Amelogenins bind to GlcNAc of the dentine-enamel matrix proteins (Ravindranath, R. M. H., Moradian-Oldak, J., Fincham, A. G. (1999) J. Biol. Chem. 274, 2464–2471). The hypothesis that amelogenins may interact with the peptides that mimic GlcNAc is tested. GlcNAc-mimicking peptide (SFGSGFGGGGY) but not its variants with single amino acid substitution at serine, tyrosine, or phenylalanine residues inhibited hemagglutination of amelogenins and the terminal tyrosine-rich amelogenin polypeptide (TRAP). The binding affinity of SFGSGFGGGGY to amelogenins was confirmed by dosimetric nin polypeptide (TRAP). The binding affinity of SFGSGFGGGGY to amelogenins was confirmed by dosimetric binding of amelogenins or TRAP with [3H]peptide, specific binding in varying concentrations of the peptide, Scatchard plot analysis, and competitive inhibition with the unlabelled peptide. The ability of the peptide or GlcNAc to stoichiometrically inhibit TRAP binding of [14C]GlcNAc or [3H]peptide indicated that both the peptide and GlcNAc compete for a single binding site. Using different fragments of amelogenins, we have identified the peptide-binding motif of amelogenin to be the same as the GlcNAc-binding “amelogenin trityrosyl motif peptide.” The GlcNAc-mimicking peptide failed to bind to the amelogenin trityrosyl motif peptide when the tyrosyl residues were substituted with phenylalanine or when the third proline was replaced with threonine, as in some cases of human X-linked amelogenesis imperfecta. This study documents that molecular mimicry may play a role in stability and organization of amelogenin during amelogenesis.

Dental enamel is derived through the biomineralization of an extracellular organic matrix secreted by the ameloblast cells of the inner enamel epithelium. Although ameloblasts synthesize several other proteins, including cytokeratin 14 prior to synthesis of amelogenins (1), the amelogenins constitute some 90% of the secretory stage enamel matrix proteins (2–4). Previously, we have hypothesized that amelogenins may bind to sugar residues of enamel matrix glycoproteins facilitating the biomineralization process (5). The hypothesis was supported by identification of a stoichiometric interaction specifically between amelogenins and the GlcNAc residues of glycoconjugates (5). Further, we have identified the glycobinding locus of the amelogenin structure in a highly conserved motif (-PYPSPGY-) located at the carboxyl-terminal of the tyrosine-rich amelogenin polypeptide (TRAP). Remarkably, this trityrosyl motif has a striking structural similarity to the GlcNAc-binding domain of several GlcNAc-specific lectins (5–7).

Recent observations have indicated that the GlcNAc-binding motif of several lectins such as wheat germ agglutinin (WGA) and lectins from Datura stramonium, Lycopersicon esculentum, Solanum tuberosum, and Wisteria floribunda also recognize and bind to a specific peptide sequence that mimics GlcNAc found in cytokeratins (8). The present study is based on the hypothesis that comparable interactions between the amelogenins and GlcNAc-mimicking peptides (GMP) may occur during amelogenesis with implications for the understanding of the control of normal enamel development and of the molecular lesions that underlie enamel pathologies such as the condition of amelogenesis imperfecta.

In this investigation, we demonstrate that a conserved GMP motif of cytokeratins specifically binds to the amelogenin trityrosyl motif peptide (ATMP). Further, we have directly tested the likely biological relevance of GMP-amelogenin interactions showing that loss of function mutations of the ATMP sequence correlates with loss of interaction with GMP, specifically that the substitution of a proline residue of ATMP (with threonine) as recently observed in a case of human X-linked amelogenesis imperfecta (AJ) (9) strongly abrogates the GMP-amelogenin interaction.

