LOCALIZATION OF PROTECTIVE EPITOPES OF THE AMINO TERMINUS OF TYPE 5 STREPTOCOCCAL M PROTEIN

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It has been known for many years that protective immunity against group A streptococcal infections is directed against the surface M protein of these organisms. Purified M proteins are immunogenic and evoke opsonic antibodies in laboratory animals and humans (1-7). In addition to type-specific, protective immunity, M proteins from several serotypes of streptococci also elicit antibodies that crossreact with human tissues, particularly the myocardium (7-10). These findings are of considerable concern to investigators attempting to develop M protein vaccines that would protect against infection by strains of group A streptococci that may give rise to acute rheumatic fever and rheumatic heart disease. The fear has been that the vaccines themselves may evoke autoimmune responses that could lead to host tissue injury.

To avoid the potential immunological toxicity of intact M proteins, and also to minimize the total amount of protein included in streptococcal vaccines, studies have been undertaken to determine the protective immunogenicity of natural and synthetic peptides of several serotypes of M protein (11-16). Peptides as small as 35 amino acid residues obtained by cyanogen bromide cleavage of type 24 M protein evoked opsonic antibodies in laboratory animals (11). Chemically synthesized peptides representing regions of types 5, 6, and 24 M proteins, ranging in size from 10 to 35 amino acids, have proven to be effective immunogens (12-16). None of the peptides representing very limited regions of intact M molecules has evoked tissue-crossreactive antibodies. These studies have provided definitive evidence that the entire M protein molecule is not necessary to elicit protective immunity, and that type-specific epitopes may be separated from tissue-crossreactive regions of M protein.

The present study was undertaken to determine the precise location of protective epitopes within the amino terminus of type 5 M protein, a rheumatogenic serotype with worldwide distribution. We report here that a chemically synthesized peptide representing the first 35 amino terminal residues of type 5 M protein was immunogenic in rabbits and evoked opsonic antibodies against type 5 streptococci that did not crossreact with myocardial tissue. Using a set of overlapping synthetic subpeptides, a major protective region was localized to a...
13-residue peptide within the 35-residue peptide. We believe these studies have relevance to the development of a safe and effective streptococcal vaccine.

Materials and Methods

Extraction and Purification of M Protein. M proteins were purified from limited peptic digests of types 5, 6, 19, and 24 group A streptococci (2-4). The purified M proteins (pep M) were judged to be homogeneous by SDS-PAGE (3).

Chemical Synthesis of Peptides. Overlapping polypeptides identical to the amino-terminal amino acid sequence of pep M5 (17) were synthesized by an automated peptide synthesizer (Beckman Instruments, Fullerton, CA) by a solid-phase method (18) as previously described (14). The peptides were purified by HPLC and sequenced by automated Edman degradation (13-15). The peptides synthesized for this study are designated SM5(1-35), SM5(14-35), SM5(21-35), SM5(26-35), and SM5(14-26). An additional peptide representing amino acid residues 14-26 was synthesized with a carboxyl-terminal cysteine, SM5(14-26)C, which allowed coupling to a carrier molecule using a bifunctional crosslinking reagent.

Conjugation of Synthetic Peptides. SM5(1-35), SM5(26-35), and SM5(14-26) were conjugated to lysylated tetanus toxoid as previously described (15). SM5(14-26)C was conjugated to KLH using succinimidyl-4-(N-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) (Pierce Chemical Co., Rockford, IL) as described (19), with the modifications of Rothbard et al. (20).

Immunization of Rabbits. New Zealand White rabbits were injected subcutaneously with 100 μg of the conjugated peptides, which had been emulsified in CFA (13-15). Blood was obtained before the initial injection, and at 2-wk intervals thereafter. At 4 and 10 wk, the animals were given booster injections of 100 μg of the conjugated peptide in (0.02 M phosphate, 0.15 M NaCl, pH 7.4). Rabbit antisera against the natural M5 protein was similarly prepared by immunizing with 100 μg doses of pep M5 or against whole type 5 streptococci by injecting 10⁸ streptococci (heat-killed, 56°C for 30 min) intracutaneously in 1 ml PBS. Booster injections of the same dose were given every 4 wk for 12 wk; one-tenth of the dose was given intraperitoneally, followed several hours later by intravenous injection of the remainder of the organisms.

