A hallmark of biological systems is a reliance on protein assemblies to perform complex functions. We have focused attention on mammalian enamel formation because it relies on a self-assembling protein complex to direct mineral habit. The principle protein of enamel is amelogenin, a 180-amino acid hydrophobic protein that self-assembles to form nanospheres. We have used independent technical methods, consisting of the yeast two-hybrid (Y2H) assay and surface plasmon resonance (SPR), to demonstrate the importance of amelogenin self-assembly domains. In addition, we have analyzed mutations in amelogenin observed in patients with amelogenesis imperfecta who demonstrate defects in enamel formation. Assessments of self-assembly of these mutant amelogenins by either SPR or Y2H assay yield concordant data. These data support the conclusion that the amelogenin amino-terminal self-assembly domain is essential to the creation of an enamel extracellular organic matrix capable of directing mineral formation. It also suggests that a pathway through which point mutations in the amelogenin protein can adversely impact on the formation of the enamel organ is by disturbing self-assembly of the organic matrix. These data support the utilization of the Y2H assay to search for protein interactions among extracellular matrix proteins that contribute to biomineralization and provide functional information on protein-protein and protein-mineral interactions.

The self-assembly of a protein complex is a hallmark of biological systems. From processive enzymatic reactions to extracellular matrices, cells achieve control over their microenvironment through the assembly of a mixture of proteins. Protein assemblies are created with a specific stoichiometry. Such assembly precision is achieved through the interaction of protein motifs contained within the constituent protein members. The yeast two-hybrid (Y2H) assay has been used successfully to study protein assembly and offers distinct advantages over many biochemical techniques used previously. An example of the value of the Y2H assay can be seen in the assembly properties of type X collagen. Type X collagen is a homotrimer of α1(X) chains encoded by the COL10A1 gene, which is expressed in hypertrophic chondrocytes during the process of endochondral ossification. Dwarfism in domestic pigs is a result of a single change (Gly590 → Arg) to the α1(X) chain of type X collagen (1). In humans, a similar phenotype has been described for a mutation at the equivalent position (Gly590 → Glu) of type X collagen. These patients have Schmid metaphyseal chondrodysplasia, which is a mild skeletal disorder associated with dwarfism and growth plate abnormality. The Y2H assay and in vitro assembly experiments demonstrated that the amino acid substitution interfered with the ability of the mutated collagen molecules to engage in trimerization (1). In this example, confirmation of a valid animal model for a human genetic disease has been possible in part by the Y2H assay. At the same time, the Y2H assay has given unique information about assembly dynamics that add to the genetic, radiologic, histologic, and biochemical data previously available. Although biochemical methods can be used to detect dimerization, they often lack specificity, they may not be quantitative, they are not easy to perform, and they do not facilitate the genetic analysis of protein-protein interactions under physiological conditions.

We have focused attention on mammalian enamel as an example of the self-assembly of an extracellular matrix protein complex. In the case of enamel, a complex mixture of proteins self-assemble to form supramolecular complexes that are capable of guiding the crystal habit of hydroxyapatite crystallites (2–4). The crystals organize in such a way that the final product resists wear from repeated use during mastication, resisting non-catastrophic failure in a wet and bacterial laden environment (5). The principal protein of enamel is amelogenin, a hydrophobic protein that has been shown to undergo self-assembly to form nanospheres (6). Two domains in amelogenin (an A domain consisting of amino-terminal residues 1–42 and a B domain consisting of carboxyl-terminal residues 157–173) were subsequently identified as mediating amelogenin self-assembly by using the Y2H assay (7). In the present study, we corroborate the importance of these two self-assembly domains to the process of amelogenin self-assembly using surface plasmon resonance (SPR). We further define altered assembly dynamics for changes in single residues in the A domain, which are altered in individuals affected with X-linked amelogenesis imperfecta (AIH1). These mutations result in a Thr21 → Ile alteration (8) and a Pro41 → Thr alteration (9). Amelogenesis imperfecta is a hereditary disease of enamel and, when linked to the amelogenin locus, severely reduces the ability of amelogenin to self-assemble as measured by either the Y2H assay or by SPR.

