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Identification of B- and T-Cell Epitopes within the Fibronectin-Binding Domain of the SfbI Protein of *Streptococcus pyogenes*

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The fibronectin-binding repeats of the SfbI protein of *Streptococcus pyogenes* constitute the minimal domain able to confer protection against lethal infection. We investigated the presence of B- and T-cell epitopes within this region in congenic mice. One linear B-cell epitope was recognized by BALB/b and BALB/k mice, whereas two epitopes were found in BALB/c animals. A unique T-cell epitope was recognized by all three mouse strains. All identified epitopes clustered in a 30-amino-acid fragment. These results suggest that this polypeptide may be suitable for incorporation into a polyepitope-based vaccine formulation against *S. pyogenes*.

The human pathogen *Streptococcus pyogenes* can cause localized infections resulting in noninvasive diseases like pharyngitis, impetigo, or scarlet fever. These infections can lead to severe poststreptococcal diseases, such as rheumatic fever and acute poststreptococcal glomerulonephritis. In addition, *S. pyogenes* can cause highly invasive diseases, such as sepsis, necrotizing fasciitis, and toxic shock-like syndrome (3, 25, 32). The incidence of *S. pyogenes* infections and their sequelae, as well as the emergence of strains that are resistant to macrolides (e.g., erythromycin), has been steadily increasing (7, 11, 13, 18). In the last decade, several approaches have been pursued to develop a vaccine against *S. pyogenes*. The M protein, a major virulence factor of *S. pyogenes*, constitutes the best-characterized vaccine candidate (2, 4, 17). However, M protein mainly triggers serotype-specific immunity and may lead to cross-reaction with host tissues. In the last few years the C5a peptidase, a surface-bound peptidase that cleaves C5a (10), and the extracellular cysteine protease, which cleaves fibronectin and converts interleukin-1β precursor to biologically active interleukin-1β (12), have been proposed as candidate antigens. The use of peptides encompassing conserved B- and T-cell epitopes of the M protein constitutes a new approach to prevent diseases caused by different M serotypes and the potential side effects resulting from immune cross-recognition (1, 4, 5, 6, 20). However, peptides are usually poorly immunogenic and need to be administered with depot-forming adjuvants (19, 30) or bacterial toxins or their derivatives (2, 24). They can also be generated using approaches in which multiple copies of the epitopes are displayed (17, 31).

It has been shown that intranasal vaccination with the fibronectin-binding protein I (SfbI) of *S. pyogenes* stimulates humoral and cellular immune responses, which are able to protect against lethal challenge with *S. pyogenes* (9, 22). The SfbI protein plays a central role in the virulence process; is highly conserved, surface located, and expressed by a large number of clinical isolates from different serotypes (approximately 73%); and does not lead to cross-reactivity with host tissues (16, 26–29). Additional studies have identified the domain encompassing the fibronectin-binding repeats (148 amino acids) as the minimal fragment able to confer protection (Fig. 1). In an attempt to further characterize the vaccine-relevant immune determinants of the SfbI protein, we mapped the linear B-cell and T-cell epitopes located within the fibronectin-binding repeats.

Identification of linear B-cell epitopes within the fibronectin-binding repeats of the SfbI protein. A vaccine should be able to stimulate efficient immune responses in individuals, who are genetically different. Thus, initial studies were performed to investigate whether efficient humoral immune responses against the fibronectin-binding domain were stimulated in congenic BALB/c, BALB/b, and BALB/k mice, after intranasal immunization (11 μg on days 1, 14, and 21) with a polypeptide encompassing the fibronectin-binding domain. The obtained results demonstrated that similar immunoglobulin G (IgG) responses were obtained in all mouse strains, which were characterized by a dominant IgG1 isotype response pattern (data not shown). Thus, subsequent work was carried out to characterize the linear B-cell epitopes recognized by sera from vaccinated animals by using two complementary approaches.

