An Archaeal Protein with Homology to the Eukaryotic Translation Initiation Factor 5A Shows Ribonucleolytic Activity

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To identify proteins that are involved in RNA degradation and processing in Halobacterium sp. NRC-1, we purified proteins with RNA-degrading activity by classical biochemical techniques. One of these proteins showed strong homology to the eukaryotic initiation factor 5A (eIF-5A) and was accordingly named archaeal initiation factor 5A (aIF-5A). Eukaryotic IF-5A is known to be involved in mRNA turnover to bind RNA. Hypusination of eIF-5A is required for sequence-specific binding of RNA. This unique post-translational modification is restricted to Eukarya and Archaea. The exact function of eIF-5A in RNA turnover remained obscure. Here we show for the first time that aIF-5A from Halobacterium sp. NRC-1 exhibits RNA cleavage activity, preferentially cleaving adjacent to A nucleotides. Detectable RNA binding could be shown for aIF-5A purified from Halobacterium sp. NRC-1 but not from Escherichia coli, while both proteins possess RNA cleavage activity, indicating that hypusination of aIF-5A is required for RNA binding but not for its RNA cleavage activity. Furthermore, we show that the hypusinated form of eIF-5A also shows RNase activity while the unmodified protein does not. Charged amino acids in the N-terminal domain of aIF-5A as well as in the C-terminal domain, which is highly similar to the cold shock protein A (CspA), an RNA chaperone of E. coli, are important for RNA cleavage activity. Moreover our results reveal that activity of aIF-5A depends on its oligomeric state.

Maturation of the 5’-end of tRNA in Archaea is catalyzed by the ribonucleoprotein complex RNase P as in all other organisms (1). Processing at the tRNA 3’-end is performed by RNase Z in all domains of life (2). The helix-bulge-helix endoribonuclease that removes introns from archaeal transcripts is also involved in rRNA processing (3). It is likely but not proven to date that exosome-like protein complexes which are present in some Archaea are also involved in rRNA processing, because this was demonstrated for the eukaryotic exosome (4). The archaeal exosome consists of a central hexameric ring of 3’ to 5’ exoribonucleases with RNase PH domains and additional exoribonucleases and RNA-binding proteins (4). In Sulfolobus solfataricus and Methanothermobacter thermotogotrophicus, two proteins, each containing an RNase PH-like domain, assemble into a hexameric ring with 3’ to 5’ phosphorolytic exoribonuclease activity. This complex is associated with a putative hydrolytic 3’ to 5’ exoribonuclease, an RNA-binding protein, and a DnaG homologue (5–8). The archaeal exosome polyadenylates RNA substrates in vitro and degrades polyadenylated substrates (9, 10), but the in vivo function has not been elucidated. The eukaryotic exosome is also involved in mRNA degradation (11, 12).

The genome of Halobacterium sp. NRC-1 does not encode homologues of the central subunits of the exosome. It was shown that the control of mRNA stability is involved in regulation of gene expression in Haloarchaea (13), but almost nothing is known about the enzymes involved in mRNA processing and degradation. Bioinformatic searches for RNA-degrading enzymes revealed that the genome of Halobacterium encodes a homologue to the bacterial 3’ to 5’ exoribonuclease RNase R and a protein with very limited homology to the bacterial endoribonuclease RNase E. In the Gram-negative bacteria Escherichia coli, Rhodobacter capsulatus, and Pseudomonas syringae RNase E organizes degradosome complexes, which are involved in mRNA turnover (14–16). In all three degradosomes 3’ to 5’ exoribonucleases and helicases are associated with RNase E. It cannot be excluded that enzymes with no or very low similarity to RNases of bacteria or eukaryotes are involved in RNA degradation/processing in Archaea.

