Analysis of Factors Affecting Surface Expression and Immunogenicity of Recombinant Proteins Expressed by Gram-Positive Commensal Vectors

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Several key protein structural attributes were altered in an effort to optimize expression and immunogenicity of a foreign protein (M protein from *Streptococcus pyogenes*) exposed on the surface of *Streptococcus gordonii* commensal bacterial vectors: (i) a shorter N-terminal region, (ii) the addition of a 94-amino-acid spacer, and (iii) the addition of extra C-repeat regions (CRR) from the M6 protein. A decrease in the amount of cell surface M6 was observed upon deletion of 10 or more amino acid residues at the N terminus. On the other hand, reactivity of monoclonal antibody to surface M6 increased with the addition of the spacer adjacent to the proline- and glycine-rich region, and an increase in epitope dosage was obtained by adding another CRR immediately downstream of the original CRR. The results obtained should facilitate the design of improved vaccine candidates using this antigen delivery technology.

Streptococcus gordonii is a commensal bacterium found in the human oral cavity. Recently *S. gordonii* has been used in a vaccine vector system which utilizes surface expression of heterologous antigens to induce mucosal and systemic immunity (16). For example, the *emm6.1* gene encoding the M6 protein from the pathogenic *Streptococcus pyogenes* has been recombined into the recipient strain of *S. gordonii*, and the recombinant bacterium translocates and anchors the protein to its surface (19).

The aim of this study was to optimize expression of the conserved region of the M6 protein on the surface of *S. gordonii* and, if possible, increase the immune response against the molecule in mice immunized with the vaccine. Our approach involved testing three types of changes to the *Δemm6.1* gene: (i) trimming the N-terminal region to test its effect on translocation and anchoring of the protein to the surface of the bacterium; (ii) adding an alpha-helical spacer between the epitope C-repeat region (CRR) and the C-terminal anchor motif (8) in order to potentially increase accessibility of the surface-displayed target peptide to the immune system; and (iii) replicating the target CRR epitope to increase the epitope dosage.

MATERIALS AND METHODS

Bacteria, growth conditions, and DNA manipulations. The bacterial strains used in this study are described in Table 1. The streptococci were grown in brain heart infusion (BHI) (Difco, Detroit, Mich.) or in Todd Hewitt broth (Difco) supplemented with 0.2% yeast extract with or without 1.5% agar. Antibiotics were used at the following concentrations: ampicillin, 50 µg/ml in *Escherichia coli*; kanamycin, 500 µg/ml; erythromycin, 5 µg/ml and streptomycin, 500 µg/ml in *S. gordonii*. Standard procedures were used for gene fusions and mutagenesis in *E. coli* vectors (14).

Antiserum. To detect surface expression of the M6 protein, a mouse monoclonal antibody (MAb), MAb 10F5, was used. It binds to an epitope in the CRR of the M6 protein, between amino acid residues 275 and 289 (13).

Construction of recombinant *S. gordonii*. The recombinant plasmid pSMB104, a derivative of pSMB55 (19), was used as a template for these studies. It is a 5.66-kb *E. coli* plasmid that does not replicate in *S. gordonii*. The plasmid contains *emm6Δ104*, which encodes the signal peptide, the first 16 N-terminal amino acids, and the last 220 C-terminal amino acids of the M6 protein. To reduce the N-terminal region, the last 10 amino acids of the N-terminal region of pSMB104 were looped out, yielding the plasmid pL6 (Fig. 1).