Enzyme-linked immunosorbent assay (ELISA) studies were performed using streptavidin-coated microtiter plates (Pierce), which were incubated with 16-mer biotinylated overlapping peptides (5 μg/well) spanning the first two fibronectin-binding repeats of the SfbI protein with an offset of 4 amino acids (Fig. 1). Plates were then incubated with the sera from immunized animals, and bound antibodies were detected as previously described (23). As shown in Fig. 1 and 2, only one epitope was recognized by sera from BALB/b and BALB/k mice, which corresponds to the NH_2-terminal part of each repeat (positions 377 to 387 and 414 to 424, respectively). To further confirm these results, immunoblot studies were performed using cellulose paper membranes (1Chr; 3MM; Whatman) spotted with 15-mer peptides (8), which encompass all four fibronectin-binding repeats (positions 373 to 515 of the SfbI sequence, accession no. X67947) with an offset of 3 amino acids (Table

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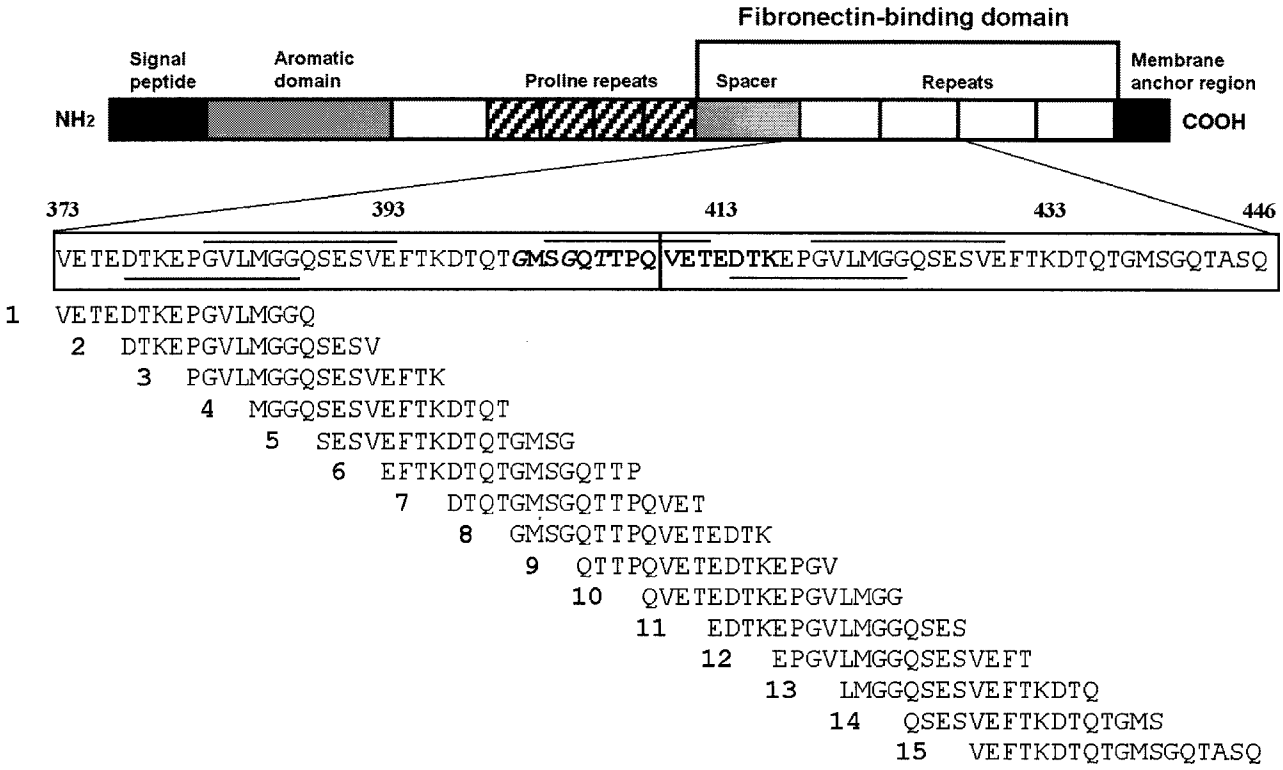