To identify enzymes involved in RNA processing and degradation in Halobacterium we performed a biochemical screen for such activities. Among several proteins with RNase activity identified by this approach was the gene product with the OE
number2 OE3487R (Swiss-Prot Q9HP78), which shows high similarity to the eukaryotic initiation factor 5A (eIF-5A). Because the eIF-5A protein was isolated initially from a ribosomal fraction of rabbit reticulocytes, it was considered a translational initiation factor (17). However, many different functions have been assigned to eIF-5A, especially in higher eukaryotes. e.g. its involvement in the control of cell proliferation and apoptosis suggested it as a target for anticancer strategies (18). A single point mutation in eIF-5A of yeast gives a temperature-sensitive phenotype. This strain shows reduced translation by 30% but also impaired mRNA decay at the non-permissive temperature (19). Another temperature-sensitive mutation in eIF-5A of yeast can be suppressed by overexpression of the gene for PAB1 (poly(A)-binding protein), indicating an involvement in mRNA turnover (20). A SELEX (systematic evolution of ligands by exponential enrichment) approach revealed that eIF-5A is capable of sequence-specific RNA binding to an AAAUGU or UAACCA element (21). This sequence-specific RNA interaction requires the hypusination of eIF5-A, which is a unique post-translational modification restricted to Eukarya and Archaea. Modification of a single lysine residue is catalyzed by deoxyhypusine synthase, which covalently links an amino-buty1 group, derived from spermidine that is subsequently hydroxylated by deoxyhypusine hydroxylase (22–24). Both, the genes for eIF-5A and for deoxyhypusine synthase are essential in yeast, and their disruption leads to a lethal phenotype (25, 26). Affinity co-purification and PCR differential display identified 20 RNA sequences that bind to eIF-5A, some of which contained the previously selected RNA sequence motifs. All identified RNA sequences had the potential to form extensive stem-loop structures (27).

We isolated an eIF5-A homologue, designated aIF-5A from Halobacterium cell extracts because of its RNA-degrading activity, which has not been reported for its eukaryotic homologue. Our biochemical characterization revealed that aIF5-A possesses RNA-degrading activity in vitro that does not require high salt concentrations and is inhibited at magnesium concentrations above the physiological range. Recombinant Histagged proteins expressed in either E. coli or Halobacterium show identical cleavage patterns, suggesting that hypusination is not required for a specific association of aIF-5A and RNA with subsequent cleavage in vitro. However only the recombinant protein expressed in Halobacterium binds to RNA in electrophoretic mobility shift assays, indicating that hypusination stabilizes the RNA protein complex. Interestingly recombinant human eIF-5A shows RNA binding (21) and also hypusine-dependent ribonucleolytic activity. Here we characterize the in vitro RNA-degrading activity of aIF-5A and show that mRNA is cleaved at identical positions in vivo.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture Conditions, and Transformation—E. coli M15 (REP4) cells were grown on standard I medium (Difco) at ampicillin and kanamycin concentrations as specified by Qiagen (Qiagen). E. coli strains JM109 and JM110 were grown on standard I medium containing 200 mg/liter ampicillin (JM109) or 100 mg/liter streptomycin (JM110). Halobacterium sp. NRC-1 (ATCC number: 700922) was grown in ATCC medium 2185 at 37 °C (small scale cultures, < 200 ml) or 42 °C (200–500 ml liquid or solid media cultures) at aerobic conditions. Selection for transformants of Halobacterium sp. NRC-1 was done on plates containing 10 mg/ml mevinolin. The prodig for mevinolin was a gift from Merck Sharp and Dohme Ltd. and was converted into the active form according to the manufacturer. Protein expression in E. coli harboring pQE30 vectors was induced by adding 0.8–1 mM isopropyl-1-thio-β-D-galactopyranoside to exponentially grown cultures at A600 = 0.6–0.8. Cells were kept growing for 3–4 h at 37 °C and 180 rpm on a shaker and harvested by centrifugation (5000 x g, 4 °C, 20 min).

