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AUF1 and HuR Proteins Stabilize Interleukin-8 mRNA in Human Saliva

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Abstract

Human saliva contains thousands of mRNAs, some of which have translational value as diagnostic markers for human diseases. We have found that more than 30% of the mRNAs detected in human saliva contain AU-rich elements (ARE) in their 3' untranslated regions (3'UTR). Since AREs are known to contribute to RNA turnover by forming complexes with ARE-binding proteins, we hypothesized that salivary mRNA stability is mediated by ARE-binding proteins in human saliva. To test this hypothesis, we monitored the *in vitro* degradation of a radiolabeled ARE-containing salivary mRNA (IL-8) in salivary protein extracts. The degradation of IL-8 mRNA was accelerated by competition for saliva ARE-binding proteins through the addition of excess unlabeled IL-8 mRNA fragments containing 4 tandem AREs. UV cross-linking and immunoprecipitation experiments revealed 2 ARE-binding proteins, AUF1 and HuR, associated with IL-8 mRNA in saliva. These results demonstrate that ARE-binding proteins contribute to the stability of ARE mRNAs in human saliva.

Keywords

Human saliva; mRNA stability; AU-rich elements; AUF1; HuR

INTRODUCTION

Human saliva mRNA biomarker discovery is an emerging field for noninvasive diagnostic applications. Discoveries resulting from the mRNA profiling of saliva from healthy humans and persons with oral cancer have opened up a new area of research for the study of salivary gene expression (Li *et al.*, 2004a,b). In addition, the mRNA profiling of saliva by real-time PCR analysis has been used in forensic applications (Juusola and Ballantyne, 2005, 2007; Setzer *et al.*, 2008). Most recently, salivary amylase mRNA was identified as a biomarker for sleep disorders (Seugnet *et al.*, 2006). Of interest is that salivary amylase mRNA is more

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informative than amylase activity as a biomarker for sleep deprivation. At present, the mechanisms that regulate salivary RNA stability are not clear. We have shown that mRNA in the saliva is associated with certain macromolecules and is protected against ribonucleases (Park *et al.*, 2006). However, detailed studies on the stability of salivary RNA are required before we can understand the molecular mechanisms underlying protection of RNA from RNases in human saliva.

There are several RNA-binding proteins that are either directly or indirectly involved in mRNA stability in higher eukaryotes. *Cis*-acting adenylate-uridylate-rich elements (AREs) present in the 3' untranslated region (UTR) of certain mRNAs are known to be involved in the regulation of gene expression by affecting the mRNA stability through interaction with specific AU-rich RNA-binding proteins (Khabar, 2005). Of these, HuR and AUF1 are the best-characterized. HuR mediates stabilization of AU-rich mRNAs against exonucleases (Ma *et al.*, 1996; Keene, 1999; Li *et al.*, 2007). AUF1 is principally associated with destabilization of target mRNAs (Dreyfuss *et al.*, 2002), but several lines of evidence suggest that this protein is also implicated in mRNA stabilization through interaction with other protein macromolecules (Kiledjian *et al.*, 1997). Therefore, the level of RNA-protein interaction determines the stability of the mRNAs.

The goal of this study was to identify the molecular factors responsible for the stabilization of mRNAs in human saliva. Based on our observation that ~30% of salivary mRNAs contain an ARE motif in their 3'UTR, we hypothesize that these ARE motifs are involved in salivary mRNA stability. In this paper, we demonstrate that two ARE-binding proteins, AUF1 and HuR, are present in human saliva and are involved in the stability of AU-rich-containing mRNAs, providing the first evidence that the ARE-binding proteins can modulate mRNA stability in human saliva.