A gene fragment encoding a 184-amino-acid-region from the serum albumin binding region of streptococcal protein G (18) was generated by PCR (primers 5'-GAATTCAACAAATATGGAGTAAGTGAC-3' and 5'-GGATCCAGGTAATGCAGCTAAATTTTCATCTATC-3') with chromosomal DNA from the group G streptococcal strain D845 (Rockefeller University) as a template. Another gene fragment encoding a 94-amino-acid region from the immunoglobulin A binding protein ML2.2 (1, 2) was generated by PCR (upper primer, 5'-GAA TTCATCTGAGTTAACACAAGCAAAG-3'; lower primer, 5'-GGATCC TTGTGATCTCATTCCTTTATTTTG-3') with chromosomal DNA from the group A *S. pyogenes* strain T2/44/Rb4/119 (Rockefeller University collection) as a template. Chromosomal template DNA was prepared from overnight cultures and purified as previously described (4). Both fragments were sequenced in plasmid pCR2.1 (Invitrogen, Carlsbad, Calif.). Each fragment was then subcloned in frame into the *EcoRI-BamHI* site of the polylinker region that was looped into pL6 and pSMB104 upstream of the proline-glycine rich region (6), yielding the plasmids pL6:sG184, pL6:sA94, and pL16:sA94, respectively (Fig. 1).

The CRR of the M6 protein was extracted by PCR (primers 5'-AAGCTTACGCCCCCTTAAACAAGAATTAG-3' and 5'-AAGCTTTTCAACTGTGTTTCTTAGCTTC-3', respectively) using pSMB104 as a template, sequenced in plasmid pCR2.1, and subcloned in frame at a *HindIII* site downstream of the original CRR in pL16:sA94, yielding the plasmid pL16:sA94XCRR (Fig. 1).

Frozen cells of naturally competent *S. gordonii* GP251 were prepared and transformed with each of the plasmids as described previously (20). Plating and scoring of transformants on multilayered plates were also done as described previously (21); erythromycin was added to the overlay at a concentration of 5 µg/ml.

Competition ELISA. The recombinant streptococcal strains were prepared for competition enzyme-linked immunosorbent assay (ELISA) as described previously (3). The resulting cell suspensions were used to compete for the binding of MAb 10F5 to recombinant M6 protein in competition ELISAs as described by Jones et al. (12, 13). Samples from each strain were run in duplicate.

Immunogenicity studies. The intranasal and oral inoculation of mice was performed essentially as described by Medaglini et al. (16), with some modifi-

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TABLE 1. *S. gordonii* strains

Strain	Relevant properties	Reference
GP204	Spontaneous Sm ^r mutant of wild-type V288 (ATCC 35105)	20
GP251	Recombinant recipient strain contains the cat gene flanked by 145 bp of <i>emm6.1</i> gene and 202 bp of <i>ermC</i> gene; Cm ^r	19
L16	Recombinant strain GP1223 that expresses M6 protein (<i>S. pyogenes</i>) residues 1–16 fused to residues 222–441 and contains an <i>aphIII</i> gene; Km ^r Sm ^r	10
L6	Recombinant strain that expresses M6 protein (<i>S. pyogenes</i>) residues 1–6 fused to residues 222–441; Em ^r Sm ^r	This study
L6:sA94	L6 + sA94 spacer; Em ^r Sm ^r	This study
L6:sG184	L6 + sG184 spacer; Em ^r Sm ^r	This study
L16:sA94	L16 (GP1223) + sA94 spacer; Em ^r Sm ^r	This study
L16:sA94XCRR	L16:sA94 + extra CRR at <i>HindIII</i> ; Em ^r Sm ^r	This study

cations. Briefly, *S. gordonii* recombinants were grown in 50 ml of BHI broth containing 500 µg of streptomycin sulfate/ml until an optical density at 650 nm of ~0.8 (late exponential phase) was reached. Cultures were placed immediately on ice for 15 min and then centrifuged at 2,800 × *g* for 15 min at 4°C. The resulting cell pellet was resuspended in 1/50 of the original volume with fresh BHI. Two days prior to immunization, 6-week-old BALB/c mice were administered streptomycin water (5 g/liter). Mice (10 per group) received two 50-µl inocula, spaced 2 days apart, with the volume divided evenly between the nares and the oral cavity. Regular tap water replaced the streptomycin water after the second inoculation. The average inoculum was ~6 × 10⁸ CFU/dose. Prior to the original inoculation and at weeks 4, 8, and 12, mice were bled from the saphenous vein and saliva was collected using a 2.5- by 25-mm polyester wick (Whatman, Clifton, N.J.). Saliva was extracted from the wick by using 400 µl of ice-cold phosphate-buffered saline and centrifuging the fluid from the wick in a micro-centrifuge. Serum and saliva samples were stored at –20°C.