EXPERIMENTAL PROCEDURES

Amelogenin Proteins—The following amelogenin polypeptides (Fig. 1) were used: (i) rM179 (20.16 kDa), a recombinant mouse amelogenin, which is identical to the native murine amelogenin, M180 (except for the lack of the amino-terminal methionine residue (10) and a phosphorylated serine at position 16) (11, 12); (ii) rM166 (18.6 kDa), as rM179 but lacking the 13 C-terminal amino acid residues (13); (iii) TRAP (5.20 kDa), a synthetic murine tyrosine-rich amelogenin polypeptide representing the N-terminal 45 amino acid residues of the M180 amelogenin; (iv) LRAP (6.82 kDa), synthetic leucine-rich amelogenin polypeptide, identical to the full-length (M180) amelogenin at its two termini but lacking the center portion of the protein (14); (v) amelogenin C-terminal peptide (ACP); (vi) P173 (25 kDa) and P148 (20 kDa) (porcine amelogenins were extracted and purified following the protocol of Fincham et al. (15) as described previously (5)); and (vii) ATMP, PYPSPGYEPFGGW and two altered ATMP peptides in one of which

1 The abbreviations used are: TRAP, tyrosine-rich amelogenin polypeptide; LRAP, leucine-rich amelogenin polypeptide; WGA, wheat germ agglutinin; GMP, GlcNAc mimicking peptide; ATMP, amelogenin trityrosyl motif peptide; T-ATMP, ATMP where proline is replaced by threonine; F-ATMP, ATMP where tyrosine is substituted by phenylalanine; ACP, amelogenin carboxyl-terminal peptide; HA, hemagglutination; HAI, hemagglutination inhibition; HSA, human serum albumin; HPLC, high performance liquid chromatography; TBS, Tris-buffered saline; Al, amelogenesis imperfecta; GM-peptide, GlcNAc-mimicking peptide; CK-14, cytokeratin-14; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin.

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‡ To whom correspondence should be addressed: Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, Los Angeles, California 90033.
Decapetides Mimicking GlcNAc—Synthetic GlcNAc-mimicking peptide (GM-peptide) with the cytokeratin amino acid sequence SFGSGFGGGY (GMp1) and its variants (GMp2 to -8) with single amino acid substitutions (Table I) were used in this investigation. These peptides are known to bind to anti-GlcNAc monoclonal antibody and also to GlcNAc-specific lectins such as WGA, U. europaeus II (UEA-II), and that of D. stramonium, expressing functional similarity to the carbohydrate (8). The atomic mass units of these synthetic GM-peptides range from 935 to 888 (Table I). In order to identify the amino acid residues of the peptide sequence critical for binding to the tyrosyl motif of amelogenins, the pentamers (first and second half) of the GMp1 (SFGSGFGGGY), “SFGS” and “FGGGY,” were synthesized and used to inhibit the amelogenin or TRAP-mediated hemagglutination.

Synthesis and Purification of Polypeptides—All of the polypeptides, (GMp variants, the two pentamers, TRAP, and LRAP) used in this investigation were synthesized by the USC microchemical Core Laboratory using an Applied Biosystems model 430A one-column peptide synthesizer with the modified Merrifield procedure (16). Peptides were purified by reversed-phase HPLC (C4–214TP54 column; Vydac/The Separations Group, Hesperia, CA) with a gradient of 35–50% B in 60 min (buffer B contained 60% (v/v) aqueous 291HS54 column; Vydac/The Separations Group, Hesperia, CA) with a gradient of buffer B (0.01 M trifluoroacetic acid, aqueous) and buffer B (0.01 M trifluoroacetic acid) and buffer B (0.01 M trifluoroacetic acid) over 30 min, at a flow rate of 1.0 ml/min. The peptide was supplied in an aqueous solution in a silanized borosilicate multidose vial with additional screw-cap under argon. The peptide was stored in the absence of light and air at 4 °C.

Positive and Negative Controls—GlcNAc, chitobiose, chitotetraose, and WGA (Sigma) were used as positive controls and β(+)-glucosamine, LRAP, and BSA (Sigma) as negative controls.