We have used the Y2H system and SPR to perform a genetic analysis of the determinants of amelogenin self-assembly. The favorable correlation between assembly parameters for amelogenin as measured by either the Y2H assay or SPR
Each test protein was diluted from stock solution in HBS-EP buffer (10 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P-20, pH 7.4) to a desired concentration. The sample was then injected at 10 μl/min at 25 °C with an 5-min contact period, followed by a 2-min dissociation period. The sensor chip was regenerated with 1 M MgCl₂ after each experiment.

Data transformation and overlay plots for all experimental interactions were prepared with BIAevaluation software 3.0 (Biacore AB). To correct for refractive index changes and nonspecific binding, the binding responses generated from flow cell 1 were subtracted from the responses generated in flow cells 2 and 3.

**Plasmid Constructs Prepared for the Yeast Two-hybrid Assay—**Wild-type and mutated amelogenin cDNAs were engineered into the GAL4 DNA-binding hybrid vector pPC97 (7). The signal peptide of amelogenin has been excluded from all constructs in this study. Briefly, the plasmids prepared above (rM180, rp(H)M180, rp(H)M180ΔB, rp(H)M180T21 → I, rp(H)M180P41 → T, and rp(H)M180T21 → I, P41 → T) were used as template DNA for PCR using the forward primer TCGGATCCTATGCCCCTACCACCT and the reverse primer TAGGAGGACGACCCTCTACCTTCTCCGG. These two primers included a BamHI (forward primer) and SacI (reverse primer) restriction endonuclease restriction sites (underlined) to allow for efficient, in-frame cloning of the PCR product into pPC97. The construct rp(H)M180ΔB includes the hemagglutinin epitope (12). Respectively, these amelogenin-containing, GAL4 DNA-binding hybrid vectors have been called p97-M180, p97-M180ΔB, p97-M180T21 → I, rp(H)M180P41 → T, and p97-M180T21 → I, P41 → T M180. DNA nucleotide sequences for each amelogenin were obtained to confirm correct orientation, framing, and sequence of the CDNA insert. The domain A-deleted amelogenin in the GAL4 binding domain plasmid had been prepared for a previous study (pMa97/3 (7)) and will be referred to in this work as p97-M180ΔA. Construct p97-M180ΔA does not contain the GAL4 DNA-binding hybrid vector. The liquid assay used to quantitate yeast two-hybrid activities was performed previously (7).

**Yeast Two-hybrid Assay—**The liquid assay used to quantitate β-galactosidase activity has been reported previously (7).

**RESULTS**

**Mutations to Amelogenin Domain A Interfere with Amelogenin Self-assembly Using the Yeast Two-hybrid Assay—**The results from the yeast liquid culture assay are presented (Table I). Yeast hybrid assay was used to compare relative interaction strengths (including standard deviations) between wild-type and mutated amelogenin proteins. For each double-transformant combination eight yeast clones were grown until an A₆₀₀ reading of 0.6–0.8 was reached and assayed for β-galactosidase activity. Wild-type amelogenin interacting with itself is calculated at unity; and other readings are reported relative to this including calculated p values. Each of the mutated amelogenin constructs studied showed a statistically significant decreased ability to interact with wild-type amelogenin; the most significant was the domain A-deleted construct followed by the Thr21 → Ile point mutation construct. Positive controls were the tumor suppressor protein p53 transformed with the SV40...
large T antigen and H-ras (wild type) cotransformed with CDC25 protein of Saccharomyces cerevisiae (14). Negative controls involve each of the amelogenin protein-hybrid constructs cotransformed with either pPC86 or pPC97 hybrids or pPC97 (for the pPC97 hybrids). All negative control combinations had no ascertainable β-galactosidase activity as measured by the liquid culture assay.

Mutations to Amelogenin Interfere with Amelogenin Self-assembly Using Surface Plasmon Resonance—The real-time interaction of wild-type and mutant amelogenins were determined using SPR at concentrations of 0.25 µM (test molecule) to the rM179 ligand. The first sample tested (rp(H)M180) was tested a second time following the completion of the experiment and gave consistent data for both passes. In addition, this experiment in its entirety was repeated in two different laboratories by different individuals and gave identical results. A representative overlay of a sensogram generated for each interaction is presented (Fig. 2). It is apparent from these data that the chip surface remains fully active between each sample. It is also apparent that the greatest disruption to the amelogenin, as measured by response units, is seen for the ΔA (rp(H)M180ΔA) test construct.