FIG. 1. Peptides spanning two fibronectin-binding repeats used for epitope-mapping studies. Linear B-cell epitopes recognized in BALB/b and BALB/k mice are underlined, whereas those detected in BALB/c mice are indicated by an overline. The T-cell epitope found in all tested mice is indicated in boldface, with the predicted anchor residues in italics.

1). In brief, after overnight incubation in blocking buffer (2 ml of Genosys blocking buffer, 5% saccharose, 8 ml of T-TBS [50 mM Tris base-buffered saline plus 0.05% Tween 20, pH 7.0]), membranes were further incubated with sera from immunized mice diluted 1:100 in blocking buffer for 3.5 h. After three washes with T-TBS, membranes were treated with alkaline phosphatase-conjugated goat anti-mouse antibodies (Dianova, Hamburg, Germany) diluted 1:500 in blocking buffer for 1.5 h. After washing, bound antibodies were detected by incubating membranes in 50 μl of 1 M $MgCl_2$–40 μl of 5-bromo-4-chloro-3-indolylphosphate–60 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in 10 ml of CBS (8 g of NaCl, 0.2 g of KCl, 2.1 g of 10 mM citric acid–1-hydrate in 1 liter). Densitometric analysis of the spots was carried out using the Multi-Analyst analysis software (Bio-Rad Laboratories, Inc.), and results were expressed as relative densities (optical density per square millimeter).

The obtained results demonstrated that the linear B-cell epitope recognized by BALB/b and BALB/k mice corresponds to the sequence DTKEPGVLMGG (Fig. 1 and 3). Peptides encompassing this region showed similar antibody binding rates, whereas binding was drastically reduced when the NH_2-terminal aspartic acid or the COOH-terminal glutamic acid was lost (Fig. 1). In contrast, two epitopes were recognized by sera from mice of the BALB/c haplotype. The first one was similar to the one recognized by BALB/b and BALB/k mice and is located between positions 382 and 393 and positions 419 and 430 (GVLMGGQSESVE), respectively. Peptides encompassing this sequence showed the strongest antibody binding rates in both ELISA and Western blot studies (Fig. 2A and 3A), and antibody binding rates were dramatically reduced by loss of the NH_2-terminal glycine or the COOH-terminal glutamic acid. The second linear B-cell epitope recognized in BALB/c mice is located in the interrepeat region between positions 403 and 412 (SGQTTPQVET), and also between positions 440 and 449, in which two mismatches are located (SGQTASQVET). Thus, it seems that these two residues are not essential for antibody recognition. By using the antigenic index algorithm (PROTEAN software from DNAStar, Inc., Madison, Wis.) it was shown that all detected epitopes were localized in regions of high probability for antigen determinants. Preliminary studies suggest that similar regions are also recognized by sera from patients convalescing from *S. pyogenes* infections. When tested by ELISA, antibodies were present in the sera (n = 10), which were able to recognize linear B-cell epitopes encompassed within positions 389 to 416 of SfbI. However, to prove the statistical significance of these initial data, it would be essential to analyze a larger number of samples obtained from patients with a different form of the disease.

