of growth; Co-culture, strain MGAS1563 co-cultured with D562 human epithelial cells. Culture supernatants were analyzed using purified rabbit anti-SlaA antibody raised against purified recombinant SlaA.

![Figure 6](http://www.jbc.org/)

**Figure 6.** Western immunoblot analysis showing time course of *in vitro* expression of SlaA occurring on co-culture of GAS with D562 human epithelial cells. Supernatants obtained at 0, 1, 2, and 3 h after co-culture are shown. A, GAS serotype M2 strain MGAS12508. B, GAS serotype M3 strain MGAS1563. C, GAS serotype M6 strain MGAS10394. D, GAS serotype M28 strain MGAS6180.

| PL      | Fatty acid | Label position | Assay † | Products detected | Indication                        |
|---------|------------|----------------|---------|-------------------|----------------------------------|
| PC      | Dipalmitoyl| ¹⁴C Dpalmitoyl  | V       | LPC, FA ‡         | Able to use vesicle as substrate ‡ |
| PC      | Dipalmitoyl| ¹⁴C Dpalmitoyl  | MM      | LPC, FA           | Able to use PC as substrate     |
| PC      | sn-1 Palmitoyl | sn-2-¹⁴C Arachidonyl | MM | FA               | Able to use arachidonic acid as substrate |
| PC      | sn-2 Arachidonyl | sn-2-¹⁴C Arachidonyl | MM | FA               | Able to use arachidonic acid as substrate |
| SM      | Ceramide   | ¹⁴C Choline     | MM      | None              | Not active against ceramide backbone |
| PS      | Dioleoyl   | ¹⁴C Serine      | MM      | LPS               | Able to use PS and oleic acid as substrates |
| PE      | sn-1 Palmitoyl | sn-2-¹⁴C Arachidonyl | MM | FA               | Able to use PE as substrate     |
| PE      | sn-2 Arachidonyl | sn-2-¹⁴C Arachidonyl | MM | FA               | Able to use PE as substrate     |
| PI3P    | sn-1 Palmitoyl | sn-2 C5 BODIPY | MM      | None              | Not active against PI3P         |

† V, vesicle assay; MM, mixed micelle assay.

‡ Products observed in this vesicle assay are comparatively much less than products in the mixed micelle assay, with otherwise the same conditions.
groups I, II, III, V, IX, X, XI, and XII enzymes, group XIV enzymes have relatively few cysteine residues, only one conserved residue (Cys) in the Ca$^{2+}$-binding loop, and very low sequence conservation in the active site region. Of note, the crystal structure of the Streptomyces enzyme showed conservation of the His-Asp catalytic center motif, but absence of a loop architecture in the Ca$^{2+}$-binding region. The SlaA enzyme appears to represent a unique type of sPLA$_2$ in that it is made by a prokaryote, has low cysteine content like group XIV enzymes, but has an active site and Ca$^{2+}$-binding loop sequence most similar to group I and II enzymes. SlaA also differs from other sPLA$_2$s in molecular mass. The great majority of previously described PLA$_2$s have an inferred molecular mass (mature protein) of 12.4–15 kDa (30), whereas the inferred molecular mass of mature SlaA is 18.6 kDa. The additional amino acids responsible for this increase in inferred molecular mass are located at the N-terminal region immediately after the secretion signal. Based on these aggregate amino acid sequence features and the assessment that SlaA hydrolyzes the ester protein) of 12.4–15 kDa (30), whereas the inferred molecular mass of mature SlaA is 18.6 kDa. The additional amino acids responsible for this increase in inferred molecular mass are located at the N-terminal region immediately after the secretion signal. Based on these aggregate amino acid sequence features and the assessment that SlaA hydrolyzes the ester bond of a range of phospholipids at the sn-2 position, we propose that SlaA be designated as the first member of a novel group of the PLA$_2$ superfamily of enzymes.

Insights into Substrate Specificity—The number of phospholipid species is too numerous to investigate the ability of SlaA to cleave all possible substrates. Hence, we focused on potential physiologically relevant substrates. Specifically, we addressed whether SlaA distinguished between the sn-1 and sn-2 positions, cleaved saturated and/or unsaturated fatty acyl groups, and used substrates with varying physiologically relevant head groups. Our analysis revealed that SlaA functions on saturated, monounsaturated, and polyunsaturated fatty acyl groups. Most significantly, we found that SlaA was able to cleave arachidonic acid, a known precursor to eicosanoids participating in the inflammatory cascade. Furthermore, we found that SlaA was able to cleave three common phospholipid head groups (Table III).