Hemagglutination Inhibition (HAI) Assay—The HAI assays were performed in 8 × 12-microtiter plates, with U-bottomed wells, after assessing the HA activity of the recombinant amelogenin/TRAP molecules as described earlier (5). Previously, we have screened a variety of mammalian erythrocytes and selected mouse erythrocytes for effective HA by amelogenins including both rM179 and TRAP. We have selected the concentration of rM179/TRAP that gives two-half agglutination. All GMps were diluted (1:10 in Tris-buffered saline (TBS), pH 6.3) in Eppendorf tubes and were warmed to 30 °C. To each well, 12.5 µl of peptide solutions were added. The final concentration of peptides/well was adjusted to and ranged from 1 µg/ml to 10-fold dilution. 6 µg/12.5 µl of amelogenin or 3 µg/12.5 µl of TRAP (diluted in trifluoroacetic acid (0.04%) + TBS, pH 6.3), or TBS (pH 6.3) capable of two-well agglutination was added to each well and incubated for 1 h at 25 °C. After incubation, 25 µl of a 1.5% suspension of mouse erythrocytes (purchased from Crane Laboratories, Inc., Syracuse, NY) in TBS, pH 7.2, was added to all the wells. The plates were covered with parafilm, subjected to gentle low speed vortex for 10 s, and incubated at 25 °C, and scoring was done after 2 h. The HAI titers were reported as the reciprocal of the lowest concentration of the inhibitors giving complete HAI (button formation) after 2 h.

Dose-dependent Binding of 14C-GlcNAc to TRAP Molecules—A known amount of [14C]GlcNAc (2 × 10^4 cpm) in 100 µl of TBS (pH 7.2) was added to polypropylene microcentrifuge tubes containing 100 µl of increasing amounts of TRAP molecules in 0.04% trifluoroacetic acid. The mixture was incubated under constant agitation for 90 min at 25 °C and precipitated with 1 ml of cold ethanol (200 proof; Gold Shield Chemical Co., Hayward, CA) for 4°C for 20 min. The tubes were then centrifuged for 15 min at 12,000 × g in a Beckman Microfuge 12, and the supernatant was removed. The unbound 14C-labeled GlcNAc was removed completely by repeated vortex mixing and washing three times with cold ethanol. Washing three times with ethanol did not affect the bound peptides. The final pellets were dissolved in 1 N NaOH, and the radioactivity was measured 15 min after adding 4 ml of scintillation.

**Fig. 1.** Amino acid sequence of recombinant amelogenin (rM179) and synthetic polypeptides representing different regions of the amelogenin. The bar chart shows the nature and the characteristics of amelogenin polypeptides. The boxed sequence is the amelogenin tyrosyl motif.

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<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>rM 179</td>
<td>Full length with 179 amino acid residues</td>
</tr>
<tr>
<td>rM 166</td>
<td>Full length without 13 amino acid residues of C-terminal</td>
</tr>
<tr>
<td>TRAP</td>
<td>45 amino acid residues of N-terminal</td>
</tr>
<tr>
<td>LRAP</td>
<td>33 amino acid residues of N-terminal plus 26 amino acid residues of C-terminal</td>
</tr>
<tr>
<td>ACP</td>
<td>13 amino acid residues of C-terminal</td>
</tr>
</tbody>
</table>

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**Amelogenins Bind GlcNAc-mimicking Peptide**

39655

http://www.jbc.org/ by guest on October 4, 2017
fluid (Bio-safe 11, Research Products International Corp., Mount Prospect, IL) in a β-counter (Beckman, LS-1801). WGA was used as a positive control and BSA as a negative control.

**Inhibition of \(^{14}C\)GlcNAc Binding to rM179 and TRAP Molecules with Unlabeled GlcNAc or GMps—** For the competitive binding inhibition, 100 μl of unlabeled GMps or GlcNAc (TBS, pH 7.2) at different concentrations were prepared in duplicate in microcentrifuge tubes. To each concentration of the GMps or GlcNAc, 100 μl of \(^{14}C\)GlcNAc (2 × 10^6 cpm in TBS, pH 7.2) was added and mixed. 100 μl of rM179 (7.5 nmol) or TRAP (20 nmol) in 0.04% trifluoroacetic acid were added to the mixture and incubated for 90 min at 25 °C. After incubation, the proteins were precipitated with 1 ml of cold ethanol at 4 °C for 20 min and centrifuged for 15 min at 12,000 × g. The unbound radioactive GlcNAc was removed completely by repeated vortex mixing and washing three times with ethanol. The final pellets were dissolved in 50 μl of 1 n NaOH, and the bound radioactivity was measured with 4 ml of scintillation fluid (Bio-safe 11) in a β-counter as mentioned earlier (5). Glucosamine was used as a negative control. The values were expressed as percentage of bound \(^{14}C\)GlcNAc to rM179 or TRAP.