MATERIALS & METHODS

Participants

Saliva samples were obtained from healthy volunteers from the Division of Otolaryngology, Head and Neck Surgery, at the Medical Center, University of California, Los Angeles (UCLA), CA, in accordance with a protocol approved by the UCLA Institutional Review Board. All participants gave written informed consent, and the ethics committee of UCLA approved the study. The mean age of the volunteers was 31 yrs (range, 26–43 yrs). The volunteers had no history of malignancy, immune deficiencies, autoimmune disorders, hepatitis, or HIV infection.

Preparation of Salivary Protein Extracts

The unstimulated supernatant of the whole saliva was collected as described previously (Li *et al.*, 2004a,b). Further, the supernatant saliva samples were concentrated 10X by means of Amicon ultracentrifugal filters (Millipore, Bedford, MA, USA) with a molecular-weight cut-off of 5 kDa. The concentrated saliva was recovered and then stored at –80°C until further use.

RNA Substrates and Synthesis

The pCMV6 vector containing full-length IL-8 ORF under control of the T7 promoter was obtained from OriGene Technologies, Inc. (Rockville, MD, USA). We prepared the 1700-nt full-length IL-8 mRNA by linearizing the vector using Bgl II. We prepared β -actin mRNA (approximately 1300 nt of the transcript) by linearizing the pGEM3 vector containing the full-length cDNA under the control of the T7 promoter (1.8 kb inserted using a Pst I site) with Hind III. The remaining IL-8 ARE-containing short transcripts (see Appendix for RNA sequences) were a kind gift from Helmut Holtmann (Winzen *et al.*, 2004). Labeled RNA (10^7 to 10^8 cpm/

µg) was synthesized with the use of a Maxiscript *in vitro* transcription kit (Ambion, Austin, TX, USA), and prepared according to the manufacturer's protocol.

RNA Decay Assay

The mRNA decay assay was conducted as described previously (Ford and Wilusz, 1999; Viswanathan *et al.*, 2003), with slight modifications (for details, see the Appendix).

UV Cross-linking Assay

The UV cross-linking assay was performed with 10 µg of saliva protein extracts as described previously (Moraes *et al.*, 2006).

RNA-Protein Co-immunoprecipitation

Following UV cross-linking, samples were incubated with RNase A (30 U/sample) for 20 min at 37°C. Next, a 200-µL quantity of Net2 buffer (50 mM Tris, pH 7.6, 75 mM NaCl, 0.05% NP-40) was added to the samples, which were then incubated for 1 hr at 4°C with anti-AUF1 (Upstate Biotechnologies, Lake Placid, NY, USA) or anti-HuR (Zymed Laboratories, San Francisco, CA, USA) antibodies. Antigen-antibody complexes were collected on protein-A sepharose beads and washed 5 times with Net2 buffer. The immunoprecipitated cross-linked proteins were separated on a 10% SDS polyacrylamide gel. The gels were dried and scanned with a phosphor imager.

Preparation of Recombinant HuR

Recombinant GST-HuR was prepared according to a previously described method (Abdelmohsen *et al.*, 2007).

RESULTS

Enriched AU-rich mRNAs in Human Saliva

We have previously shown, through microarray analysis, that saliva from healthy individuals contains more than 3000 transcripts (Li *et al.*, 2004b). Of these transcripts, 185 (core transcriptome) are commonly present in all persons' saliva. Here, we found that 30% (56/185) of these mRNAs contain the nanomeric "WWAUUUAAW" ARE motif (Appendix Table 1) at their 3' UTR, and that 60% (110/180) contain the pentameric "AUUUA" motif. These ARE motifs were confirmed by means of the ARED database (Bakheet *et al.*, 2006). Next, we determined whether the previously identified 7 salivary oral cancer mRNA biomarkers contained ARE motifs (Li *et al.*, 2004a). Interestingly, 5 of the 7 salivary oral cancer mRNA biomarkers (71%) contained ARE at the 3' UTR, *i.e.*, DUSP1, H3F3A, IL1β, IL-8, OAZ1, S100P, and SAT (Appendix Table 2). It should be noted that approximately 5–8% of mRNAs in the human transcriptome contain putative ARE sequences (Bakheet *et al.*, 2006). The salivary transcriptome therefore contains a five-fold greater content of ARE-containing mRNAs, suggesting that these elements may contribute to a common feature of salivary mRNA that may account for their collective presence in the saliva.