Identification of T-cell epitopes within the fibronectin-binding repeats of the SfbI protein. To identify T-cell epitopes located within the fibronectin-binding repeats, spleen cells recovered from immunized mice were restimulated in vitro using the 15 individual peptides described for Fig. 1. Preliminary analysis performed using different concentrations of each pep-

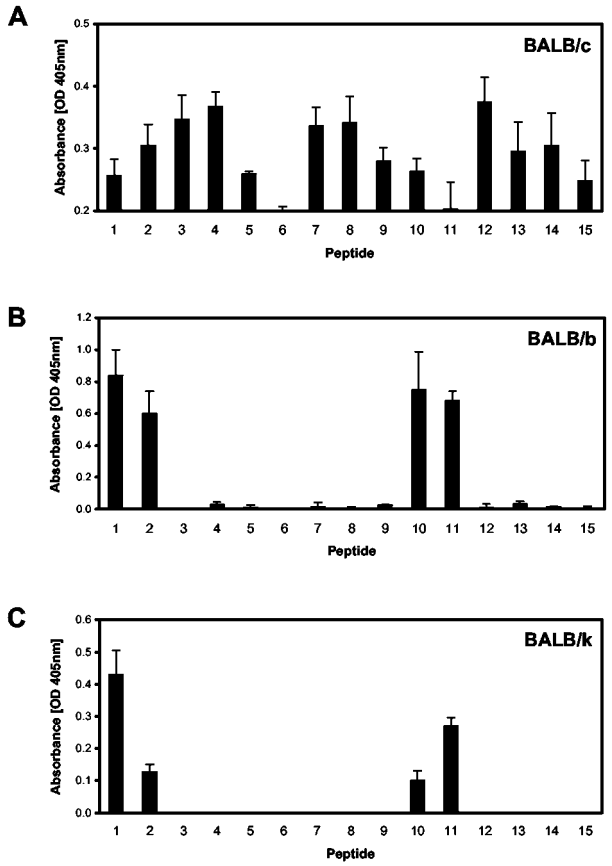

FIG. 2. Identification of B-cell linear epitopes within the fibronectin-binding repeats of the SfbI protein. Peptide-specific serum IgG responses stimulated after vaccination were determined by ELISA in BALB/c, BALB/b, and BALB/k mice (n = 5). Standard errors of the means are indicated by vertical lines. OD, optical density.

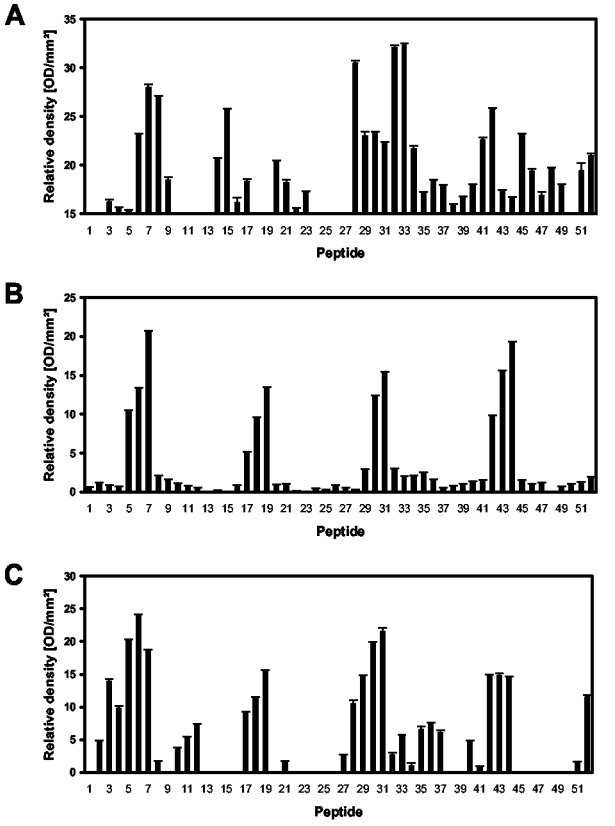

FIG. 3. Identification of B-cell linear epitopes within the fibronectin-binding repeats of the SfbI protein. Peptide-specific serum IgG responses stimulated after vaccination were determined by immunoblot analysis in BALB/c (A), BALB/b (B), and BALB/k (C) mice (n = 5). Standard errors of the means are indicated by vertical lines. OD, optical density.