The real-time interaction of the various test molecules was determined at concentrations of 0.25 and 0.50 µM to the rM179 ligand. For each analysis at these two concentrations, the sensogram was calculated at the same selected time points; threshold, response, and final response. Threshold was measured 5 s before the end of the injection of the sample, the response was measured 10 s after the end of the injection, and the final response point was measured after an additional 10 s (an example is illustrated in Fig. 3A). The data for both these concentrations are presented in Fig. 3B. At a 0.25 and 0.50 µM concentration, rp(H)M180 produced the highest binding response whereas rp(H)M180ΔA yielded the smallest binding response (Fig. 3B).

**DISCUSSION**

Amelogenin is an extracellular matrix protein, the amino acid sequence of which is highly conserved across species. Following the initial description of amelogenin proteins in the early 1960s, scientists have attempted to gain information about its secondary and tertiary structure. Amelogenin CD spectrum suggests that the amelogenin amino terminus contains β-sheet structure, whereas the central region and carboxyl-terminal region exhibits a random-coil conformation (15). It has been suggested that that amelogenin protein fails to retain stable secondary or tertiary structure (16); however, stable supermolecular structures are observed and take the form of 10–15-nm diameter “nanospheres” (6). How amelogenin as-

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**TABLE I**

Yeast two-hybrid assay results comparing relative interaction strengths (including standard deviations) between wild-type and mutated amelogenin proteins

| GAL4 DNA binding domain fusion vector | GAL4 activation domain fusion vector | Interaction (relative strength) | p value |
|--------------------------------------|-------------------------------------|-------------------------------|---------|
| p97-M180                             | p86-M180                            | 1.00                          |         |
| p97-M180ΔA                           | p86-M180                            | 0.08 ± 0.02                   | <0.002  |
| p97-M180TB                           | p86-M180                            | 0.75 ± 0.16                   | <0.002  |
| p97-M180T11 → I                      | p86-M180                            | 0.57 ± 0.14                   | <0.002  |
| p97-M180P11 → T                      | p86-M180                            | 0.74 ± 0.20                   | <0.002  |
| p97-M180T11 → I,P41 → T              | p86-M180                            | 0.83 ± 0.18                   | <0.005  |
| p53                                  | SV40 large T antigen                 | 0.66 ± 0.17                   |         |
| H-ras                                | CDC25                               | >16                           |         |

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**FIG. 2.** A representative overlay of a sensogram generated for each recombinant protein at a concentration of 250 nM. The sensor-bound protein was rM179. The greatest to the least responsive units were recorded for each recombinant protein in the following order rp(H)M180 (M180), rp(H)M180T21 → I (T21), rp(H)M180P41 → T (P41), rp(H)M180T21 → I,P41 → T (T21P41), rp(H)M180ΔB (ΔB), and rp(H)M180ΔA (ΔA).
relative quantitative data for wild-type amelogenin as well as for mutated amelogenins to interact with wild-type amelogenin. Previously AFM and DLS studies gave data related to amelogenin multimolecular assemblies (i.e. nanospheres), and these data included assembly dynamics and size distributions (12). The Y2H assay provides data related to the ability of two molecules to interact and is both qualitative and quantitative (7). Surface plasmon resonance provides additional information relating to how proteins react, in real time, to other proteins or protein complexes. The aqueous environment chosen to dilute the test protein for the SPR studies is similar to the aqueous environment of the enamel matrix as determined previously, i.e. HBS-EP buffer at pH 7.4 (including 150 mM NaCl) approximates closely these ionic values previously determined for maturing enamel (pH 7.26, with 140 mM Na\(^+\) and 150 mM Cl\(^-\)) (18). Although the recombinant proteins prepared for the SPR studies are non-phosphorylated, a characteristic apparent in some animal species (19), the use of recombinant proteins to study amelogenin biochemistry is widely accepted and well documented (4, 11, 12, 17).