M Protein Antibody Assays. Sera were tested for the presence of the M protein antibodies by an ELISA using unconjugated synthetic peptides or pep M proteins as solid-phase antigens, as previously described (7-21). In some cases, ELISAs were performed using type 5 streptococci as particle-phase test antigens (22). ELISA inhibition experiments were performed by incubating a constant dilution of antiserum with increasing concentrations of synthetic peptides or pep M5, as soluble inhibitors (15-21). Opsonic antibodies were detected by in vitro opsonophagocytic assays (9), and the peptide specificity of opsonic antibodies directed against type 5 streptococci was determined by opsonization inhibition tests using the synthetic peptides as soluble inhibitors (7, 13).

Affinity Purification of SM5(1-35) Antibodies from Antiserum against Whole Type 5 Streptococci. Antibodies raised against whole type 5 streptococci that reacted with SM5(1-35) were affinity purified over a column of CH-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) to which SM5(1-35) had been covalently linked (7, 9). The purified antibodies were dialyzed against PBS and concentrated to the original volume of serum by membrane filtration (YM 30 membrane; Amicon Corp., Danvers, MA).

Detection of Heart-crossreactive Antibodies. Indirect immunofluorescence tests for heart-crossreactive antibodies were performed using purified sarcolemmal membranes or thin sections of human heart tissue, as described elsewhere (7). Antibodies crossreactive with myosin were detected by ELISA (10).

Results

Amino Acid Sequences of Chemically Synthesized Peptides. The primary structures of the overlapping peptides synthesized for this study were determined by
automated Edman degradation to assure their identity with the native pep M5 molecule (Table I). All of the peptides were identical to the corresponding regions of the primary structure of pep M5 (17).

**Immunogenicity of SM5(1-35) and SM5(26-35).** Initial experiments were performed to determine the immunogenicity of SM5(1-35), the longest polypeptide synthesized for the study, and SM5(26-35), the shortest polypeptide, both of which had been covalently linked to tetanus toxoid. All six rabbits immunized with SM5(1-35) developed significant levels of antibody against the immunogen and native pep M5, as determined by ELISA (Table II). All six rabbits also developed significant levels of opsonic antibodies against type 5 streptococci, as determined by in vitro opsonophagocytic tests (Table II). None of the immune sera crossreacted with human heart tissue or myosin, as determined by indirect immunofluorescence tests and ELISA, respectively. These results show that
SM5(1-35) is immunogenic and evokes antibodies that recognize protective epitopes of type 5 M protein on the surface of the streptococcus.

Three rabbits were also immunized with SM5(26-35). The immune sera contained significant levels of antibody against SM5(1-35) and pep M5 (Table III). In contrast to the SM5(1-35) immune sera, SM5(26-35) failed to evoke opsonic antibodies against type 5 streptococci, although the ELISA titers against pep M5 were equivalent for both sets of antisera. These results suggested that the antibodies raised against SM5(26-35) were binding to epitopes of pep M5 that may not be exposed on the surface of type 5 streptococci. To test this hypothesis, a particle-phase ELISA was performed using whole type 5 streptococci as the test antigen. None of the SM5(26-35) immune sera reacted with type 5 streptococci (Table III), confirming that the antibodies were directed against pep M5 epitopes that were not exposed on the type 5 M protein in its native conformation on the surface of the organism.

**Peptide Specificity of Pep M5-reactive Antibodies Evoked by SM5(1-35).** To determine the location of epitopes within SM5(1-35) that evoked antibodies that reacted with native pep M5, ELISA inhibition tests were performed using the overlapping synthetic peptides as soluble inhibitors of SM5(1-35) antibody binding to pep M5 (Fig. 1). As expected, increasing concentrations of SM5(1-35), the immunizing antigen, and pep M5, the solid-phase test antigen, completely inhibited the binding of SM5(1-35) antibodies to pep M5 (Fig. 1). Interestingly, SM5(14-35) also completely inhibited the binding of antibodies to pep M5, suggesting that all of the relevant epitopes of SM5(1-35), or those that evoke pep M5–reactive antibodies, reside in the SM5(14-35) peptide. SM5(21-35) partially inhibited antibody binding, whereas SM5(26-35) had no effect (Fig. 1). Although these data were obtained using antiserum from one rabbit immunized with SM5(1-35), similar patterns of inhibition were obtained from all six immune sera (data not shown).