TABLE 1. Peptides spanning the four fibronectin-binding repeats used to identify linear B-cell epitopes by immunoblot analysis

No.	Positions	Peptide sequence	No.	Positions	Peptide sequence
1	361–375	KLPNETGFSGNMVET	27	439–453	MSGQTASQVETEDTK
2	364–378	NETGFSGNMVETEDT	28	442–456	QTASQVETEDTKEPG
3	367–381	GFSGNMVETEDTKEP	29	445–459	SQVETEDTKEPGVLM
4	370–384	GNMVETEDTKEPGVL	30	448–462	ETEDTKEPGVLMGGQ
5	373–387	VETEDTKEPGVLMGG	31	451–465	DTKEPGVLMGGQSES
6	376–390	EDTKEPGVLMGGQSE	32	454–468	EPGVLMGGQSESVEF
7	379–393	KEPGVLMGGQSESVE	33	457–471	VLMGGQSESVEFTKD
8	382–396	GVLMGGQSESVEFTK	34	460–474	GGQSESVEFTKDTQT
9	385–399	MGGQSESVEFTKDTQ	35	463–477	SESVEFTKDTQTGMS
10	388–402	QSESVEFTKDTQTGM	36	466–480	VEFTKDTQTGMSGQT
11	391–405	SVEFTKDTQTGMSGQ	37	469–483	TKDTQTGMSGQTTPQ
12	394–408	FTKDTQTGMSGQTTP	38	472–486	TQTGMSGQTTPQVET
13	397–411	DTQTGMSGQTTPQVE	39	475–489	GMSGQTTPQVETEDT
14	400–414	TGMSGQTTPQVETED	40	478–492	GQTTPQVETEDTKEP
15	403–417	SGQTTPQVETEDTKE	41	481–495	TPQVETEDTKEPGVL
16	406–420	TTPQVETEDTKEPGV	42	484–498	VETEDTKEPGVLMGG
17	409–423	QVETEDTKEPGVLMG	43	487–501	EDTKEPGVLMGGQSE
18	412–426	TEDTKEPGVLMGGQS	44	490–504	KEPGVLMGGQSESVE
19	415–429	TKEPGVLMGGQSESV	45	493–507	GVLMGGQSESVEFTK
20	418–432	PGVLMGGQSESVEFT	46	496–510	MGGQSESVEFTKDTQ
21	421–435	LMGGQSESVEFTKDT	47	499–513	QSESVEFTKDTQTGM
22	424–438	GQSESVEFTKDTQTG	48	502–516	SVEFTKDTQTGMSGF
23	427–441	ESVEFTKDTQTGMSG	49	505–519	FTKDTQTGMSGFSET
24	430–444	EFTKDTQTGMSGQTA	50	508–522	DTQTGMSGFSETVTI
25	433–447	KDTQTGMSGQTASQV	51	511–525	TGMSGFSETVTIVED
26	436–450	QTGMSGQTASQVETE	52	514–528	SGFSETVTIVEDTRP

