Identification of the Actin and Plasminogen Binding Regions of Group B Streptococcal Phosphoglycerate Kinase

Background: Phosphoglycerate kinase (PGK) on the surface of group B streptococcus (GBS) binds actin and plasminogen. Site-directed mutagenesis identified amino acids involved in binding actin and plasminogen. PGK residues 126, 127, 130, 133, 204, and 208 bind actin and plasminogen. These results may allow future research to determine the role of PGK in GBS virulence.

Results: Site-directed mutagenesis identified amino acids involved in binding actin and plasminogen. PGK residues 126, 127, 130, 133, 204, and 208 bind actin and plasminogen.

Conclusion: PGK residues 126, 127, 130, 133, 204, and 208 bind actin and plasminogen. Site-directed mutagenesis identified amino acids involved in binding actin and plasminogen. These results demonstrate that the lysine residues at amino acid positions 126, 127, 130, 133, 204, and 208 along with the glutamic acid residue at amino acid position 133 are necessary for actin and plasminogen binding by GBS-PGK.

Significance: These results may allow future research to determine the role of PGK in GBS virulence.

Phosphoglycerate kinase (PGK), present on the surface of group B streptococcus (GBS), has previously been demonstrated to bind the host proteins actin and plasminogen. The actin and plasminogen binding sites of GBS-PGK were identified using truncated GBS-PGK molecules, followed by peptide mapping. These experiments identified two actin and plasminogen binding sites located between amino acids 126–134 and 204–208 of the 398-amino acid-long GBS-PGK molecule. Substitution of the lysine residues within these regions with alanine resulted in significantly reduced binding to both actin and plasminogen. In addition, conversion of the glutamic acid residue at amino acid 133 to proline, the amino acid found at this position for the PGK protein of Streptococcus pneumoniae, also resulted in significantly reduced binding to actin and plasminogen. These results demonstrate that the lysine residues at amino acid positions 126, 127, 130, 204, and 208 along with the glutamic acid residue at amino acid position 133 are necessary for actin and plasminogen binding by GBS-PGK.

Group B streptococcus (GBS) is a significant cause of neonatal bacterial pneumonia, sepsis, and meningitis (1–4). GBS is also a major cause of invasive disease in the adult population (5, 6). Surface expressed and secreted proteins are crucial for GBS virulence (7) as they can mediate the following: adherence to host cells (8–14), crossing of host barrier tissues (15–17), and immune evasion (18). Glycolytic enzymes, in addition to being contributing to GBS virulence. Similar to GAPDH and α-enolase from other streptococcal species, confirming the role of PGK in GBS virulence has been hampered by its role in glycolysis. Due to their essential role in metabolism, traditional knock-out mutagenesis is not possible. Site-directed mutagenesis, creating a glycolytically active enzyme that lacks the ability to bind host proteins, has previously been used to demonstrate a role for GAPDH and α-enolase in virulence of other streptococcal species (26, 51). The objective of this work was to identify point mutations within the pgk gene that significantly reduce binding of GBS-PGK to actin and plasminogen without affecting its glycolytic activity.
Actin and Plasminogen Binding by GBS-PGK

EXPERIMENTAL PROCEDURES

Production and Purification of Full-length and Truncated Recombinant GBS-PGK Molecules—The previously characterized GBS strain NCS13 (37, 38, 52) was incubated for 16 h at 35 °C in Todd Hewitt broth. Genomic DNA, isolated from this overnight culture, was used as a template to PCR amplify the pgk gene. The full-length gbs-pgk gene was PCR amplified using primers PGK-bamF (TTCGGATCCTGCTAAACTTGGTAGTTG) and PGK-pstR (TTCTCTGCAATTGATACTGCT). The truncated gbs-pgk gene spanning amino acids 83–303 (TJB 1) was PCR-amplified using primers PGK1-bamF (TTCGGATCCTCAGCTCAAGATGTTG) and PGK3-hinR (TTCTAAACTTGGTCAAGATGTTG) and PGK3-hinR (TTCTAAGCTTGGTAGTTTAGGACCGATGCTAAG). The resulting truncated gbs-pgk gene spanning amino acids 1–165 (TJB 2) was PCR-amplified using primers PGK-bamF and PGK4-hinR (TTCTCTGCTGATACTGCT). The truncated gbs-pgk gene spanning amino acids 166–398 (TJB 3) was PCR-amplified using primers PGK2-bamF (TTCGGATCCTCGCTAAACTTGGTAGTTG) and PGK3-hinR (TTCTAAAGCTGTTTACTGCTAAGCA). The resulting amplified fragments were ligated into the plasmid pQE 30 and transformed into chemically competent Escherichia coli M15 carrying the plasmid pREP4. Full-length and truncated rGBS-PGK molecules were expressed and purified as described previously (38).