Halobacterium sp. NRC-1 cells containing pBPH-M for expression of recombinant proteins were harvested at room temperature (5000 x g, 20 min) after shifting exponentially grown cultures from aerobic to anaerobic conditions overnight and back to aerobic conditions for 6–8 h.

Transformation of Halobacterium sp. NRC-1 was performed as previously described (28) with the modification that regeneration of the cells after transformation was done for 1.5–2 days at 37 °C on a shaker instead of only overnight.

Plasmid Construction—The following primers were used for PCR amplification (nucleotides resulting in restriction sites underlined): for expression of full-length aIF-5A with pQE30 in E. coli: (5′-GCGGATCCATGGCGAAAGAGCAGAAG- GAAGT-3′) and (5′-CGGAGCCTTAAACCGGTGGCCGC- GCTGGA-3′), full-length aIF-5A with pBPH-M in Halobacterium sp. NRC-1: (5′-GGCCATATGGCGAAGAAGGGACAGAAGGAGAAAGG-3′) and (5′-GGCAGCATGCACCTCTCGAGAGAATCGTGTAA-3′), N-terminal domain of aIF-5A with pQE30 in E. coli: (5′-ACATGCTGGCGAAAGAGCAGAAGGAGAGAAG-3′) and (5′-CCCAAGCTGATCCATGCGAAAGAAGGAGAGAAGGAGAG-3′) and (5′-CCCAAGCTGATCCATGCGAAAGAAGGAGAGAAGGAGAG-3′) and (5′-AATGTGCTGATCCATGCGAAAGAAGGAGAGAAGGAGAG-3′). Site-directed mutagenesis appropriate primers were used to perform overlay PCR with plasmid DNA as template, used for heterologous protein expression in E. coli. Primers were designed either introducing or eliminating a restriction site at the mutation site to allow easy screening of the transformants. Resulting PCR products were ligated into pQE30 for protein expression in E. coli or into pBPH-M for expression in Halobacterium sp. NRC-1. Accuracy of the plasmid constructs was confirmed by sequencing. Vectors for protein expression in E. coli were either used directly in the cloning host (E. coli JM109) or propagated in E. coli M15 (REP4) cells. Plasmids for protein expression in Halobacterium sp. NRC-1 were shuttled by E. coli JM110 to demethylate DNA before transformation.
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In Vitro Transcription of RNAs and Enzymatic Assays—Generation of DNA templates used for in vitro transcription is described under supplemental materials. In vitro transcription was done using MEGShortscript™ T7 transcription kit (Ambion) or by incubating T7 RNA Polymerase (NEB) according to the manufacturer’s instructions except that 2.5 mM of each nucleotide was used for nonradioactive in vitro transcription. For producing radioactively labeled RNA substrates, 2.5 mM rUTP was replaced by 0.5 mM rUTP and 20 μCi of [α-32P]rUTP (3000 Ci/mmol, Hartmann Analytic or Amersham Biosciences). Radioactively labeled RNA transcripts were purified on a 10% polyacrylamide-8M urea gel. Bands were cut and their radioactivity was determined with a Betaplate (Packard). The DNA template were loaded on the same gel to map the positions of the cleavage sites.

In Vitro Transcription—RNA Isolation

Transcription reactions were performed in a total volume of 40 μl containing 50 mM Tris-HCl, pH 7.5, 10 mM of each salt buffer (3M NaCl, 1M KCl), 2 mM β-mercaptoethanol, 50 mM sodium phosphate buffer, pH 7.5) by sonication and affinity chromatography using Ni-NTA-agarose under high salt conditions (3 M NaCl, 1 M KCl). Protein samples were concentrated, and imidazol was removed using Vivaspin 500 (Vivascience) or Centricon® (Millipore). Nontagged (wt) protein was purified from Halobacterium sp. NRC-1 as follows: cells were lysed osmotically in 50 mM Tris-HCl, pH 7.5 and by sonication; lysate was ultracentrifuged for 1 h at 40,000 rpm (45 Ti, Beckman) and 4 °C; proteins were precipitated with ammonium sulfate (85% saturation), dissolved in 50 mM Tris-HCl, pH 7.5, and dialyzed against the same buffer containing additionally 100 mM KC1 and 1 mM EDTA to remove ammonium sulfate; dialysate was loaded onto a heparin column (Amersham Biosciences); bound proteins were eluted with an increasing KC1 gradient; aIF-5A eluted at ~200 mM KC1 and was further purified by gel filtration.