Excess IL-8 AU-rich mRNA Leads to Rapid Degradation of Full-length IL-8 RNA *in vitro*

To evaluate the significance of AREs in salivary mRNA stability, we used human saliva extracts to examine decay of ARE-containing salivary mRNAs. IL-8 mRNA was selected, since it is an ARE-containing salivary mRNA present in the normal salivary transcriptome core. Radiolabeled full-length endogenous IL-8 mRNA slowly degrades in the presence of human salivary extract, with more than 60% of transcript remaining after 5 min of incubation with salivary extracts (Fig. 1B). To determine whether ARE motifs and potential ARE-binding proteins are important for IL-8 mRNA stability, we performed competition assays with a 60-

nucleotide (nt) stretch of IL-8 mRNA containing 4 AREs. Competition for potential ARE-binding proteins by the addition of an excess cold 60-nt ARE fragment led to increased and more rapid degradation of the full-length transcript. After 1 min in the presence of the 60-nt ARE fragment, IL-8 mRNA degradation had already exceeded that seen at the five-minute timepoint in the absence of competitor (Fig. 1C). The addition of equimolar concentrations of β -actin mRNA or unlabeled 60-nt ARE mutant fragment (60-nt IL-8 AREmt), in which point mutations had been introduced into the AREs, did not decrease IL-8 mRNA stability (Figs. 1B, 1C). To confirm that ARE motifs contribute to IL-8 mRNA stability, we tested the effect of ARE mutation on the degradation of the 60-nt ARE fragment. As expected, mutation of ARE motifs in IL-8 mRNA (Figs. 1D, 1E) accelerated degradation. Interestingly, the degradation rate of an IL-8 mRNA fragment harboring mutation in only 1 ARE (IL-8 ARE 3g-mt) was comparable with that of the un-mutated fragment, suggesting that the 3 AUUUA motifs are needed for IL-8 mRNA stability in saliva. These results suggest that the ARE motif is important for RNA stability in saliva.

AUF1 and HuR Bind and Stabilize IL-8 Transcripts

To identify the potential proteins that bind to and stabilize salivary IL-8 mRNA, we performed UV cross-linking experiments. Analysis of cross-linked RNA-protein revealed that 2 proteins, at around 45 kDa and 36 kDa, were associated with IL-8 mRNA, but not with IL-8 mRNA harboring ARE mutations (Fig. 2). We performed immunoprecipitation of the cross-linked RNA-protein complexes with AUF1 or HuR antibodies, to determine whether these species could be positively identified. Both AUF1 and HuR antibodies specifically precipitated the 45- and 36-kDa species, respectively, from samples containing IL-8 AREs, but not from samples containing IL-8 ARE mutants or β -actin mRNAs (Fig. 2, lanes 5 and 8). Non-specific antisera did not precipitate any cross-linked material (lanes 7 and 10). Thus, analysis of our data indicates that AUF1 and HuR proteins bind to IL-8 AREs in saliva lysates. Further, immunodepletion of AUF1 and HuR proteins accelerated the degradation of IL-8 mRNA in an *in vitro* decay assay (Appendix Figs. 2A, 2B, 2C). The results suggest that both HuR and AUF1 are important for stabilization of AU-rich mRNAs in human saliva, and that HuR has a more significant role in stabilizing IL-8 mRNA (Appendix Fig. 2).