Interaction of Full-length and Truncated rGBS-PGK with Actin and Plasminogen—Actin and plasminogen binding by full-length and truncated rGBS-PGK molecules were assayed using ELISA as described previously (38). Actin (1 μg/well; Sigma-Aldrich) and plasminogen (0.1 μg/well; Sigma-Aldrich) were resuspended in 0.1 M sodium carbonate solution (pH 9.5) and immobilized to wells of a 96-well polystyrene plate (Maxisorp; NUNC, Thermo Fisher Scientific, Nepean, ON, CA) via 8 h of incubation at room temperature. Wells incubated with sodium carbonate solution containing no protein were used as a negative control. Wells were washed 1 × 10 min with Tris-buffered saline (TBS) and incubated for 16 h with blocking buffer (5% BSA, 0.1% Tween 20 in TBS) at room temperature. Wells were then washed 3 × 10 min with TBS and incubated with full-length or truncated rGBS-PGK molecules (15 or 0 μg/ml; in blocking buffer) for 1 h at room temperature. After this incubation, wells were washed 3 × 10 min with TBS and incubated with rabbit anti-rGBS-PGK antibodies (1:300 in blocking buffer) (38) for 1 h at room temperature. Another wash, 3 × 10 min, with TBS was performed, and the plates were incubated with goat anti-rabbit-IgG alkaline phosphatase conjugated antibodies (1:200 in blocking buffer; Sigma-Aldrich) for 1 h at room temperature. Wells were then washed 3 × 10 min with TBS and developed for 16 h at 4 °C. Membranes were washed 3 × 15 min with TBS before developing with SigmaFast BCIP/NBT (Sigma-Aldrich) for 5 min. Membrane development was stopped with three changes of distilled water.

Peptide Mapping—A peptide mapping procedure, similar to that described previously (27), was used to identify the actin and plasminogen binding regions of GBS-PGK. The GBS-PGK region corresponding to amino acids 83–303 (TJB 1) was divided into 21 peptides (1–21); each peptide was 20-amino acids in length and overlapped the previous peptide by 10 amino acids (Peptide 2.0, Inc. Chantilly, VA). Peptides were ordered as crude peptides, and their amino acid sequences are listed in Table 1. Peptides were fixed to nitrocellulose membranes (15 μg/spot) using the biodot apparatus (Bio-Rad). The membranes were washed 15 min with TBS, incubated 30 min with blocking buffer (5% BSA, 0.1% Tween 20 in TBS), washed 15 min with TBS, and incubated with either actin or plasminogen (20 μg/ml in blocking buffer; Sigma-Aldrich) for 16 h at 4 °C. A positive control, rGBS-PGK was also fixed as a spot to the nitrocellulose membrane (15 μg). As a negative control, duplicate membranes containing immobilized peptides were incubated with blocking buffer only for 16 h at 4 °C and assayed in parallel with the experimental membranes. After incubation, the membranes were washed 15 min with TBS and incubated with either mouse anti-actin (clone C4; Millipore, Billerica, MA) or mouse anti-plasminogen (clone 3E6; Sigma-Aldrich) antibodies (1:1000 in blocking buffer) for 2 h at 4 °C. Membranes were washed 3 × 15 min with TBST (0.1% Tween 20 in TBS), 2 × 15 min with TBS before incubating with goat anti-mouse-IgG alkaline phosphatase conjugate (1:10,000 in blocking buffer; Sigma-Aldrich) for 1 h at room temperature. Membranes were washed 3 × 15 min with TBST and 2 × 15 min with TBS before developing with SigmaFast BCIP/NBT (Sigma-Aldrich) for 5 min. Membrane development was stopped with three changes of distilled water.