RESULTS

aIF-5A from Halobacterium sp. NRC-1 Exhibits RNA-cleaving Activity, Which Does Not Require Hypusination—To identify RNA-degrading proteins from Halobacterium sp. NRC-1 we used fractions of cell extracts for in vitro RNA degradation experiments. We synthesized different radiolabeled small RNA molecules by in vitro transcription as substrates. These transcripts differ in length from about 50 to 600 nt (Table 1) and contain double-stranded and single-stranded regions (Figs. 3A and 6A, and supplemental Fig. S3, A–K). Secondary structure prediction was surprisingly, we only observed very low degradation activity. When
TABLE 1
List of RNA substrates used including sources of plasmids and lengths of resulting in vitro transcripts

| RNA name | Plasmid name | Description | Linearized with: | Transcript length | Reference |
|----------|--------------|-------------|------------------|-------------------|-----------|
| sub1a_ (NotI) | pDrive | Religated cloning vector with T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | NotI | 136 nt | Qiagen |
| sub1b_ (HincII) | pDrive:OE2664F | Cloning vector with OE2664F (VNG1185G) insert downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | HincII | 74 nt | BamHI |
| sub1c_ (BamHI) | pDrive:OE2664F | Cloning vector with OE2664F (VNG1185G) insert downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | BamHI | 48 nt | This work |
| sub2_(oe2664) | pDrive:OE2664F | Cloning vector with OE2664F (VNG1185G) insert downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | HincII | 308 nt | This work |
| sub3_(gvp a/c) | pDrive::gvpA/C | Cloning vector with gvp A/C (VNG6029G, VNG6031G) insert downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | HindIII | 379 nt | This work |
| sub4_(37b4) | pUC18:H9Rc37b4 | Helices 8–11 of R. capsulatus 23S rRNA (DNA region 109–205 (E. coli numbering)), Ap' | HindIII | 189 nt | (29) |
| sub5a_(cut) | dsRNA pDrive cut | Cloning vector with two times 21 complementary nucleotides linked with sequence: (TGCCGC) downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | Small | 48 nt | This work |
| sub5b_(nocut) | dsRNA pDrive nocut | Cloning vector with two times 21 complementary nucleotides linked with sequence: (TGCCGC) downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | Small | 48 nt | This work |
| sub6_(IF-binding) | – | Antisense DNA oligo hybridized with T7 promoter oligo to obtain RNA with single-stranded regions containing putative elf-5A binding sites | – | 48 nt | This work |
| sub7_(pzbp) | pZBP | 168 bp BstEll-Psitl-fragment of R. capsulatus pssl-gen in pGEM3Zf+; Ap' | HindIII | 225 nt | (30) |
| sub8_(alf-5a) | pDrive::alf-5A | Cloning vector with alf-5A (VNG1768G) insert downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | HindIII | 584 nt | This work |
| sub9_(gvp a) | pDrive::gvpA | Cloning vector with gvpA (VNG6029G) insert downstream of T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap'; Km' | HindIII | 382 nt | This work |
| sub10_(halo5S) | pSP72::rrib | Cloning vector with rrib (VNGr04) insert downstream of T7 RNA-polymerase promoter and start site; β-lactamase coding region; LacZ-Peptide; MCS; Ap' | Xhol | 142 nt | This work |
| sub11_(SK_XbaI) | pBluescript IISK | Cloning vector with T7 RNA-polymerase promoter and start site; LacZ-Peptide; MCS; Ap' | XbaI | 93 nt | Stratagene (21) |

we omitted MgCl₂ in the buffer, the Halobacterium extracts showed cleavage of some substrates when the concentration of NaCl or KCl was lower than 200 mM.