Recombinant HuR Protects IL-8 mRNA against Degradation by Salivary Proteins *in vitro*

Since HuR clearly binds to IL-8 salivary mRNA, we next determined the effect of recombinant HuR binding on IL-8 mRNA stability as measured by *in vitro* degradation assays. To test whether HuR is important for IL-8 mRNA stability, we performed *in vitro* decay assays with saliva proteins and increasing amounts of recombinant GST-HuR. The addition of increasing amounts of recombinant GST-HuR resulted in dose-dependent protection against IL-8 mRNA degradation (Figs. 3A, 3B). IL-8 mRNA stability was unaffected by 100 ng GST-HuR, while larger amounts of GST-HuR in the range of 250 to 500 ng greatly enhanced the stability of IL-8 transcripts. BSA and non-specific GST proteins did not alter degradation of IL-8 transcripts incubated in HuR-depleted saliva proteins. Analysis of these data clearly shows that HuR promotes IL-8 mRNA stability in saliva.

Detection of ARE-binding Proteins in Human Salivary Glands and Saliva

The large concentration of ARE-containing mRNAs in saliva, coupled with the finding that ARE-binding proteins confer stability to ARE-containing mRNAs, prompted us to determine whether the major salivary glands produce ARE-binding proteins. Four AUF1 isoforms have been identified (Lu and Schneider, 2004), and all were present in human submandibular (SM) and parotid (PA) glands (Fig. 4) as well as in saliva (Appendix Fig. 1). HuR was also present in human salivary glands (Fig. 4) as well as in the saliva (Appendix Fig. 1). Interestingly, the ARE mRNA decay-promoting protein TTP (Tristetraprolin) was present in both salivary

glands, but was completely absent from saliva (Appendix Fig. 1). These findings suggest that salivary glands are sources of saliva ARE-binding proteins.

DISCUSSION

Saliva is a non-invasive body fluid that can be used for the identification of biomarkers for disease detection. Our laboratory (Li *et al.*, 2004b) and other groups (Juusola and Ballantyne, 2005; Gomes *et al.*, 2006) have shown that human salivary secretions contain mRNAs that can be used as biomarkers for oral cancer, periodontal disease, and forensic applications. We have previously shown that the association of RNAs with certain macromolecules confers stability against nucleases present in the saliva (Park *et al.*, 2006). Here, we provide evidence that a mechanism of salivary mRNA stability in the majority of salivary mRNA is mediated by AU-rich elements. Using IL-8 mRNA as an example, we have shown that ARE-containing transcripts are stabilized in the presence of human salivary protein lysates and are associated with HuR and AUF1, as shown by UV cross-linking and co-immunoprecipitation experiments. Importantly, immuno-depletion experiments revealed that both HuR and AUF1 promoted the stability of ARE mRNAs in the saliva. The former appeared to be particularly important for IL-8 mRNA stability, as shown by *in vitro* reconstitution experiments.

ARE-containing genes comprise 5–8% of the human transcriptome (Bakheet *et al.*, 2006) and encode proteins that are involved in diverse biological processes. We have found that more than 30% of the normal saliva RNA transcripts contain AUUUA motifs, making up the majority of the salivary transcriptome. Analysis of oral cancer transcriptomic biomarkers revealed that 70% (5/7) of the ARE-containing mRNAs, suggesting that the ARE sequence contributes to their stability and function. It is tempting to speculate that the half-life of these salivary mRNAs is determined by cooperative interaction with ARE-binding proteins regulating their stabilization and destabilization. Findings demonstrating enhanced mRNA stability in tumor cells suggest that altered recognition of ARE sequences in neoplasia may lead to improper function of mRNAs (Schuler and Cole, 1988; Xu *et al.*, 1997). Here we show that binding of AUF1 and HuR to the IL-8 mRNA enhanced stability of the ARE transcripts in saliva.