Table 1: Peptides used in study

| Peptide | Sequence | Amino acids | Bound by |
|---------|----------|-------------|----------|
| PGK 1   | aklgqdlfvgtygztaklee | 83–102 | Actin and plasminogen |
| PGK 2   | gtvrgaklesaanaledegv | 93–112 | Actin and plasminogen |
| PGK 3   | ainaledgevlgvltreff | 103–122 | Actin and plasminogen |
| PGK 4   | vlgkkkesnkdleegkwyall | 123–142 | Actin and plasminogen |
| PGK 5   | eelgkwasldgfdgfdvdaf | 133–152 | Actin and plasminogen |
| PGK 6   | gdlqfvdafgtaahasn | 143–162 | Actin and plasminogen |
| PGK 7   | gtaahhasmgvisanveka | 153–172 | Actin and plasminogen |
| PGK 8   | vginanrekavagflleini | 162–182 | Actin and plasminogen |
| PGK 9   | vfglleneiayiqeavetp | 173–192 | Actin and plasminogen |
| PGK 10  | aiygeavetpserpflaig | 183–202 | Actin and plasminogen |
| PGK 11  | erkvlaigkNNkvgvdgivi | 192–212 | Actin and plasminogen |
| PGK 12  | slktdlkdigvdgtdgig | 202–222 | Actin and plasminogen |
| PGK 13  | enlkdaklNlkgigmgntyf | 213–232 | Actin and plasminogen |
| PGK 14  | lggmmtyflfkgqkjegi | 223–242 | Actin and plasminogen |
| PGK 15  | ykaqgieignsdvtedldv | 233–252 | Actin and plasminogen |
| PGK 16  | aklgqdlfvgtygztaklee | 243–262 | Actin and plasminogen |
| PGK 17  | slveeldklakdikleksng | 253–272 | Actin and plasminogen |
| PGK 18  | dtergeavseflgdigpgk | 284–303 | Actin and plasminogen |

pared with those obtained from standard curves containing decreasing amounts full-length or truncated rGBS-PGK (10–0.3125 ng/well; supplemental Fig. S1) to determine the amount of full-length and truncated rGBS-PGK molecules remaining in the wells.

Table 1: Peptides used in study

| Peptide | Sequence | Amino acids | Bound by |
|---------|----------|-------------|----------|
| PGK 1   | aklgqdlfvgtygztaklee | 83–102 | Actin and plasminogen |
| PGK 2   | gtvrgaklesaanaledegv | 93–112 | Actin and plasminogen |
| PGK 3   | ainaledgevlgvltreff | 103–122 | Actin and plasminogen |
| PGK 4   | vlgkkkesnkdleegkwyall | 123–142 | Actin and plasminogen |
| PGK 5   | eelgkwasldgfdgfdvdaf | 133–152 | Actin and plasminogen |
| PGK 6   | gdlqfvdafgtaahasn | 143–162 | Actin and plasminogen |
| PGK 7   | gtaahhasmgvisanveka | 153–172 | Actin and plasminogen |
| PGK 8   | vginanrekavagflleini | 162–182 | Actin and plasminogen |
| PGK 9   | vfglleneiayiqeavetp | 173–192 | Actin and plasminogen |
| PGK 10  | aiygeavetpserpflaig | 183–202 | Actin and plasminogen |
| PGK 11  | erkvlaigkNNkvgvdgivi | 192–212 | Actin and plasminogen |
| PGK 12  | slktdlkdigvdgtdgig | 202–222 | Actin and plasminogen |
| PGK 13  | enlkdaklNlkgigmgntyf | 213–232 | Actin and plasminogen |
| PGK 14  | lggmmtyflfkgqkjegi | 223–242 | Actin and plasminogen |
| PGK 15  | ykaqgieignsdvtedldv | 233–252 | Actin and plasminogen |
| PGK 16  | aklgqdlfvgtygztaklee | 243–262 | Actin and plasminogen |
| PGK 17  | slveeldklakdikleksng | 253–272 | Actin and plasminogen |
| PGK 18  | dtergeavseflgdigpgk | 284–303 | Actin and plasminogen |
AAATTGACTGTTAAGAAGCTT and PGK-hinR, ligated into the plasmid pUC19 and transformed into chemically competent <i>E. coli</i> DH5α. The plasmid was recovered using the Qiapin miniprep kit (Qiagen) and subjected to mutagenesis using the GeneTailor site-directed mutagenesis system (Invitrogen) following the manufacturer’s instructions. The plasmid was first methylated for 1 h at 37 °C, followed by mutagenesis through PCR amplification using the following primers: PGK-M1 (TTGAGATTTGAGGTGCCTGGCGAGATCTGCGAATGCGACCGAAGATTTCCTTGTGAATTTGTTAAATACC) and PGK-M1ol (ACCTGCTACATTTTCAACAACCGTGTGTGTTTCAACCC); PGK-M2 (CTTCTTGTGGTGGGTGTTGCGAGAATCTGG) and PGK-M2ol (GTGGTTTATCGAAAACCTTCTTGAAACGGTTATGCGCATGATGCAGTTTCTTTCTTACCG); and PGK-M3ol (AGAAGGTCTTCTGATACCAATCAATCTTTA) and PGK-M3 (TTGGAAGATGTTGAGCCGTCACCTTGCGCCGACATCAACG) and PGK-M4ol (TGCTGTCAACATCTTACGTGATACACCGAGATTTCCTTGTGAATTTGTTAAATACC) and PGK-M5 (TTGAGATTTGAGGTGCCTGGCGAGATCTGCGAATGCGACCGAAGATTTCCTTGTGAATTTGTTAAATACC); PGK-M5ol (ACCTGCTACATTTTCAACAACCGTGTGTGTTTCAACCC); PGK-M6ol (GAGAAGGTCTTCTGATACCAATCAATCTTTA) and PGK-M6 (TTGGAAGATGTTGAGCCGTCACCTTGCGCCGACATCAACG) and PGK-M7ol (ACCTGCTACATTTTCAACAACCGTGTGTGTTTCAACCC); PGK-M7 (TTGGAAGATGTTGAGCCGTCACCTTGCGCCGACATCAACG) and PGK-M8ol (ACCTGCTACATTTTCAACAACCGTGTGTGTTTCAACCC). Amplification was carried out using the following cycles: 94 °C for 2 min; 20 cycles of 94 °C 30 s, 55 °C 30 s, 68 °C for 4 min; and 68 °C 10 min. Following the mutagenesis reaction, the plasmid was transformed into One-Shot® MAX Efficiency® DH5α™-TM (Invitrogen). Plasminogen Binding Domains—To identify the actin and plasminogen binding sites, the predicted program, and the amino acids identified to be involved in binding to actin and plasminogen were highlighted to determine their relative location within the GBS-PGK molecule.