Taken together, the data outlined thus far suggest that all of the immunogenic protective epitopes of SM5(1-35) reside between amino acid residues 14 to 26: (a) all of the pep M5 antibodies evoked by SM5(1-35) were inhibited by SM5(14-
Peptide specificity of pep M5-reactive antibodies raised against SM5(1-35) as determined by inhibition of ELISA. A constant dilution of anti-SM5(1-35) was incubated with increasing concentrations of pep M5 (■), SM5(1-35) (●), SM5(14-35) (▲), SM5(21-35) (▲), or SM5(26-35) (○) used as soluble inhibitors of antibody binding to the natural pep M5 antigen, which was immobilized on plastic plates. The highest concentration of the synthetic peptides was 40 nM, while the highest concentration of pep M5 was 10 μg/ml.

ELISA inhibition by the subpeptide SM5(14-26) of pep M5-reactive antibodies raised against SM5(1-35). A constant dilution of anti-SM5(1-35) was incubated with increasing concentrations of either pep M5 (●) or SM5(14-26) (○) as soluble inhibitors of antibody binding to pep M5, which was the solid-phase antigen. The highest concentration of SM5(14-26) was 40 nM, and the highest concentration of pep M5 was 10 μg/ml.

35); (δ) none of the pep M5 reactive antibodies was inhibited by SM5(26-35); and (ε) SM5(26-35) evoked antibodies that were nonopsonic. ELISA inhibition experiments with SM5(1-35) as the solid-phase antigen and pep M5 as the soluble inhibitor revealed that not all of the antibodies were inhibited by pep M5, indicating that a portion of the antibodies were peptide specific (data not shown).

Antigenicity of SM5(14-26). To test the hypothesis that the protective epitopes of SM5(1-35) were located between amino acid residues 14 and 26, this peptide was synthesized and tested for its ability to inhibit type-specific and opsonic antibodies evoked by SM5(1-35). Increasing concentrations of SM5(14-26) completely inhibited SM5(1-35) antibody binding to pep M5, as determined by ELISA inhibition studies (Fig. 2).

Opsonization-inhibition assays were also performed to test for the presence of protective epitopes within SM5(14-26). Opsonic antibodies against type 5 streptococci that were evoked by SM5(1-35) were completely inhibited by SM5(14-26) (Table IV). Total inhibition was also observed with SM5(1-35), the immunogen, and pep M5, the natural peptide (Table IV). Opsonization of type 5 streptococci by the remaining SM5(1-35) antisera was similarly inhibited by
SM5(14-26), indicating that the immunodominance of this region of the polypeptide was not the result of individual variation in immune responses.

Because our previous studies have shown that SM5(1-20) evoked opsonic antibodies against type 5 streptococci whereas SM5(20-40) did not (15), we also used these two peptides as inhibitors of opsonic antibodies raised against SM5(1-35) (Table IV). Opsonization of type 5 streptococci by one of the antisera (8426) was totally inhibited by SM5(1-20), but not at all by SM5(20-40) (Table IV). Taken together with the finding that SM5(14-26) also completely inhibited opsonization by this antiserum, the data suggest that all of the opsonic antibodies are directed against the region between amino acid residues 14 and 20. Inhibition studies using another antiserum showed that opsonization of type 5 streptococci was partially inhibited by SM5(1-20) and almost totally inhibited by SM5(20-40), indicating that the opsonic antibodies in this antiserum were directed against epitopes located in the 14-20 and the 20-26 regions of the SM5(1-35) peptide.