To simplify our data, we present the following relationship among the studied amelogenins. For the Y2H we determined the strength of interaction for wild-type amelogenin (p86-M180) interacting with the wild-type or altered amelogenins in the pPC97 cassette to be: M180 > (M180T21 → I\(P41\) → T \(≥\) M180A\(P41\) \(≥\) M180T21 \(≥\) I \(≥\) M180T21 \(≥\) T) \(≥\) (M180T21 → \(I\) \(P41\) → T \(≥\) M180A\(B\) \(≥\) M180A\(ΔA\)). The ratio of magnitude of interaction recorded for the two extreme readings (M180: M180A\(ΔA\)) is \(≈\) 10:1. In a similar fashion, we can present that the SPR response activity for wild-type amelogenin (rM179) interacting with wild-type and altered amelogenin complexes. In the case of SPR the relationship of response activity (for the histidine-tagged recombinant proteins) would be M180 > (M180T21 \(≥\) I \(≥\) M180P41 \(≥\) T) \(≥\) (M180T21 \(≥\) \(I\) \(P41\) \(≥\) T \(≥\) M180A\(B\) \(≥\) M180A\(ΔA\)) where the ratio of response units of the two extreme readings (M180: M180A\(ΔA\)) is \(≈\) 10:1. Thus, data from the Y2H and SPR techniques are largely concordant.

The study here has demonstrated that the most dramatic mutation, with respect to interaction and nanosphere assemblies, occurs with the removal of the A domain (amino-terminus 42 amino acids). This domain is roughly equivalent to the tyrosine-rich amelogenin peptide (amino-terminal 45 amino acids of mouse amelogenin). This is not a surprising result and emphasizes previous DLS data in which we found that the A domain is required for protein-to-protein interactions leading to nanosphere self-assembly whereas the absence of the A domain led to inhibition of the self-assembly process (12). Dynamic light scattering showed that the A domain-deleted construct (rpm(H)M180\(ΔA\)) in solution produced a very heterogeneous size distribution (12). To equate this DLS conclusion to our SPR data, it is clear that the A domain-deleted construct reacted weakly with the sensor-bound wild-type amelogenin. Presumably this interaction was mediated through the intact B domain of the sensor-bound protein. Although some self-assembly of the mutated protein may occur prior to passing over the sensor, there appears to be a significant population of monomeric (mutated) amelogenins. This has been determined by generating a series of sensorgrams for selected protein combinations (wild-type interacting with serial dilutions of the A domain-deleted construct) for which kinetic data were determined and matched that of the 1:1 Langmuir binding model (data not shown) (13).

Removal of the domain B (p97-M180A\(B\)) resulted in a loss of 75% \(β\)-galactosidase activity, compared with wild-type amelogenin, as determined by the yeast assay. Surface plasmon resonance spectroscopy data showed that the interaction of rpm(H)M180A\(B\) with rM179 produced a significantly reduced response when compared with the rpm(H)M180 to rM179 amelogenin interaction using identical molar concentrations (Figs. 2 and 3B). The removal of the hydrophilic carboxyl terminus may promote hydrophobic interaction between amelogenin molecules. It has previously been postulated that the amelogenin hydrophilic carboxyl terminus is exposed on the surface of the amelogenin nanosphere, making the nanosphere soluble in an aqueous environment and thus preventing the fusion of neighboring nanospheres (12). It has been shown that disruption of the carboxyl terminus would then encourage interactions with neighboring molecules and molecular assemblies. Indeed, DLS data have demonstrated that amelogenin
assemblies lacking domain B are unstable and progressively fuse with neighboring assembled amelogenin nanospheres (12).

Finally, there is a question of correlating the \textit{in vitro} data derived here to \textit{in vivo} data already available. Biomaterials inspired by designs of nature comprise the newly emergent discipline of biomimetics. We anticipate that identifying and understanding the role for the various domains within amelogenin to direct its self-assembly into nanospheres will improve our understanding of the structural hierarchy of the native material and also provide the knowledge base for an enamel biomimetic. With the goal of predicting protein-to-protein and protein-to-mineral interactions using \textit{in vitro} approaches, we can then correlate such outcomes to the data derived from \textit{in vivo} experiments using transgenic animals bearing engineered amelogenin protein constructs. Ultimately such a correlation will allow the construction of a biomimetic matrix that can direct the formation of hydroxyapatite into a highly ordered material mimicking the unique properties of the original enamel composite ceramic and do so at physiologic parameters of temperature and ion concentration.

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