**Dose-dependent Binding of \(^{3}H\)Glp1 to Amelogenins by Western Blot Analysis and Autoradiography—** Purified recombinant murine amelogenins (rM179 and rM166) were resolved via SDS-polyacrylamide gel electrophoresis using 15% resolving and 3.5% stacking gels (17) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.; Immobilon-P Transfer Membrane) at 100 mA for 1 h using a semidry transblot apparatus (Hoefer Scientific Instruments, San Francisco) (18). Protein transfer was assessed by staining the PVDF strips with 0.1% Fast Green (Sigma) in 40% acetic acid and 10% methanol, and the membranes were washed five times with phosphate-buffered saline (pH 6.0) for 18 h at 25 °C, after blocking the nonspecific binding of \(^{3}H\)Glp1 was removed completely by repeated vortex mixing and washing four times with ethanol, which did not affect the bound peptides. The final pellets were dissolved in 1 n NaOH, and the bound radioactivity was measured 15 min after adding 4 ml of scintillation fluid (Amersham Pharmacia Biotech) in a β-counter. BSA and WGA were used as negative and positive controls, respectively.

**Binding of \(^{3}H\)-Labeled GMp1 to Amelogenins by Western Blot Analysis and Autoradiography—** Purified recombinant murine amelogenins (rM179 and rM166) were resolved via SDS-polyacrylamide gel electrophoresis using 15% resolving and 3.5% stacking gels (17) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.; Immobilon-P Transfer Membrane) at 100 mA for 1 h using a semidry transblot apparatus (Hoefer Scientific Instruments, San Francisco) (18). Protein transfer was assessed by staining the PVDF strips with 0.1% Fast Green (Sigma) in 40% acetic acid and 10% methanol, and the strips were compared with Coomassie Blue-stained protein bands (19). Replicate wells treated with \(^{3}H\)Glp1 (7 × 10^6 dpm/ml) resuspended in phosphate-buffered saline (pH 6.0) for 18 h at 25 °C, after blocking the membrane with phosphate-buffered saline, 1% HSA for 1 h at 37 °C. The membranes were washed five times with phosphate-buffered saline, 0.1% HSA (20). After washing and drying, the membranes were exposed to hyperfilm-\(^{3}H\) (Amersham Pharmacia Biotech) for 18 h at 25 °C, and the films were developed manually.

**Purification of Synthetic GMps—** GMp with the amino acid sequence SFSGFSGGGGGY (GMp1) and its variants (GMp2 to -8) with single amino acid substitution (marked in boldface type in Table I) were purified by reverse phase HPLC, and a typical profile of GMp1 is illustrated in Fig. 2. HAI of rM179 and TRAP by GMps—** The peptide binding specificity of purified rM179/TRAP molecules was probed by peptide inhibition of HA. GlcNAc-mediated HAI was used as a positive control. Table I shows the nature of GMps that inhibited HA of amelogenin and of TRAP. Of the peptides tested, only GMp1 and GMp4 inhibited the HA of rM179. None of the other GMps inhibited the HA of amelogenins even at 1000-fold higher concentrations. The substitution of the serine or phenylalanine residues of GMp1 significantly affected the binding. Both GMp1 and GMp4 inhibited the HA at 150 nM concentration, which is the same as with the dimer and tetramer of GlcNAc. The binding of GlcNAc to rM179 is 10-fold less than that of GMp1, suggesting that GlcNAc is the preferred ligand of rM179 than GMp1.