Binding of Mutant rGBS-PGK to Immobilized Actin and Plasminogen—Non-mutated rGBS-PGK along with enzymatically active mutated rGBS-PGK molecules were assayed for binding to actin and plasminogen using ELISA as described above. As a control for nonspecific binding, of either rGBS-PGK or anti-rGBS-PGK antibodies to BSA, 96-well polystyrene plates with no immobilized protein were used. A second control, wells containing immobilized actin or plasminogen but not incubated with rGBS-PGK, was also used.

Experiments were performed a total of nine times, and the A405 measurements from the control plates were subtracted from the A405 measurements from the experimental plates to provide a final A405 measurement. These A405 measurements were compared with those obtained from standard curves containing decreasing amounts mutated and non-mutated rGBS-PGK (10–0 ng/well; supplemental Fig. S3) to determine the amount of rGBS-PGK molecules remaining in each well.

Binding of actin and plasminogen to immobilized rGBS-PGK and mutant rGBS-PGK molecules—Binding of actin and plasminogen to immobilized rGBS-PGK and mutant rGBS-PGK molecules were also assayed as previously described (38). Non-mutated rGBS-PGK along with mutant rGBS-PGK molecules, that were found to be enzymatically active, were immobilized to wells of a 96 well polystyrene plate (0.5, 0.25 and 0.125 μg/well) and the wells were incubated 16 h with blocking buffer (5% BSA, 0.1% tween 20 in TBS). Wells were then washed with TBS and incubated with either actin or plasminogen (20 μg/ml in blocking buffer) for 1 h. After this incubation, wells were washed 3× with TBS and incubated with either mouse anti-actin or mouse anti-plasminogen antibodies (1:300 in blocking buffer) for 1 h. Another wash (3×) with TBS was performed, and the plates were incubated with goat anti-mouse IgG alkaline phosphatase-conjugated antibodies (1:200 in blocking buffer; Sigma-Aldrich) for 1 h. Wells were washed 3× with TBS and developed for 30 min with 4-nitrophenol phosphate before stopping the reaction with NaOH (3 n). The A405 was measured using an Athos LP40 microplate reader. Experiments were performed a total of six times. To control for nonspecific binding of the antibodies to the rGBS-PGK molecules, A405 values from wells incubated with 0 μg/ml actin or plasminogen were subtracted from the A405 values obtained from the experimental wells.

Modeling GBS-PGK and Visualization of Actin and Plasminogen Binding Domains—To predict the protein structure, the amino acid sequence of GBS-PGK was submitted to the I-TASSER site as described previously (54–56). The predicted model structure of GBS-PGK was viewed using the RasMol program, and the amino acids identified to be involved in binding to actin and plasminogen were highlighted to determine their relative location within the GBS-PGK molecule.