In humans, salivary nucleic acid has been viewed as unstable due to the presence of strong salivary RNases (Eichel *et al.*, 1964; Bobek *et al.*, 1993). Nevertheless, the protein-associated RNAs are protected against ribonucleases (Moallem *et al.*, 1998), with decreased protein-binding rendering RNAs susceptible to RNase activity. Thus, protein-RNA interactions are vital for RNA stability. We have previously shown that secreted salivary RNA is protected from degradation, whereas mRNA that is exogenously (*in vitro*-transcribed) added to saliva is rapidly degraded (Park *et al.*, 2006). The current study extends these findings by identifying the salivary proteins (AUF1 and HuR) that bind to and stabilize salivary ARE-containing RNAs, regulating salivary mRNA stability. HuR has been shown to bind to and stabilize many different ARE-containing mRNAs in different cell systems (Ma *et al.*, 1996; Keene, 1999; Dixon *et al.*, 2001). In salivary gland carcinomas, HuR was overexpressed and stabilized Cox-2 mRNA (Cho *et al.*, 2007). We showed that recombinant HuR promoted stabilization of IL-8 mRNA in salivary lysates, suggesting that it regulates ARE mRNA stability in the saliva. AUF1 is a cytosolic protein that reportedly associates with other proteins to stabilize mRNAs such as α -globin mRNA (Kiledjian *et al.*, 1997; Chkheidze *et al.*, 1999). The additive function of AUF1 and HuR may possibly determine the rate of AU-rich mRNA decay in human saliva, although it is currently unknown whether different mRNA species may have different stabilities in saliva. Although HuR and AUF1 stabilize salivary ARE mRNAs, it is unclear whether these proteins are associated with mRNAs prior to their passage from the salivary ductal fluid into the oral cavity. Future study of protein-RNA interactions in secretions from the three major glands will circumvent buccal cell and other contamination and will enable us to obtain a more complete understanding of the results from these salivary RNA studies. In conclusion, the

results presented here provide a novel link between the mRNA stability and ARE-binding proteins in human saliva.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Addition of AUUUA-containing competitor accelerates IL-8 RNA degradation *in vitro*. **(A)** Sequences of RNA used for the study. **(B)** Representative autoradiograms showing *in vitro* decay of full-length, radiolabeled IL-8 mRNA in the absence of competitor or in the presence of unlabeled 60-nt IL-8 ARE competitor (100 ng), unlabeled mutated IL-8 ARE competitor (100 ng), or a control 950-nt β -actin mRNA (100 ng). **(C)** Kinetics of transcript degradation. **(D)** Representative autoradiographs showing decay of 60-nt IL-8 ARE-containing fragments in which all 4 AREs were intact (IL-8 ARE), fragments in which a single ARE was mutated (3g mt), or fragments in which all AREs were mutated (All mt). **(E)** Kinetics of transcript degradation. The data are from three independent experiments; values are means $\pm$ SD.

Figure 2.

IL-8 mRNA associates with AUF1 and HuR in human saliva protein lysates. Salivary lysate was UV-cross-linked with *IL-8* ARE, *IL-8* ARE mutant, or β -actin. Cross-linked RNA-protein complexes were then immunoprecipitated with anti-AUF1 (**lanes 4–6**) or anti-HuR (**lanes 8 and 9**) and analyzed by SDS-PAGE. **Lanes 1–3** show the cross-linked extract prior to immunoprecipitation. NS, non-specific serum.

Figure 3. Recombinant HuR protects IL-8 mRNA against *in vitro* degradation by human saliva lysates. **(A)** Representative autoradiographs showing decay of IL-8 mRNA in the presence of increasing concentrations of recombinant HuR. **(B)** Autoradiographs showing decay in the presence of equal amounts of BSA or non-specific recombinant GST fusion protein. **(C)** Effect of recombinant HuR on the kinetics of transcript degradation. The data are from three independent experiments; values are means $\pm$ SD.

Figure 4.

AUF1 and HuR protein levels in human salivary glands and saliva. Western blot analysis of AUF1 isoforms and HuR in the submandibular gland (SM), the parotid gland (PA), and the saliva (SL).