The data presented thus far indicate that all of the antibodies evoked by SM5(1-35) that reacted with pep M5 and type 5 streptococci were actually directed against the region represented by SM5(14-26). We wished to determine whether this region of type 5 M protein was also immunodominant and evoked protective antibodies in animals immunized with whole type 5 streptococci, a situation that more closely resembles the immune response during natural infection. Antiserum against intact type 5 organisms was affinity purified over a Sepharose-SM5(1-35) column, and the purified antibodies were used in opsonization-inhibition assays (Table V). The SM5(1-35)-reactive antibodies opsonized type 5 streptococci, and the opsonization was completely inhibited by SM5(1-35), pep M5, and SM5(14-26). These data indicate that the immune response to the amino terminus of type 5 M protein on the surface of the organism is similar to the immune response against SM5(1-35). Although opsonization of type 5 streptococci by the affinity-purified antibodies was completely inhibited by SM5(14-26), control experiments using unfractionated antiserum clearly indi-
TABLE V
Peptide Specificity of Opsonic Antibodies Evoked by Intact
Type 5 Streptococci

| Antiserum                  | Inhibitor       | Percent opsonization of type 5 streptococci |
|---------------------------|-----------------|---------------------------------------------|
| 7903 [affinity-purified over SM5(1-35)] | None            | 52                                        |
|                           | SM5(1-35)       | 10                                         |
|                           | SM5(14-26)      | 10                                         |
|                           | Pep M5          | 6                                          |
| 7903 (unfractionated)     | None            | 88                                         |
|                           | SM5(1-35)       | 88                                         |
|                           | SM5(14-26)      | 86                                         |
|                           | Pep M5          | 10                                         |

TABLE VI
Type-specific, Opsonic Antibodies Evoked in Rabbits Immunized with SM5(14-26)C

| Antiserum   | ELISA titer against: | Percent phagocytosis of type 5 streptococci |
|-------------|-----------------------|---------------------------------------------|
|             | SM5(1-35)             | SM5(14-26)                                 |
| 8516 preimmune | <200                  | <200                                        |
| 8516 14-wk   | 6,400                 | 25,600                                      |
|             | 12,800                |                                            |
| 8517 preimmune | <200                  | <200                                        |
| 8517 14-wk   | 25,600                | 25,600                                      |
|             | 25,600                |                                            |
| 8518 preimmune | <200                  | <200                                        |
| 8518 14-wk   | 12,800                | 25,600                                      |
|             | 12,800                |                                            |

cated the presence of additional opsonic epitopes that were not represented in SM5(1-35) but that were present in pep M5 (Table V).

Immunogenicity of SM5(14-26). To determine the immunogenicity of SM5(14-26), rabbits were immunized with the synthetic peptide that had been covalently linked to tetanus toxoid. SM5(14-26) in the conjugated form proved to be an ineffective immunogen, possibly because the carrier molecule was covalently linked in such a way that it masked or altered the M protein epitopes. We therefore synthesized an additional peptide SM5(14-26)C, which was crosslinked to KLH via the carboxyl-terminal cysteine residue (19, 20). All three rabbits immunized with this conjugate developed significant levels of antibodies against SM5(14-26), SM5(1-35), and pep M5, as measured by ELISA, and all three opsonized type 5 streptococci, as determined by in vitro opsonization tests (Table VI).

The protective immunogenicity of SM5(14-26)C and SM5(1-35) was confirmed in indirect bactericidal tests (Table VII). Type 5 streptococci were rotated in whole human blood, to which was added either preimmune or immune serum. The immune sera against both synthetic peptides contained bactericidal antibodies, as indicated by a significant reduction in CFU after a 3-h rotation in the test mixture (Table VII).

Type-specific and Crossreactive Antibodies Evoked by Synthetic Peptides of Type 5 M
TABLE VII
Bactericidal Antibodies Against Type 5 Streptococci Evoked in Rabbits
by SM5(1-35) and SM5(14-26)

| Antiserum       | Immunizing antigen | Number of colonies of type 5 streptococci surviving after 3 h growth in test mixture (inoculum of 6 CFU) |
|-----------------|--------------------|-----------------------------------------------------------------------------------------------|
| 8518 preimmune | SM5(14-26)C        | >2,000                                                                                           |
| 8518 14-wk      |                    | 210                                                                                             |
| 8514 preimmune | SM5(1-35)          | >2,000                                                                                           |
| 8514 14-wk      |                    | 94                                                                                              |
| 8329 preimmune | Pep M5             | >2,000                                                                                           |
| 8329 14-wk      |                    | 0                                                                                               |