In contrast, GMp1 is the most potent inhibitory peptide of TRAP-HA. The binding of GMp1 to the TRAP molecule is 20-fold stronger than that of GlcNAc, suggesting that GMp1 is the preferred ligand of TRAP. TRAP-HAI of GMp1 occurred at a concentration as low as 500 pM, whereas 500 nM of GMp4 is required to cause such inhibition. The inhibitory potency of GMp1 is 1000-fold greater than that of GMp4 or GMp2. The substitution of serine (as in GMp5 and GMp7) or phenylalanine residues (as in GMp3, GMp6, or GMp8) or changing the terminal tyrosine (as in GMp2 and GMp4) least affected HAI. The binding affinity for GlcNAc versus GMp1 appears to change with processing of the larger amelogenins (rM179) to TRAP.

**Inhibition of rM179/TRAP-mediated HA by SFSG and FGGGY of GMp1—** To identify and minimize the amino acid sequence responsible for binding to the tyrosyl TRAP motif of amelogenins, the pentamers were used to inhibit the amelogenin- or TRAP-mediated HA. The pentamer, FGGGY, did not affect amelogenin- or TRAP-mediated HA, whereas SFSG, representing the amino-terminal half of the GMp1 inhibited the TRAP-mediated HA partially compared with the full-length GMp1 (see Table I), suggesting that SFSG may mediate most of the functional amino acid sequence of GMp1 recognized by TRAP. The loss of binding affinity of the peptide when GMp1 is spliced into two halves indicates that the binding site of amelogenin or TRAP recognizes more than 5 amino acid residues of GMp1.

**Direct Binding of \(^{14}C\)GlcnAc to TRAP—** In order to select the maximum concentration of rM179 or TRAP required for
GlcNAc, respectively. GMp1 and GMp4 dosimetrically inhibited the binding of [14C]GlcNAc to rM179 and TRAP. The relative inhibitory potency of GMp1 is 2.5-fold more efficient than cold GlcNAc to rM179 and TRAP—mediated HA. None of the other peptides inhibited the interaction of TRAP with [14C]GlcNAc in the solid matrix assay. Therefore, GMp1 is the more preferred peptide ligand inhibiting the lectin-like activity of rM179 and the TRAP molecule.

**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid sequence of peptides</th>
<th>GMp</th>
<th>Atomic mass units</th>
<th>rM179</th>
<th>TRAP</th>
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<tr>
<td></td>
<td></td>
<td>Minimal concentration required</td>
<td>Relative inhibitory potency</td>
<td>Minimal concentration required</td>
</tr>
<tr>
<td>SFGSGFGGGY</td>
<td>GMp1</td>
<td>935.15</td>
<td>150 nM</td>
<td>10</td>
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<tr>
<td>SFGSGFGGGK</td>
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<tr>
<td>SFGSGKGGGY</td>
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<tr>
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<td>GMp7</td>
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<tr>
<td>BSA</td>
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<td>60 kDa</td>
<td>150 μM</td>
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</table>

which GMp1 is more potent than GlcNAc in inhibiting TRAP-mediated HA. None of the other peptides inhibited the interaction of TRAP with [14C]GlcNAc in the solid matrix assay. Therefore, GMp1 is the more preferred peptide ligand inhibiting the lectin-like activity of rM179 and the TRAP molecule.

**Fig. 2.** Analytical reverse phase HPLC profile of GMp1 (atomic mass units 935.15). For GMps, C18 column (Vydac/The Separations Group, Hesperia, CA) was used.

GMp inhibition of [14C]GlcNAc binding, the dosimetry of [14C]GlcNAc interaction with rM179/TRAP was determined. The binding of [14C]GlcNAc increases with the concentration of rM179 (5) and TRAP (Fig. 3). Based on the results, we selected 7.5 nmol of rM179 and 20 nmol of TRAP for inhibition of binding of [14C]GlcNAc by GMps.