After ultracentrifugation of cell-free extracts, ammonia sulfate precipitation, dialysis, and affinity chromatography (heparin-Sepharose) we found four peaks of RNA-degrading activity distributed over the eluted fractions. For each of the activity peaks, fractions were combined, and the proteins concentrated (Centricon®, Millipore). Four different protein fractions were then separately applied to an anion exchange column, and the eluted fractions were analyzed in regard to protein pattern and RNA degradation of standard substrates. We were able to purify a protein to near homogeneity that showed RNAse activity in vitro. MALDI-TOF analysis identified this protein as alf-5A.

Subsequently we expressed an N-terminally His₆-tagged version of alf-5A in E. coli as well as a C-terminally His₆-tagged version in Halobacterium. The proteins were isolated as described and activities were studied by in vitro degradation experiments. Expression of the His₆-tagged protein and purification from Halobacterium should be pursued in hypusinase since the gene for the key enzyme of the modification step (OE3059F: gene for deoxyhypusine synthase (EC 2.5.1.46), Swiss-Prot Q9HPX2) is present in Halobacterium, while E. coli is unable to catalyze this modification. As shown in Fig. 1A, the two alf-5A preparations cleave substrate sub1a_ (NotI) producing cleavage products with identical lengths. Thus, hypusinase is not required for cleavage of this substrate in vitro. All other substrates listed in Table 1 with the exception of the 44-nt sub1c_ (BamHI) (data not shown) and the 48-nt sub5b_ (nocut) substrate (Fig. 6B) were also cleaved by alf-5A.

Archaeal IF-5A contains two domains connected by a short hinge. The N-terminal part contains the hypusine residue, an SH3 (Src homology 3)-like barrel motif as well as a KOW motif (36). The C-terminal domain has 10–12% sequence identity and a striking structural similarity to CspA (37), the major cold shock protein of E. coli, which is known to be an RNA chaperone (38). Like CspA, the C-terminal domain of alf-5A possesses a fold that is classified as oligomer binding (OB) fold (39). We expressed His₆₉-tagged versions of both domains in E. coli and used the purified proteins for RNA degradation assays. None of the domains alone showed RNA cleavage (Fig. 1B). When both domains were combined either before (br) or after refolding (ar), again no significant RNA cleavage activity was observed. We conclude that both domains together are required for RNA binding or catalysis and that a covalent linkage is required to fulfill these functions.

We further characterized the RNA cleavage activity of alf-5A in regard to requirement for cations, polycations and compatible solutes. Cleavage activity of the recombinant proteins expressed in Halobacterium as well as the non-tagged protein purified from Halobacterium was maximal at around 120 mM KCl and even lower if it was purified from E. coli. All three proteins showed no ribonucleolytic activity when ion concentrations exceeded ~300 mM, shown for the recombinant protein expressed in E. coli and incubated with sub1a_ (NotI) in Fig. 1C. This is considerably lower than the intracellular KCl concentration that was estimated to be ~4.5 M.
for *Halobacterium* (40). No difference in cleavage activity was observed when NaCl was used instead of KCl.

The presence of 2 mM EDTA did not inhibit ribonucleolytic activity. We conclude that no divalent metal ions are required for the ribonucleolytic activity of aIF-5A. Divalent metal ions showed inhibitory effects in concentrations in the mM range (4 mM or higher for Mg$^{2+}$ and 2 mM or higher for Zn$^{2+}$, data not shown).