Serum immunoglobulin G (IgG) levels were determined by standard ELISA, as described previously (9). Reciprocal endpoint titers were determined at two-fold over background on duplicate samples. Salivary IgA levels were analyzed by chemiluminescence ELISA on triplicate samples. Briefly, 96-well black microplates (Packard, Meriden, Conn.) were coated with 100 µl of a 5-µg/ml solution of recombinant M6 protein in 50 mM carbonate buffer, pH 10, overnight at room temperature. For IgA standards, microplate wells were coated with 1 µg of anti-mouse IgA (Sigma, St. Louis, Mo.)/ml under the same conditions. Plates were washed five times with phosphate-buffered saline containing 0.05% Brij-35 and blocked with casein block (Pierce, Rockford, Ill.) with 0.05% Brij-35 added for 60 min at 37°C. Saliva samples (1:2 dilution) or mouse IgA standard dilutions (mouse myeloma TEPC 15; Sigma) were added (50 µl) in blocking solution and incubated at 37°C for 3 h. Plates were washed, and goat anti-mouse IgA horse-radish peroxidase conjugate (1:4,000; Sigma) was added (50 µl) and incubated for 2 h at 37°C. After a final wash, a chemiluminescent substrate (Power Signal; Pierce) was added, and plates were read within 10 min with a Tecan SpectraFluor Plus (Tecan USA, Research Triangle Park, N.C.). Values for M6-specific IgA concentrations were determined by extrapolating luminescence readings in the IgA standard curve.

RESULTS AND DISCUSSION

Surface expression of M6 CRR. Previous studies have demonstrated the use of the *S. gordonii* vector system to express a variety of foreign proteins (5, 7, 15–17, 22). However, optimization of this system for its use as a vaccine vector has yet to be undertaken. Here we assess the contribution of three parameters (N-terminal leader length, extension of the expressed protein further from the bacterial surface, and epitope duplication) on the expression and immunogenicity of foreign proteins.

We first assayed the effect of altering the N terminus of the protein to be expressed on the cell surface. In the original construction the N-terminal 58 amino acids of the M6 protein were appended to the CRR of the M6 protein. During the process of translocation across the plasma membrane, the N-terminal 42 amino acids are removed by the leader peptidase (11), leaving 16 amino acids of M6 sequence attached. Al-

FIG. 1. Schematic representation of the M6 recombinants based on the L16 backbone. NTR, N-terminal region; triangle, cleavage point; CRR, C repeat region of the M6 protein; Pro/Gly, proline- and glycine-rich region.

FIG. 2. Competition ELISA with *S. gordonii* recombinant strains expressing surface-anchored M protein versus purified M6 protein. The graphs show percent inhibition of binding of MAb 10F5 to recombinant M6 protein by decreasing concentrations of cells. (A) Strains used were L16, L6, L6:sA94, L6:sG184, and GP204, which is used as a negative control. (B) Strains used were L16, L16:sA94, and L16:sA94:XCRR. Undil., undiluted.

though the protein was efficiently expressed in this configuration, it was of interest to determine if the amount of M6 sequences could be reduced to avoid including extraneous epitopes that could potentially induce deleterious cross-reactions (13). As shown in Fig. 2A, trimming the residual M6 protein sequences from 16 amino acids (L16) to 6 amino acids (L6) did not affect expression of the CRR segment of the M6 protein on the cell surface as measured by competition ELISA. Further deletions of the remaining 6 amino acids resulted in a marked reduction in foreign protein expression (data not shown).