RESULTS

Interaction of Full-length and Truncated rGBS-PGK with Actin and Plasminogen—To identify the actin and plasminogen binding region of GBS-PGK, full-length and truncated rGBS-PGK molecules were assayed for binding to actin and plasminogen immobilized to 96-well plates. Due to the increased binding of rGBS-PGK to plasminogen compared with actin (38), it was necessary to immobilize 10-fold less plasminogen to the 96-well plates compared with actin to obtain quantifiable results. It was not possible to assess binding of TJB 2 (amino acids 1–165) to either actin or plasminogen, as the anti-rGBS-PGK antibodies did not bind this construct (supplemental Fig.
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S1). Also, TJB 3 (amino acids 166–398) was unstable and could not be purified at a high enough concentration to assay binding activity to actin or plasminogen. However, TJB 1 (amino acids 83–303) was found to bind nearly identical amounts of actin and plasminogen compared with the full-length rGBS-PGK molecule (Fig. 1). Based on this result, further experiments to identify the actin and plasminogen binding sites of GBS-PGK focused on amino acids 83–303.

Peptide Mapping—The GBS-PGK region spanning amino acids 83–303 (TJB 1) was further assayed for binding to both actin and plasminogen using dot blots of 21 peptides spanning this region. Actin binding to peptides 8, 13, and 14 could clearly be visualized along with faint spots corresponding to actin binding to peptides 4, 5, 12, and 15 were observed (Fig. 2A). Plasminogen binding to peptides 4, 5, 8, 13, and 14 were clearly visualized along with a faint spot corresponding to plasminogen binding to peptide 18 (Fig. 2B). These results suggest that the actin binding site of GBS-PGK involves amino acids 123–132, 153–172, and 193–242, whereas the plasminogen binding site of GBS-PGK involves amino acids 123–132, 153–172, 213–222, and possibly 253–272. Because five of the peptides were found to bind both actin and plasminogen, it is possible that the binding sites within GBS-PGK for these two proteins are similar. As a result, mutations resulting in loss of binding to plasminogen may also result in loss of binding to actin. Since it has previously been demonstrated that plasminogen binding by GBS-PGK involves lysine residues within the GBS-PGK molecule (38), lysine residues within the identified regions of GBS-PGK were specifically targeted for mutagenesis.

Site-directed Mutagenesis—Within the amino acid sequence of the peptides identified to bind both actin and plasminogen, three lysine-rich motifs were identified. The first lysine-rich motif was found to span amino acids 126–130 (KKESK), the second motif was found to span amino acids 204–208 (KVSDK), and the third motif was found to span amino acids 218–221 (KADK). In addition, peptide 8 contained a positively charged region spanning amino acids 156–159 (HRAH) that was found to be localized near the first lysine-rich motif. A total of seven mutant rGBS-PGK molecules, summarized in Table 2, were generated targeting these four regions of the GBS-PGK protein. PGK-M1 contained mutations converting the lysine
residues at amino acids 126, 127, and 130 to alanine. PGK-M2 contained mutations converting the lysine residues at amino acids 204 and 208 to alanine. PGK-M3 contained mutations converting the lysine residues at amino acids 218 and 221 to alanine. PGK-M4 contained mutations converting the histidine residues at amino acids 156 and 159 along with the arginine residue at amino acid 157 to alanine. PGK-M5 contained a mutation converting the lysine residue at amino acid 130 to glutamic acid. Because PGK from *Streptococcus pneumoniae* was not identified as a plasminogen binding protein (24), the GBS-PGK protein sequence (57) was compared with the PGK protein sequence from *S. pneumoniae* using BLAST. This analysis revealed that the PGK protein from *S. pneumoniae* contains a proline instead of a glutamic acid at amino acid 133, near the lysine-rich motif spanning amino acids 126–130. Based on this observation, the glutamic acid residues at amino acids 133 and 134 were also targeted for mutation. PGK-M6 contained the glutamic acid to proline mutation at amino acid residue 133 seen in the *S. pneumoniae* PGK molecule. PGK-M7 contained mutations converting the lysine residues at amino acids 126, 127, and 130 along with the glutamic acid residues at amino acids 133 and 134 to alanine. Following mutagenesis, the mutant rGBS-PGK molecules were assayed for enzymatic activity. PGK-M2 and PGK-M4 were found to have sharply reduced enzymatic activity (27 and 17%, respectively; supplemental Fig. S2) compared with non-mutated rGBS-PGK. These mutant proteins were not assayed for binding to actin or plasminogen as they would not be usable for future experiments