We also tested the effect of polyamines on the RNA degrading activity of aIF-5A. Polyamines are known to bind to RNA and may help to make stable sites accessible for degrading activities or to mask labile sites thereby increasing their stability. Interestingly, the hypusine modification with its positive charges resembles spermidine and may have a similar function in RNA binding (21, 27). All tested polyamines stimulated RNA degradation by aIF-5A to different extent and inhibited degradation at higher concentrations. The maximal increase of ribonucleolytic activity of aIF-5A was reached at 1 mM putrescine, 4 mM spermidine or 120 mM ornithine (supplemental Fig. S2, 0.5 mM spermidine or 10 mM ornithine. Almost no activity was detected above 20 mM putrescine, 4 mM spermidine, or 120 mM ornithine (supplemental Fig. S1, A–C). To investigate whether the stimulating and inhibitory effect of polyamines may be of biological relevance we representatively quantified RNA cleavage assays containing varying concentrations of spermidine.

While a stimulating effect was only 20–40%, the inhibitory effect is obvious, because less than 30% of RNase activity remains at 4 mM spermidine (supplemental Fig. S2D). The effect of polyamines on the activity of a protein from *Halobacterium* is rather surprising because *Halobacterium* was reported to lack any polyamines (41). However, genome information indicates the presence of polyamines such as putrescine and spermidine from arginine in microorganisms; OES205R: ornithine carbamoyl-transferase (EC 2.1.3.3), Swiss-Prot Q48296).

**Determination of Sites Cleaved by Halobacterium sp. NRC-1 aIF-5A on Different Artificial Substrates**—The binding of RNA has been described for the eukaryotic IF-5A (21, 27); however, no cleavage activity has been reported. To determine cleavage sites we performed primer extension analysis after incubation of the substrates with aIF-5A (Fig. 2, A–C). Controls without the addition of aIF-5A were run to identify bands which are not due to the generation of new 5′-ends by RNA cleavage but rather by stop of reverse transcription at secondary structures. Among 32 unambiguously identified cleavage sites within different substrates, 18 were located between a 5′-C and a 3′-A. Seven of the remaining 14 cleavage sites were also located between a 5′-pyrimidine and a 3′-purine base (C/G: 2, U/A: 5). Four sites were found between two pyrimidine bases (C/C: 2, C/U: 2), two sites were found between two purine bases (G/A: 2), and only one site was located between a 5′-purine and 3′-pyrimidine base (A/U). Beside the preference for cleavage between C and A, no consensus sequence could be identified when sequences of nine nucleotides flanking the cleavage site were analyzed by a multiple sequence alignment program (ClustalW, data not shown). Interestingly, not all CA bonds of a substrate molecule were cleaved by aIF-5A, and control reactions with NaOH revealed that cleavages did not occur at bonds that were most susceptible to hydrolysis. To verify our hypothesis that CA bonds within single-stranded regions are the primary target sites for the ribonucleolytic activity of aIF-5A, we generated two short RNA substrates which form a stable stem-loop structure. Both substrates are derived from the cloning vector pDrive and differ only in one base within the sequence forming the loop which is: (5′-UCCAGC-GC-3′) for the sub5a (cut) and (5′-UGCCGC-GC-3′) for the sub5b (nocut) substrate (Fig. 3A). Fig. 3B shows that only the sub5a (cut) substrate is cleaved by aIF-5A generating two bands migrating nearly at the same position indicating that the ribonucleolytic activity of aIF-5A is restricted to single-stranded CA bonds. The sub5b (nocut) substrate lacking single-stranded CA bonds

