Archives of Oral Biology (2005) **50**, 141–145

Available online at www.sciencedirect.com

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Archives of Oral Biology

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Studies on Pax9–Msx1 protein interactions

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Accepted 22 September 2004

KEYWORDS
Pax9;
Msx1;
Protein–protein interaction;
Tooth agenesis;
Co-immuno-precipitation;
GST interaction assay

Summary Pax9 belongs to the Pax family of transcriptional regulators that are defined by a highly conserved DNA-binding region, the paired domain. Drosophila, mouse and human genetics have shown that Pax proteins play multiple roles in tissue patterning and organogenesis by mediating their functions in a highly tissue-specific manner. Members of the Pax family, Pax9 and Pax1, act synergistically during vertebral formation. However, only Pax9 is essential for tooth formation. Furthermore, mutations of *PAX9* are associated with human tooth agenesis. The highly tooth-specific molecular functions of Pax9 suggest that its activity is tightly regulated. Most likely, this occurs through interactions with other protein factors. Among the regulatory molecules that are expressed in dental mesenchyme, the Msx1 homeoprotein is of particular interest. The closely overlapping expression patterns of Pax9 and Msx1 are consistent with a role in epithelial–mesenchymal interactions. To demonstrate that Pax9 interacts with Msx1 physiologically in vivo and in vitro, we performed co-immunoprecipitation and GST interaction assays. Our results indicate that there is a physical association between the two proteins. Our biochemical data, coupled with human genetic studies and expression analysis in a mouse model, indicate a functional relationship between Pax9 and Msx1 during tooth development.

Introduction

Tooth agenesis or the developmental absence of teeth is a common genetic disorder that involves the patterning of the human dentition. Our current understanding of the molecular mechanisms underlying odontogenesis implicates a number of transcription factors and signaling molecules (i.e. growth factors and their receptors). Several lines of evidence indicate that synergistic and antagonistic interactions of signaling molecules are recursively utilized in tooth development. This leads to the local activation or inhibition of transcription factors in tooth epithelium and mesenchyme.[1] Msx1 and Pax9 are among the best studied tooth mesenchymal transcription factors that appear to

Abbreviations: GST, glutathione S-transferase; CMV, cytomegalovirus

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doi:10.1016/j.archoralbio.2004.09.011

have master regulatory functions in the early phases of odontogenesis.[1–3] Their parallel expression patterns are consistent with a role in epithelial–mesenchymal interactions. Pax9 (−/−) and Msx1 (−/−) tooth organs each arrest at the bud stage. While Msx1 is reduced in Pax9 null mutants, Pax9 expression is normal in Msx1 (−/−) mice implying that Pax9 is upstream to Msx1.[4–6] While this is suggestive of a molecular relationship between the two transcription factors, the precise mechanism remains unclear.

Human genetic studies of tooth agenesis further underscore the importance of understanding possible molecular interactions between Pax9 and Msx1. We and other laboratories have shown that novel mutations in human *PAX9* and *MSX1* each result in posterior tooth agenesis.[7–17] While *PAX9* mutations are dominantly associated with molar agenesis, *MSX1* mutations mainly affect premolars. Sufficient phenotypic variability exists in families with mutations in either gene to suggest modifying effects. Independent association studies have provided evidence of a statistically significant interaction between PAX9 and MSX1.[18] These data add to the mounting evidence from in vivo mouse and human genetic studies that suggests a functional relationship between Pax9 and Msx1 in tooth development.

Based on recent advances in our understanding of the molecular events during tooth development, we hypothesize that Pax9 interacts with Msx1 at the post-transcriptional level during tooth development. By clarifying the relationship between Pax9 and Msx1 on the protein level, these studies will advance our understanding of how transcription factors work together to mediate changes during tooth morphogenesis. In this report, we demonstrate that the Pax9 paired domain protein forms dimeric complexes with Msx1 homeoprotein in vivo and in vitro. Based on these biochemical data, we propose that such knowledge is critical to our understanding of how aberrant gene functions contribute to the pathogenesis of tooth agenesis, the most common defect in the patterning of the human dentition.

Materials and methods

Plasmid constructs

The mammalian expression vector pCMV–Pax9 with cMyc epitope tag and GST-fused expression plasmid pGEX–Pax9 are described elsewhere.[19] A murine Msx1 cDNA clone comprising the full-length coding sequence region was kindly provided by Dr. John Rubenstein (University of California at San Francisco). Expression plasmids containing the CMV promoter linked to the full-coding sequence of Msx1 was constructed in pCMV–Tag2b (Stratagene, CA). The Flag epitope is in frame with the N-terminus of Msx1. To construct GST-fused expression plasmid pGEX–Msx1, we subcloned full-length Msx1 cDNA into *Bam*HI–*Sal*I sites of the GST-fused expression vector (Amersham Pharmacia Biotech, NJ). To construct Pax9 and Msx1, the full-length Pax9 and Msx1 cDNAs were cloned into *Bam*HI–*Sal*I sites of the plasmids pGEM3Zf(+) (Promega, WI).

Protein preparations

Proteins were produced by in vitro transcription–translation using the TNT Quick coupled transcription/translation system (Promega, WI). Recombinant proteins were GST fusion proteins. Production of GST fusion proteins was described previously.[19] As noted previously, *Escherichia coli* BL21 competent cells were transformed with the recombinant plasmid and used to inoculate LB media. Cells were grown at 37 °C and protein expression induced by the addition of IPTG. Cells were harvested by centrifugation and protein purification performed on a glutathione column.

Co-immunoprecipitation

COS7 cells were maintained as described previously[19] and cotransfected with cMyc–Pax9 and either FLAG–Msx1 using Fugene6 (Roche Molecular Biochemicals, IN) as provided by the manufacturer. After 24 h, cells were resuspended in lysis buffer (50 mM Tris, pH 8.0, 400 mM NaCl, 1% Triton-X-100) with protease inhibitor cocktail tablets (Roche) and incubated for 30 min on ice. Cell lysates were added to 20 μl of anti-Flag M2 affinity gel (Sigma, MO) and rotated at 4 °C overnight. The affinity gels were washed with the lysis buffer five times and eluted with Laemmli buffer, and analyzed by western blotting with 1:200 dilution of goat anti-Pax9 polyclonal antibody (Santa Cruz, CA) or 1:1000 dilution mouse anti-Flag M2 monoclonal antibody (Sigma–Aldrich, MO). The signals were detected by incubating the membranes with ECL reagents (Amersham, NJ) according to instructions from the manufacturer.

GST interaction assay

The GST interaction assay was performed by incubating approximately 0.1 μg of GST–Pax9 protein bound to glutathione–sepharose resin with about 100 μl of the ^{35}S radiolabeled Msx1 and vice versa. Binding reactions were maintained at 25 °C for 10 min glutathione–sepharose resin-bound