TABLE VIII
Type-specific and Crossreactive Antibodies Evoked in Rabbits Immunized with SM5(1-35) or SM5(14-26)

| Antiserum | Immunizing antigen | ELISA titer against: | Pep M5 | Pep M6 | Pep M19 | Pep M24 |
|-----------|--------------------|----------------------|--------|--------|---------|---------|
| 8513      | SM5(1-35)          | 3,200                | 200    | 1,600  | <200    |         |
| 8514      |                    | 6,400                | 400    | 1,600  | <200    | <200    |
| 8515      |                    | 6,400                | 400    | 3,200  | <200    | <200    |
| Preimmune pool |                | <200                | <200   | <200   | <200    | <200    |
| 8516      | SM(14-26)C         | 25,600               | 200    | 800    | <200    |         |
| 8517      |                    | 51,200               | 200    | 400    | <200    | <200    |
| 8518      |                    | 51,200               | 400    | 200    | <200    | <200    |
| Preimmune pool |                | <200                | <200   | <200   | <200    | <200    |

Protein. Because previous studies (7–10, 23) have shown that pep M5 contains not only type-specific but also crossreactive epitopes, we wished to test the immune sera raised against the synthetic peptides for the presence of antibodies directed against heterologous M proteins (Table VIII). SM5(1-35) evoked significant levels of crossreactive antibodies against pep M19, very low levels against pep M6, and none against pep M24, as measured by ELISA. The SM5(14-26)C immune sera did not crossreact significantly with any of the heterologous antigens tested. Although SM5(1-35) appeared to contain epitopes that were shared with pep M19, none of the antisera opsonized type 19 streptococci, suggesting that the crossreactive antibodies may have been directed against nonprotective M protein epitopes on the surface of the organisms.

Discussion
The ability to identify limited regions of M protein molecules that contain protective epitopes, as opposed to tissue-crossreactive and nonprotective epitopes, may be an important step in developing safe and effective group A
streptococcal vaccines. We have recently shown that pepsin extracted M protein fragments from types 5, 6, and 19 streptococci contain epitopes that crossreact with sarcolemmal membrane proteins of human myocardium (7–9) and myosin (10), thus making the pep M proteins unsuitable for vaccine preparation. Previous studies (11–16) have been designed to determine the minimum peptide structures of several M proteins that retain protective immunogenicity and not tissue crossreactivity. Chemically synthesized peptides of type 24 M protein, ranging in size from 13 to 35 amino acid residues, were found (12, 14) to evoke protective antibody responses in laboratory animals. Most recently, synthetic peptides representing the amino terminus of type 6 M protein were also shown (16) to produce protective, and not heart-crossreactive, immunity. Similar studies (15) have been performed with synthetic peptides of type 5 M protein which showed that SM5(1-20) evoked protective antibodies, whereas SM5(20-40) did not.

The present study was undertaken to identify precisely the primary structure of the amino terminus of type 5 M protein that contains protective antigenic determinants. We first showed that SM5(1-35) was immunogenic and evoked opsonic antibodies in rabbits. Using overlapping peptides, we identified three immunologically distinct regions of SM5(1-35): the amino terminus, which did not evoke M5-reactive antibodies; the carboxy terminus, which evoked M5 antibodies that were nonopsonic; and the region between residues 14 and 26, which contained the immunodominant protective epitopes of SM5(1-35). Further studies using SM5(1-20) and SM5(20-40) showed that all of the opsonic antibodies in one SM5(1-35) antiserum were completely inhibited by SM5(1-20), suggesting that at least one protective epitope resides between residues 14 and 20. Opsonization of type 5 streptococci by another antiserum was partially inhibited by SM5(1-20), and almost totally inhibited by SM5(20-40), indicating that another protective epitope is located between residues 20 and 26. Our previous finding (15) that SM5(20-40) did not evoke opsonic antibodies, yet in the present study was capable of inhibiting opsonization by antibodies raised against SM5(1-35) most likely reflects differences between immunogenicity and antigenicity of the synthetic peptides. SM5(14-26)C, when linked to an appropriate carrier molecule, evoked opsonic antibodies against type 5 streptococci. Thus, by using a set of overlapping synthetic peptides representing type 5 M protein, we have identified a discrete region of the molecule, composed of only 13 amino acids, that retains protective immunogenicity and does not evoke host tissue-crossreactive antibodies.