Insights into SlaA Structure-Activity Relationships Revealed by Site-directed Mutagenesis—Alanine replacement mutagenesis provided important insights into structure-activity relationships in SlaA, particularly with respect to amino acid residues putatively involved in enzyme catalysis and Ca$^{2+}$ binding. The crystal structure of SlaA has not been solved, and molecular modeling using SWISS-MODEL was unsuccessful due to the low sequence identity between SlaA and sPLA$_2$ enzymes with structures deposited in the Protein Data Bank. The results of the site-directed mutagenesis studies support the idea that H137 is the catalytic residue, consistent with amino acid sequence alignments (Fig. 1) and known structure-activity relationships in other sPLA$_2$s enzymes (30). In addition, with the exception of Gln-135, all targeted conserved amino acid residues within the putative active site region had greatly decreased or total loss of enzyme activity. We note that Gln-135 has no known involvement in the catalytic function of sPLA$_2$s enzymes (31).

If SlaA has a His-Asp catalytic dyad like other sPLA$_2$s, based on amino acid sequence alignments it is possible that Asp-177 represents the catalytic dyad Asp residue. To test this idea, we replaced Asp-177 with an Ala residue. However, despite extensive attempts, we were unable to purify sufficient recombinant mutant protein for conducting enzyme assays. Replacement of amino acid residues thought to bind Ca$^{2+}$ (Gly-114 and Asp-138) or enhance the structural integrity of the Ca$^{2+}$-binding loop (Y112, C113, G114, and G119) resulted in loss of virtually all enzyme activity. This loss of enzyme activity could not be reversed by the addition of excess Ca$^{2+}$ to the reaction mixture (data not shown). These results are consistent with the hypothesis that SlaA requires calcium for maximum enzymatic activity (30, 36).

Previous studies have shown that tyrosine residues are located in the catalytic network of PLA$_2$s enzymes. Site-directed mutational studies have demonstrated that tyrosines function primarily to provide structural support (27). Based on amino acid sequence alignments, the most likely residues serving this function in SlaA are Tyr-141 and Tyr-160. Consistent with the idea that these two tyrosine residues are functionally important, the Y141A and Y160A mutant proteins had greatly decreased PLA$_2$ activity (Fig. 3).

Molecular Population Genetics of the slaA Gene—The slaA gene was discovered as a consequence of sequencing the genome of a contemporary serotype M3 GAS strain. The slaA gene was found to be encoded by a prophage which was recently shown to be inducible (18). These observations raised the possibility that slaA was more widely distributed in GAS than simply among serotype M3 strains. We found that the slaA gene was present in 10.8% of 1,189 GAS strains tested, including emm1, emm2, emm3, emm4, emm22, emm28, emm75, and st3757 strains. DNA sequence analysis of slaA in 129 strains revealed that with a single exception, all isolates had the identical allele of this gene. The presence of the same allele of a prophage-encoded gene in GAS strains that are otherwise highly differentiated in overall chromosomal character is strong evidence that slaA has been horizontally transferred in nature, presumably by transduction. Moreover, the lack of nucleotide variation in slaA suggests that the dissemination of slaA to diverse GAS strains has occurred very recently.

In Vitro Expression of SlaA—There is considerable emerging evidence that environmental signals have an important role in influencing the expression of prophage-encoded extracellular proven or putative virulence factors in GAS (15, 16, 18). By showing that the interaction of four GAS strains representing commonly occurring M protein serotypes with D562 pharyngeal cells resulted in greatly increased SlaA expression, we provided additional evidence supporting this idea. Furthermore, our data indicated that 92% of GAS strains tested up-regulated SlaA expression in vitro in response to interaction with D562 cells. As assessed by a time-course assay, up-regulation of production of immunoreactive SlaA occurred rapidly on co-culture with D562 pharyngeal epithelial cells. Together, the data support the hypothesis that SlaA production confers an enhanced survival capacity to GAS during human interaction in the upper respiratory tract, and perhaps other anatomic sites. The results of studies conducted with an isogenic mutant strain in which the slaA gene was inactivated are consistent with the idea that SlaA production enhances GAS survival.

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