| Mutant no./Substitutions | % PGK enzyme activity in comparison with native enzyme |
|--------------------------|-----------------------------------------------------|
| PGK-M1                   | 95%                                                 |
| Lys-126 → Ala            |                                                     |
| Lys-127 → Ala            |                                                     |
| Lys-130 → Ala            |                                                     |
| PGK-M2                   | 27%                                                 |
| Lys-204 → Ala            |                                                     |
| Lys-208 → Ala            |                                                     |
| PGK-M3                   | 69%                                                 |
| Lys-218 → Ala            |                                                     |
| Lys-221 → Ala            |                                                     |
| PGK-M4                   | 17%                                                 |
| His-156 → Ala            |                                                     |
| Arg-157 → Ala            |                                                     |
| His-159 → Ala            |                                                     |
| PGK-M5                   | 112%                                                |
| Lys-130 → Glu            |                                                     |
| PGK-M6                   | 112%                                                |
| Glu-133 → Pro            |                                                     |
| PGK-M7                   | 101%                                                |
| Lys-126 → Ala            |                                                     |
| Lys-127 → Ala            |                                                     |
| Lys-130 → Ala            |                                                     |
| Glu-133 → Ala            |                                                     |
| Glu-134 → Ala            |                                                     |

Binding of rGBS-PGK and Mutant rGBS-PGK to Immobilized Actin and Plasminogen—Binding of the mutant rGBS-PGK molecules to actin and plasminogen that had been fixed to 96-well plates was assayed using ELISA. With the exception of PGK-M3, which appeared to have significantly increased (p < 0.01) binding to both actin and plasminogen, all of the mutant rGBS-PGK molecules demonstrated significantly reduced (p < 0.01) binding to both actin and plasminogen compared with non-mutated rGBS-PGK (Fig. 3). The amount of mutated rGBS-PGK remaining in the wells was compared with the amount of non-mutated rGBS-PGK to estimate the relative binding efficiency of each mutant rGBS-PGK molecule. Compared with rGBS-PGK, the amount of PGK-M1 retained in
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FIGURE 4. Actin binding to wildtype and mutated rGBS-PGK. rGBS-PGK and mutant rGBS-PGK were fixed to a 96-well polystyrene plate and then incubated with actin (20 μg/ml). Plates were probed with anti-actin antibodies followed by anti-mouse IgG alkaline phosphatase conjugate antibodies. Each data point represents the average value of experiments performed a total of six times; error bars represent one S.D. **, p value < 0.01 compared with the same concentration of non-mutated rGBS-PGK. The amount of each mutated rGBS-PGK required to bind the same amount of actin as 0.25 and 0.125 μg of non-mutated rGBS-PGK was determined to estimate the relative loss in binding activity. Lines indicate level of rGBS-PGK binding for comparison with mutants.

 wells containing immobilized actin and plasminogen was 24 and 29%, respectively. The amount of PGK-M3 retained in wells containing immobilized actin and plasminogen was 136 and 140%, respectively. The amount of PGK-M5 retained in wells containing immobilized actin and plasminogen was 24 and 20%, respectively. The amount of PGK-M6 retained in wells containing immobilized actin and plasminogen was 11 and 16%, respectively. Finally, the amount of PGK-M7 retained in wells containing immobilized actin and plasminogen was 34 and 60%, respectively.

Binding of Actin and Plasminogen to Immobilized rGBS-PGK and Mutant rGBS-PGK Molecules—To further characterize the effect of the five mutations (PGK-M1, PGK-M3, PGK-M5, PGK-M6, and PGK-M7), decreasing amounts of the rGBS-PGK molecules (0.5, 0.25, and 0.125 μg) were fixed to wells of a 96-well plate and assayed for binding by both actin and plasminogen. With the exception of PGK-M7, all of the mutant rGBS-PGK molecules demonstrated significantly reduced (p < 0.01) binding to both actin and plasminogen, at all concentrations assayed (Figs. 4 and 5). PGK-M7 was found to have significantly reduced (p < 0.01) binding to actin at all three concentrations assayed, as well as significantly reduced (p < 0.01) binding to plasminogen when 0.25 or 0.125 μg or PGK-M7 was bound to the wells. Actin binding by PGK-M1, PGK-M5, and PGK-M6 were found to be reduced by 75% in comparison with rGBS-PGK, as 0.5 μg of these constructs were found to retain similar amounts of actin as 0.125 μg rGBS-PGK. Actin binding by PGK-M3 and PGK-M7 were found to be reduced by 50%, as 0.25 μg of these constructs retained similar amounts of actin as 0.125 μg of rGBS-PGK and 0.5 μg retained similar amounts of actin as 0.25 μg of rGBS-PGK.