**GMps Inhibition of [14C]GlcNAc Binding to rM179 or TRAP**—The results of GMps inhibition of the interaction of [14C]GlcNAc to rM179 and TRAP are presented in Fig. 4, A and B, respectively. GMp1 and GMp4 dosimetrically inhibited the binding of [14C]GlcNAc to rM179. None of the other GMps inhibited the interaction of rM179 with [14C]GlcNAc. 50% inhibition of GMp1 and GMp4 occurred at 150 μM and 130 μM, whereas inhibition by cold GlcNAc required a higher concentration (350 μM) (Fig. 4A). The relative inhibitory potencies of GMp1 and GMp4 are about 2-fold more efficient than cold GlcNAc, a finding different from that for HAI in which GlcNAc showed a 10-fold increase in inhibitory potency. However, both GMp1 and GMp4 were preferred ligands of rM179 in both assays. Furthermore, GMp1 strongly and dosimetrically inhibited the binding of [14C]GlcNAc to TRAP. The relative inhibitory potency of GMp1 is 2.5-fold more efficient than cold GlcNAc (Fig. 4B), a finding in conformity with HAI observations in which GMp1 is more potent than GlcNAc in inhibiting TRAP-mediated HA. None of the other peptides inhibited the interaction of TRAP with [14C]GlcNAc in the solid matrix assay. Therefore, GMp1 is the more preferred peptide ligand inhibiting the lectin-like activity of rM179 and the TRAP molecule.

**Fig. 3.** Dosimetry of [14C]GlcNAc binding with the synthetic and purified TRAP. To polypropylene microcentrifuge tubes containing 100 μl of GlcNAc (2 × 10⁶ cpm or 300 pmol), 100 μl of increasing concentrations of TRAP was added and incubated for 90 min on a shaker at 25 °C. After precipitation with ethanol and washing, the bound radioactivity was assessed. The mean values of triplicate analyses for each concentration were plotted. Vertical bars refer to S.D. The position of 20 nmol of TRAP chosen for further analyses is indicated on the graph as a vertical dotted line.
Specific Binding of $[^3H]GMp1$ to TRAP—Fig. 6 shows the specific binding of $[^3H]GMp1$ to TRAP as a function of increasing concentration of GMp1. The nonspecific binding was measured with unlabeled GMp1 and subtracted to obtain specific GMp1 binding. A Scatchard plot of the binding of $[^3H]GMp1$ to TRAP indicates that the peptide-binding site is homogenous with respect to the association constant.

Competitive Binding of $[^3H]GMp1$ and Unlabeled GMp1 to TRAP—Unlabeled GMp1 inhibited the binding of $[^3H]GMp1$ to TRAP stoichiometrically. The inhibition slope for GMp1 ($p < 0.001$) and GlcNAc ($p < 0.0001$) and $r^2$ for GMp1 ($-0.98$) and GlcNAc ($-0.99$) establish significant stoichiometry (Fig. 7). Comparison of the slope or the 50% inhibition showed that GMp1 is 4-fold more potent than cold GlcNAc in binding to TRAP, a finding in conformity with GMp1 inhibition of HA (Table I) and binding of $[^14C]GlcNAc$ to TRAP (Fig. 4B).

Binding of GMp1 to the GlcNAc-binding Trityrosyl Motif of Amelogenin Is Affected by Substitution of Tyrosyl Residues or Third Proline—The specific binding of GMp1 to TRAP is further confirmed in a solid matrix assay in which $[^3H]GMp1$ bound to rM179 and rM166 (lacking the 13-residue ACP) but failed to bind to ACP per se, suggesting that the ACP is not required for binding. 33 amino acids of the 45 amino acid residues of the N-terminal TRAP region are also shared by LRAP (see Fig. 1). In addition, LRAP possesses 26 amino acid residues of the C terminus of the larger (rM179) amelogenins. Failure of $[^3H]GMp1$ to bind to LRAP indicates that the N-terminal 33 amino acid residues of TRAP are not recognized by the GMp1 as was previously reported for GlcNAc (5). Therefore, the specific binding motif of GMp1 lies in the 13 amino acids of the C-terminal residues of TRAP, namely PYPSYGYEPMGGW (the trityrosyl motif or ATMP). Indeed, $[^3H]GMp1$ bound avidly to the trityrosyl motif or ATMP (Fig. 8). Fig. 8 shows that the binding of $[^3H]GMp1$ to both TRAP and ATMP is almost identical to $[^14C]GlcNAc$ (5) and is significantly greater than that to LRAP or ACP. However, substitution of the third proline in ATMP by threonine (T-ATMP) and substitution of all three tyrosine residues by phenylalanine (F-ATMP) resulted in complete loss of binding of the peptide to GMp1, identical with the observations previously made with GlcNAc (5).
Amelogenins Bind GlcNAc-mimicking Peptide