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**FIGURE 1.** RNA cleavage assays comparing RNase activity of different versions of aIF-5A (A and B) and tolerance of recombinant aIF-5A expressed in *E. coli* to potassium chloride (C). A, RNA cleavage assay comparing cleavage pattern produced by different concentrations of His$_5$–aIF-5A expressed in *E. coli* (E. coli) with aIF-5A–His$_5$ expressed in Halobacterium sp. NRC-1 (Halo). Both protein variants show identical cleavage pattern on sub1a (NotI) RNA substrate. Incubation was done for 1.5 h at 42 °C. Control reactions (−) show no degradation of RNA; M, RNA Decade$^{TM}$ Marker, B, RNA cleavage activity of full-length aIF-5A (F) and C-terminal (C) and N-terminal (N) domain of aIF-5A expressed and purified from E. coli. Substrate: sub2 (oe2664); −: control reaction; or, br C- and N-terminal protein domain mixed together after or before refolding; lanes 1 and 2, incubated for 1 or 2 h at 42 °C; protein concentration: −50–60 ng/μl, C, RNA cleavage activity of His$_5$–aIF-5A at different salt concentrations. 100 ng/μl protein was incubated for 1.5 h at 42 °C in buffer containing 50–350 mM KCl; substrate: sub1a (NotI); −: control reactions; M, RNA Decade$^{TM}$ Marker.
is not cleaved by aIF-5A and also no CA bond within the double-stranded regions are cleaved (Fig. 3C). In contrast RNase A further degrades the sub5a_(cut) substrate to faster migrating fragments, which distinguish both activities (data not shown).

We also isolated total RNA from *Halobacterium sp*. NRC-1 and performed primer extension analysis with primers specific for the mRNA of ribosomal protein S28.eR (OE2664F, SwissProt P57710, data not shown) and the central part of *gvpAC* (lane R, Fig. 2B). The pattern of 5′-ends detected was clearly different from the pattern after *in vitro* cleavage with purified aIF-5A. However, at all positions cleaved by aIF-5A *in vitro*, 5′-ends were also observed for the RNA isolated from *Halobacterium*.

To exclude that this is just an artifact due to breakage of labile RNA bonds during the procedure of RNA isolation, we added an *in vitro* transcript (sub7_(pzbp), Table 1) to the cells before RNA isolation. This transcript stems from sequences of the *puf* operon of *R. capsulatus* and is not present in *Halobacterium*. After RNA isolation primer extension was performed with a primer specific for the transcript. No additional bands were detected compared with reactions, where the sub7_(pzbp) transcript was not added to cells prior to RNA isolation. Thus the 5′-ends we determined with RNA isolated from *Halobacterium* are most likely generated *in vivo* and not during the RNA preparation.

**Identification of Amino Acids of aIF-5A Required for Substrate Binding/Cleavage**—To learn more about the mechanisms by which aIF-5A cleaves RNA substrates we attempted to identify amino acids by specific chemical modification, which are required for RNA binding or for catalysis. The inhibition of the aIF-5A catalyzed cleavage by 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC) (42) or phenylglyoxal (PGO) (43) suggested the involvement of Glu/Asp or Arg in these processes, respectively. In addition our attention focused on a single His residue in close proximity to the hypusinated Lys because the ribonucleolytic activity of aIF-5A also was inhibited by preincubation of protein samples with diethylpyrocarbonate (DEPC) (44, 45).

Fig. 4A shows the amino acid alignment of three euryarchaeal aIF-5A from *Halobacterium* sp., *Haloarcula marismortui*, and *Pyrococcus horikoshii* OT3, which was used as template to model the three-dimensional structure of aIF-5A from *Halobacterium* (Fig. 4B), two crenarchaeal IF-5A from *S. solfataricus* P2 and *Pyrobaculum aerophilum*, as well as the eukaryotic IF-5A from yeast and human, which are the most exten-
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sively characterized proteins of this family. Residues we exchanged in aIF-5A of *Halobacterium* to test the role of selected amino acid residues and homolog positions in the other shown 1F-5A are highlighted in bold with a light gray background (M1–M6, see also Fig. 4B). All mutant aIF-5A proteins were expressed as His<sub>6</sub>-tagged variants in *E. coli*. In vitro degradation assays revealed that the single His residue at position 38 (M2) does not significantly influence RNA cleavage activity of aIF-5A (not shown). Exchange of the basic amino acids lysine-lysine-arginine at positions 53–55 (M3) to the neutral amino acids alanine-alanine-glycine also did not reduce activity of aIF-5A (not shown). When arginine at position 9 (M1) was exchanged to alanine, reduced cleavage activity was observed (not shown). The same was true when arginine-lysine at positions 72, 73 (M4) were exchanged to glycine-alanine, or when arginine-lysine at positions 122, 123 (M6) were exchanged to alanine-glycine, respectively (not shown).