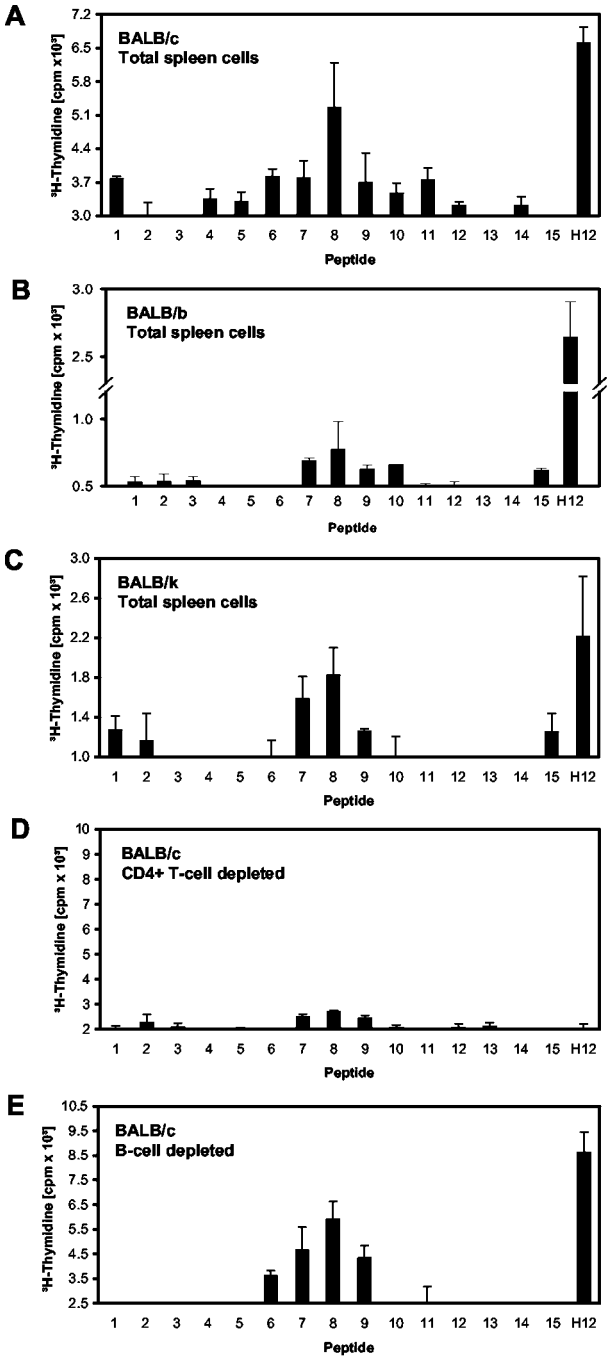

FIG. 4. Identification of T-cell epitopes within the fibronectin-binding repeats of the SfbI protein. Peptide-specific proliferative responses from immunized mice were evaluated using total spleen cells (A to C) and after depletion of either $CD4^+$ T cells (D) or B cells (E). Results are expressed as mean counts per minute of triplicate samples. Standard errors of the means are indicated by vertical lines.

tide in the range of 0 to 50 μg/ml showed that optimal responses were obtained when the peptides were tested at 50 μg/ml. Proliferation was assessed after 4 days of culture by measuring the incorporation of [^{3}H]thymidine (14, 15). The obtained results suggest the presence of one T-cell epitope located in the crossover from one repeat to the next (GMSG QTTPQVETEDTK), since the peptide encompassing positions 401 to 416 stimulated the strongest cellular response (Fig. 4A). Similar results were observed when cells from BALB/b and BALB/k vaccinated mice were tested (Fig. 4B and C). To further verify the nature of the stimulated cells, proliferation studies were performed using spleen cells depleted from either B cells or $CD4^+$ T cells. While no effect could be observed when B cells were depleted, depletion of $CD4^+$ cells significantly decreased the proliferation rates (Fig. 4D and E). This confirmed that $CD4^+$ T cells constitute the stimulated cellular population. Interestingly, a motif within this sequence is also predicted to be recognized in the human system by the SYFPEITHI algorithm (21).

In conclusion, our results indicate that efficient immune responses are stimulated in congenic mice from different haplotypes after intranasal immunization with a polypeptide encompassing the fibronectin-binding domains of the SfbI protein. This fragment also constitutes the minimal domain able to confer protective immunity. Furthermore, similar linear B-cell epitopes, as well as a T-cell epitope, were recognized in all vaccinated mouse strains. Thus, all experimentally determined linear B- and T-cell epitopes are cross-recognized and encompassed within a 30-amino-acid fragment. The obtained results provide critical information for the exploitation of the fibronectin-binding domain of the SfbI protein in the development of polyepitope-based vaccines against *S. pyogenes*.

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