Amelogenins by Amelogenins—Amelogenins, the major enamel matrix proteins, are nonglycosylated (11, 12). They consist of a single polypeptide chain of some 180 amino acids and are highly conserved across species. Further, there is some variation in amelogenin protein size between species and also as a result of alternative splicing of the primary RNA (15, 23–26). Concomitant with enamel biomineralization, the primary amelogenin is proteolytically processed from the C-terminal end to smaller fragments, among which TRAP, consisting of the 45 N-terminal residues, is prominent (27–29).

The recombinant amelogenin (rM179), which corresponds to the newly secreted murine amelogenin (M180), has the capability of preferentially agglutinating mice erythrocytes (5). Agglutination of erythrocytes or HA requires multiple (polyvalent) binding sites of a protein to interact with several erythrocytes. Our previous observations confirmed that the ATMP, a highly conserved 13-amino acid motif of the TRAP region, is the only and the specific site of GlcNAc binding.

Assuming that it is this site that is solely responsible for binding to GlcNAc residues on erythrocyte surfaces to bring about HA, then HA would not be achieved by a single amelogenin polypeptide but by an aggregate of amelogenins that creates multivalent binding sites (5). It has been well documented that both native and recombinant amelogenins possess self-assembly properties both in vivo and in vitro, forming supramo-
Amelogenins Bind GlcNAc-mimicking Peptide

A Decamer Peptide (GMp1) of Cytokeratin 14 Also Inhibits HA of rM179 and TRAP—In a similar manner to GlcNAc, a decamer (SGFSGFSGFSGF) (GMp1) was found to inhibit rM179- or TRAP-mediated HA. This amino acid sequence is found in cytokeratin-14 (CK-14) (8), a cytosolic component of ameloblasts (1), and of the epithelial cell lineage that secretes amelogenin. We have modified synthetic GMp1 by substituting different serine residues with alanine or altering the terminal tyrosine residue. All of the modified GMps, except one, failed to inhibit rM179 or TRAP-mediated HA (Table I). Failure of other GMps to inhibit HA or binding of [14C]GlcNAc to rM179 or TRAP confirms GMp1 as a specific ligand of rM179 or TRAP. The GMp4 peptide (Table I) did inhibit amelogenin- or TRAP-mediated HA but with an inhibitory potency lower than that of GMp1.

GMp1 and GlcNAc Compete for a Single Binding Site on Amelogenins—The ability of both GlcNAc and GMp1 to inhibit dosimetrically TRAP binding of [14C]GlcNAc (Fig. 4B) suggests that both GMp1 and GlcNAc compete for a single binding site on TRAP. The ability of both GMp1 and GlcNAc to inhibit dosimetrically the TRAP binding of [3H]GMp1 (Fig. 7) further reinforce the view that both GMp1 and GlcNAc are competing for one and the same binding site. These observations provide the first evidence for specific amelogenin-peptide interactions.

These findings also suggest that although amelogenins may function as lectins they are also capable of binding specifically to peptides. Some recent reports provide evidence that lectins are not solely specific for sugar residues but are also capable of binding specifically to peptides, leading to a category of peptides known as “sugar-mimicking peptides” (33–36). The list of such sugar-mimicking peptides is restricted to a few sugars such as GlcNAc (8) but may expand with the possibility of sugar-mimicking peptides simulating glycosidic linkages of dimer or of polymeric sugars (37). The Fab’ portion of anti-idiotypic sugar-specific monoclonal antibodies (8, 19, 38, 39) represents sugar-mimicking peptides. Several GlcNAc binding lectins such as D. stramonium, L. esculentum, S. tuberosum, and W. floribunda are capable of binding to GMp1 and other GMps (8), although the potency of the binding may vary with the lectin. In the present study, we show that amelogenins preferentially bind GMp1. Although GMp1 is also known to inhibit GlcNAc interaction with WGA (present study and Ref. 8), WGA interaction with GlcNAc involves multiple binding sites generated by the secondary or tertiary structure of the polypeptide (6). Amelogenins differ from WGA in that the interaction between the GlcNAc and the amelogenin lies within the primary sequence of the conserved ATMP.