The three immunologically different regions of SM5(1-35) were detected by methods designed to determine the functional activity of antipeptide antibodies. The titers of SM5(1-35) antisera against the synthetic peptide were consistently higher than those against pep M5, the natural protein, suggesting that at least some of the antibodies were peptide-specific. This was confirmed by ELISA inhibition experiments, which showed that not all of the SM5(1-35) antibodies were inhibited by pep M5. The finding that opsonic antibodies raised against whole type 5 streptococci that were affinity purified with SM5(1-35) were completely inhibited by SM5(14-26) confirms the immunodominance of this region of the amino terminus, and suggests that our findings were not artifacts of the synthetic immunogen or the coupling procedure.
Recent evidence (24) suggests that the ability of antibodies evoked by synthetic peptides to recognize epitopes within the natural protein is partly a function of the atomic mobility of the structure of its native state. Although such data are not yet available for M protein molecules, Manjula and Fischetti, and Phillips et al. (25–27) have previously shown that a consistent structural feature among the M proteins sequenced thus far is a seven-residue periodicity with respect to polar and noncharged amino acids, which led them to predict an α-helical secondary structure. The α-helical potential of pep M5 begins at residues 14–17 and is particularly high between residues 27 and 54 (25, 28). Herein, we found that the opsonic epitopes of SM5(1-35) corresponded to the region in pep M5 (residues 14–26) that displays only moderate α-helical potential (25, 28), suggesting that this secondary structural configuration may be related to immunodominance and the ability of antipeptide antibodies to react with the natural protein. We do not mean to imply that the region of SM5(1-35) encompassed by residues 14–26 contains the only opsonic epitopes of the amino terminus of type 5 M protein, but rather that it is immunodominant when animals are immunized with SM5(1-35). For example, in this study the amino-terminal 13 amino acid residues of SM5(1-35) did not evoke opsonic antibodies, yet we have recently shown (E. H. Beachy, manuscript in preparation) that a synthetic peptide copy of the first 10 amino acids of pep M5 evokes opsonic antibodies. This finding indicates that the immunogenicity of a particular primary structure may be enhanced when it is presented to the immune system in isolated form, which is consistent with data presented by other investigators (20).

Although the correlations of peptide structure with immunological function are highly speculative, we believe that such an approach may be valuable in developing an M protein vaccine. Previous data support the concept that the amino-terminal regions of M proteins evoke opsonic antibodies (15, 16). Thus far, none of the synthetic peptides of these M proteins has produced crossreactive antibodies. An effective streptococcal vaccine most likely will need to be formulated from protective fragments of M proteins representing as many as 15–20 rheumatogenic serotypes of streptococci. The ability to precisely identify protective as opposed to nonprotective and heart-crossreactive M protein epitopes will allow the disposal of the majority of the molecule, therefore minimizing the total amount of protein injected and reducing the risk of potentially harmful autoimmune reactions. We believe that studies of additional M proteins, using methods similar to those reported here, may reveal certain consistencies in the structures of various M proteins as they are related to immunological function.

Summary

We have used a set of overlapping chemically synthesized peptides representing the amino terminus of type 5 streptococcal M protein to localize protective, as opposed to nonprotective and tissue-crossreactive epitopes that might be appropriate for vaccine formulations. Rabbit antisera raised against SM5(1-35) reacted in high titer with pep M5 by ELISA and opsonized type 5 streptococci. None of the antisera crossreacted with human heart tissue or myosin. Antisera against SM5(26-35) reacted with SM5(1-35) and pep M5 but failed to opsonize type 5 streptococci. Particle-phase ELISA indicated that SM5(26-35) antibodies were
directed against nonprotective determinants of pep M5 that were not exposed on the surface of viable organisms. Opsonization and ELISA inhibition assays showed that, of the SM5(1-35) antibodies that reacted with M5, all were inhibited by SM5(14-35), whereas none was inhibited by SM5(26-35), suggesting that the protective epitopes of SM5(1-35) resided between residues 14 and 26. This was confirmed by subsequent chemical synthesis of this region; SM5(14-26) totally inhibited SM5(1-35) antibodies that reacted with pep M5 in ELISA, and completely inhibited opsonization of type 5 streptococci by SM5(1-35) antibodies. SM5(14-26) evoked high titers of type-specific, opsonic antibodies against type 5 streptococci, confirming the protective immunogenicity of this 13-residue peptide of type 5 M protein.

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