FIGURE 5. Plasminogen binding to wild type and mutated rGBS-PGK. rGBS-PGK and mutant rGBS-PGK were fixed to a 96-well polystyrene plate and then incubated with plasminogen (20 μg/ml). Plates were probed with mouse anti-plasminogen antibodies followed by goat anti-mouse IgG alkaline phosphatase conjugate antibodies. Data points represent the average of experiments performed a total of six times; error bars represent one S.D. **, statistical significance, p value < 0.01, compared with the same concentration of non-mutated rGBS-PGK. The amount of each mutated rGBS-PGK molecule required to bind the same amount of plasminogen as 0.125 and 0.25 μg of non-mutated rGBS-PGK was determined to estimate the relative loss in binding activity. Lines indicate level of rGBS-PGK binding for comparison with mutants.

DISCUSSION

In addition to its essential role in glycolysis, PGK has previously been demonstrated to bind the eukaryotic proteins actin (31, 37, 38) and plasminogen (38, 58, 59). Although actin binding by proteins expressed on the bacterial surface has not been previously characterized as a virulence characteristic, the presence of actin on the surface of a number of human cells (45–50) suggests that the presence of an actin binding protein on the GBS surface may contribute to adhesion to these cells. It has also been demonstrated that expression of GBS-PGK in the cytoplasm of HeLa cells results in disruption of the actin cytoskeleton (38), suggesting that GBS-PGK gaining access to the host cell cytoplasm may contribute to GBS internalization and intracellular survival. In contrast to actin binding, recruitment of plasminogen to the bacterial surface is a well-established virulence mechanism for a number of Gram-positive bacteria (39), including GBS (43, 44). Plasminogen recruitment has been
demonstrated to contribute to bacterial virulence through mediating adhesion to host cells (41, 60, 61). In addition, activation of the recruited plasminogen to plasmin has been demonstrated to play a role in the breakdown of extracellular matrix proteins (41-44) and may contribute to GBS paracellular invasion (62). Initial experiments demonstrated that GBS encodes several plasminogen binding proteins, one of which was identified as GAPDH (43). Further experimentation identified Skizzle as a second GBS surface expressed plasminogen binding protein (44). Although our previous results suggest that PGK may be a third plasminogen binding protein present on the GBS surface (38), we have not yet confirmed a role for PGK in plasminogen recruitment.

To determine the role of actin and plasminogen binding by surface expressed PGK in GBS virulence, it may be necessary to generate a GBS strain expressing a PGK molecule that does not bind actin and plasminogen yet retains its critical enzymatic function. A similar technique has previously been used to determine the function of α-enolase expressed on the surface of *S. pneumoniae* (26, 27). Using truncated rGBS-PGK molecules followed by peptide mapping of the middle region of GBS-PGK, we identified three lysine-rich motifs and a positively charged region that may involved in binding to actin and plasminogen. Site-directed mutagenesis targeting two of the lysine-rich motifs (126–130 and 218–221) resulted in the generation of enzymatically active rGBS-PGK molecules with altered binding to both actin and plasminogen. Amino acid residues 126–130 (KKESKNDEE) likely compose an actin and plasminogen binding site as mutations in this region (PGK-M1, PGK-M5, and PGK-M7) resulted in reduced binding under all ELISA conditions assayed (Fig. 3–5). It is not clear from our results whether these effects were due to changes in the side chains, altered charge in this region or a localized change in the tertiary structure of the protein. Further mutagenesis experiments will be necessary to fully characterize this region of the GBS-PGK molecule as an actin and plasminogen binding site.