Loss of Function “Mutations” of ATMP Correlate with Loss of Interaction with GMp1—We have directly tested the likely biological relevance of amelogenin-GMp1 interactions to correlate with loss of function mutations of ATMP with loss of interaction with GMp. One form of a known inherited enamel defect in humans, AI, manifests as mutations of the amelogenin gene. Wright et al. (40–42) have shown that hypoplastic and hypocalciﬁed AI teeth contained increased protein content (2%) compared with normal primary enamel (0.5%) and attributed the protein retention in enamel to defective mineralization. They have linked the defects in enamel formation to a variety of mutations of the X-chromosome amelogenin gene. In genetic studies on two unrelated human pedigrees for AI, separate, single amino acid changes were shown to occur within the highly conserved TRAP region. We have replaced the third proline of the synthetic ATMP polypeptide with threonine (T-ATMP), as has been found in some cases of human X-linked AI (9) and also substituted with phenylalanine (F-ATMP) all three tyrosine residues. Not only did GlcNAc fail to bind to T-ATMP and F-ATMP (5), but so did GMp1 (Fig. 8), conﬁrming that mutations of ATMP that cause enamel defects also result in loss of binding to GMp1. The failure of GMp1 to bind to phenylalanine substituted ATMP suggests the hydrophilic nature of GMp1-ATMP interaction.

The Preferred Ligand of Full-length Amelogenin and of TRAP: GlcNAc or GMp1?—Specific binding of GMp1 with rM179 and TRAP in the HAI assay and the GMp1 inhibition of binding of [14C]GlcNAc and [3H]GMp1 with rM179 and TRAP suggest that GMp1 is the preferred ligand of ATMP. However, GMp1 differed from GlcNAc in its affinity for rM179 and TRAP. Table II summarizes the relative inhibitory potencies of GlcNAc and GMp1 for rM179 and TRAP as well as the concentration of GlcNAc and GMp1 required for 50% inhibition of binding of [14C]GlcNAc or [3H]GMp1 to rM179 or TRAP. Table II shows that GMp1 is a better ligand than GlcNAc. The difference in the ligands can be attributed to the molecular size difference between GlcNAc and GMp1, which must be confirmed after comparing the binding affinity of GMp1 with GlcNAc-oligosaccharides. In the HAI assay, GlcNAc was the preferred ligand for rM179, whereas the preference changed from GlcNAc to GMp1 after proteolytic conversion to TRAP indicating a modulation of the receptor affinity of amelogenin during proteolytic processing. Purification of TRAP from enamel generally results in the isolation of two forms of the molecule, one with 45 amino acids and another with 43 amino acids lacking the C-terminal glycine 44 and tryptophan 45 (43–45). We have suggested earlier that the formation of the 43- and 45-mer TRAPs may possibly depend on whether the amelogenin is bound to GlcNAc or not, since the COOH-terminal MGGW sequence may constitute the binding pocket of GlcNAc (5). The 10-fold higher HAI potency of GlcNAc to arrest

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2 A. G. Fincham, unpublished observations.
rM17-mediated HA and the shift in HA potency (GMp1 inhibitory potency being 20-fold greater than GlcNAc) after proteolytic conversion of amelogenin into TRAP suggest that consequent upon conversion, TRAP may shift its binding affinity from GlcNAc to GMp1, the carrier of which remains to be identified.

**Does GMp1 Carrier Protein Exist within the Enamel Matrix?**—Several investigators have identified GMp1 as a conserved motif of cytokeratins (8, 46, 47). CK-14, displaying clinical symptoms of epidermolysis bullosa, has been identified immunohistochemically in secretory stage ameloblasts (1, 48, 49). Several investigators have identified GMp1 as a con-
The Enamel Protein Amelogenin Binds to the N-Acetyl-d-glucosamine-mimicking Peptide Motif of Cytokeratins
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