Gram-positive bacteria express a number of different surface-anchored proteins. Thus, it was of interest to determine if lengthening the foreign protein by insertion of an in-frame fusion (thereby extending it away from the bacterial cell wall) would affect either epitope accessibility or immunogenicity. In

order to test this hypothesis, a 94-amino-acid spacer from the immunoglobulin A binding protein ML2-2 (2) and a 184-amino-acid spacer from the serum albumin binding region of streptococcal protein G (18) were used. These sequences were chosen by virtue of their predicted alpha-helical character, which should have minimal impact on surrounding regions. This was done in both the L6 and L16 backbone. Addition of spacer elements into the L6 background appeared to increase MAb 10F5 reactivity to the expressed M protein only slightly (two- to threefold) (Fig. 2A). In contrast, the addition of the sA94 spacer in L16 increased reactivity levels markedly (six-fold) over that of the L16 construct alone (Fig. 2B). This result could potentially be due to increased protein stability, enhanced transport, or better access to the target epitope as a result of the spacer moving the CRR farther from the surface of the bacterium.

FIG. 3. Serum IgG ELISA results for pooled sera from mice inoculated with L6, L6:sA94, and L6:sG184 (A) or L16, L16:sA94, and L16:sA94:XCRR (B) collected at 0, 4, 8, and 12 weeks postinoculation.

FIG. 4. Salivary IgA ELISA results of pooled saliva from mice inoculated with L6, L6:sA94, and L6:sG184 (A) or L16, L16:sA94, and L16:sA94:XCRR (B) collected at 0, 4, 8, and 12 weeks postinfection.

The third modification tested was the effect of duplicating the target epitope in order to expose the immune system to multiple copies of the antigenic region. Since the L16 backbone with the sA94 spacer gave the highest level of surface-expressed protein in the competition ELISA, this construct was used for the epitope duplication studies. Addition of an extra CRR increased the MAb 10F5 reactivity levels twofold in the L16:sA94 background (Fig. 2B), showing the efficacy of epitope duplication.

Immunogenicity of the M6 CRR expressed on the surface of *S. gordonii* recombinants. To further test the modifications made to the *S. gordonii* vector system, each of the recombinant strains was inoculated into mice for immunogenicity studies. Blood and saliva were collected at various times from each mouse and used to determine serum IgG and salivary IgA levels, respectively. Serum IgG levels were determined by standard ELISA, as described previously (9). Reciprocal endpoint titers were determined at twofold over background on duplicate samples. Week 12 results for the L6 constructs showed an increase in IgG titers with the addition of the sA94 spacer and even more so with the sG184 spacer (Fig. 3A). In the L16 background the L16:sA94 spacer construct showed a significant increase in IgG titer over that of the L16 strain, and the addition of the extra CRR (L16:sA94XCRR) elicited a stronger IgG response in both week 8 and week 12 than was found with the constructs without epitope duplication (Fig. 3B). In addition, the L16:sA94 (Fig. 3B) construct elicited a slightly stronger response than the L6:sA94 construct (Fig. 3A).

Salivary IgA levels were analyzed by chemiluminescence ELISA on triplicate samples. The L6 constructs elicited a small but detectable mucosal response which showed a statistically significant peak at week 8 (Fig. 4A). The L6:sA94 construct displayed the highest titers (Fig. 4A) of the three L6 constructs at week 8 and had measurements similar to those for L16 and L16:sA94 IgA (Fig. 4B). In contrast, the L16:sA94XCRR construct evoked a strong IgA response in week 4 and week 8, similar to the other L16 constructs at week 8, but had an increased response in week 12 (Fig. 4B). Clearly the N-termi-

nal sequence length plays a role in the mucosal immune response.

In summary, these results suggest that surface expression and immunogenicity of proteins expressed by recombinant *S. gordonii* vectors can be enhanced by extension from the cell surface with an alpha-helical spacer and/or using epitope duplication procedures. Although N-terminal truncation had no apparent effect on expression, it significantly affected the immune response of salivary IgA and, to a lesser extent, that of serum IgG. Together, consideration of the parameters tested should provide improved future vaccine candidates for clinical study.

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