In contrast, substitutions targeting amino acids 218 and 221 (PGK-M3) gave inconsistent results based on the assay used. Although more PGK-M3 was retained in wells containing immobilized actin or plasminogen compared with non-mutated rGBS-PGK (Fig. 3), twice as much immobilized PGK-M3 was required to retain the same amount of actin or plasminogen compared with non-mutated rGBS-PGK (Figs. 4 and 5). One potential explanation for these results is that these lysine residues compose a second lower affinity binding site for actin and plasminogen. At high rGBS-PGK concentrations (ELISA assays containing immobilized actin or plasminogen), this minor binding site may compete with the major binding site for binding to actin and plasminogen. At low rGBS-PGK concentrations (ELISA assays containing immobilized rGBS-PGK), both binding sites may retain actin or plasminogen. A second potential explanation is that substitution of the lysine residues 218 and 221 with alanine (PGK-M3) resulted in changes to the GBS-PGK protein structure that affected both the ability of the protein to adhere to the 96-well plates and the reactivity with the anti-rGBS-PGK antibodies. Although it is difficult to predict the effect these substitutions would have on the tertiary structure of GBS-PGK, the reduced enzyme activity of PGK-M3 suggests that structural changes may have occurred. Potentially, PGK-M3 did not immobilize to the wells as efficiently as rGBS-PGK, but this was compensated for during the generation of the standard curves (supplemental Fig. S3) by increased reactivity with the anti-rGBS-PGK antibodies. The increased reactivity with the anti-rGBS-PGK antibodies would result in the apparent increased retention of PGK-M3 in wells containing immobilized actin or plasminogen (Fig. 3), whereas the reduced immobilization efficiency would result in reduced retention of actin or plasminogen in wells containing immobilized PGK-M3 (Figs. 4 and 5). Further experimentation including the production of a double mutant (PGK-M1,M3), co-immunoprecipitation, surface plasmon resonance, or X-ray crystallography may be necessary to determine the cause of these seemingly discrepant results.

Two mutant rGBS-PGK molecules, PGK-M1 and PGK-M5, were generated specifically targeting the lysine residues located within the lysine rich motif spanning amino acids 126–130.
Actin and Plasminogen Binding by GBS-PGK

Both of these mutations were found to have significantly reduced binding to both actin and plasminogen (Fig. 3, 4 and 5). Interestingly, while GBS-PGK has been identified as a surface expressed plasminogen binding protein (38), PGK was not identified in a previous screen to identify plasminogen binding proteins on the surface of S. pneumoniae (24). This led us to compare the amino acid sequences of PGK from GBS and S. pneumoniae. Comparison of the amino acid sequences of GBS-PGK and pneumococcal PGK revealed a difference at amino acid 133. In PGK from GBS this amino acid is glutamic acid while in the PGK molecule from S. pneumoniae this amino acid is proline. This residue was found to be located adjacent to the identified lysine rich motif spanning amino acids 126–130 of the model GBS-PGK structure (Fig. 6). PGK-M6, containing a glutamic acid to proline mutation at amino acid 133, was found to have significantly reduced binding to both actin and plasminogen. These results suggest that the glutamic acid residue at amino acid 133 of GBS-PGK may also be involved in binding to both actin and plasminogen and that the inability of S. pneumoniae PGK to bind to actin or plasminogen may correlate with substitution of the glutamic acid at position 133 with a proline residue. Finally, a fourth mutant rGBS-PGK molecule, PGK-M7, was generated converting the lysine residues 126, 127, and 130 along with the glutamic acid residues 133 and 134 to alanine. Although PGK-M7 was found to have significantly reduced binding to both actin and plasminogen compared with non-mutated rGBS-PGK, PGK-M7 binding to both actin and plasminogen was higher than PGK-M1. These results suggest that the overall charge of this region may also be important for binding to actin and plasminogen as conversion of the glutamic acid residues to alanine partially restored the binding lost by converting the lysine residues to alanine. Our mutagenesis results suggest that amino acids 126–134 (KKESSKNDDEE) of GBS-PGK are involved in binding actin and plasminogen. This actin and plasminogen binding region of GBS-PGK, which contains three lysine residues as well as four negatively charged amino acids is similar to the internal plasminogen binding region of α-enolase from S. pneumoniae (FYDKERKVVD), which contains two lysine residues and three negatively charged amino acids (27). Although our results suggest that amino acids 126–134 of GBS-PGK are involved in binding actin and plasminogen, a competition assay or x-ray crystallography experiments would be necessary to confirm that actin and plasminogen share a binding site on GBS-PGK.

Using site-directed mutagenesis targeting amino acids 126–134, we have generated four rGBS-PGK molecules with significantly reduced binding to both actin and plasminogen (PGK-M1, PGK-M5, PGK-M6, and PGK-M7). Although these mutant rGBS-PGK molecules retained their glycolytic activity, work is ongoing to replace the GBS genomic pgk gene with these mutant pgk genes to determine the role of actin and plasminogen binding by surface-expressed GBS-PGK in GBS virulence.

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