The fact that all these variants still showed cleavage activity suggested that these amino acids are involved in RNA binding rather than in catalysis or that several amino acids may contribute to an acid base catalysis. In the next step, we combined the exchanges at positions 72, 73, 122, 123 (M4 + M6) leading to a drastic reduction of cleavage activity (Fig. 5). Nevertheless cleavage was still observed at low rate and long incubation time. In addition we replaced the glutamic acid at position 117 by alanine (M5). This glutamic acid is positioned between the basic amino acids (Fig. 4B), which were found to be involved in RNA binding/cleavage. This mutant also showed clearly reduced activity but was still able to cleave the substrate (Fig. 5).

To check whether the amino acid found to be important for *in vitro* RNase activity of aIF-5A are involved in RNA cleavage or binding we performed electrophoretic mobility shift assays. Based on the published information (27) and our finding that potential target sequences for RNase activity of aIF-5A are mainly located within single-stranded regions, we constructed a 48-nt RNA substrate: sub6<sub>-IF binding</sub>, Table 1. This substrate should fold into a stable structure (Fig. 6A) containing the putative eIF-5A binding sites (two times (5'−AAAUGU-3') and once (5'-UAACCA-3')) within single-stranded regions. Using different electrophoretic systems, modes of preincubation and protein/substrate ratios we were not able to show binding of aIF-5A expressed either in *Halobacterium* sp. NRC-1 or *E. coli* (data not shown), although this substrate is well accessible for RNA binding/cleavage. This mutant also showed clearly reduced activity but was still able to cleave the substrate (Fig. 5).

To check whether the amino acid found to be important for

**FIGURE 4.** Structural organization of translation initiation factor 5A. A, multiple sequence alignment of five archaeal IF-5As (*Halobacterium* sp. NRC-1 (*Halobacterium* sp. NRC-1, Swiss-Prot Q9HP78), Haloarcula marismortui (*Halobacterium* sp. NRC-1, Swiss-Prot Q9HP78), Pyrococcus horikoshii OT3 (*Pyrococcus horikoshii OT3, Swiss-Prot Q9HP78), Pyrococcus furiosus 5010 (*Pyrococcus furiosus 5010, Swiss-Prot Q9HP78), S. solfataricus P2 (*Sulfolobus solfataricus P2, Swiss-Prot P000089), P. aerophilum (*Pseudomonas aerophilum, Swiss-Prot P000089), and P. aerophilum (*Pyrococcus aerophilum, Swiss-Prot P000089)) as well as two eukaryotic IF-5As (*Saccharomyces cerevisiae* (K1A, yeast, Swiss-Prot P19211), and human (human_eIF5A, Swiss-Prot P63241), generated with ClustalW (1.83). Asterisk, identical residues; colon, conserved substitution; dot, semiconserved substitution; -, gap. Bold and highlighted in light gray, amino acids that have been exchanged in the archaeal IF-5A from *Halobacterium* sp. NRC-1 in this study (mutations M1–M6, see also Fig. 4B); the KOW motif is highlighted in dark gray, and corresponding E-values are given. E-values were obtained by comparing each protein to the protein families data base Pfam, version 21.0 with Fragment (fs) Pfam search-type. B, two views of the putative structure of aIF-5A from *Halobacterium* sp. NRC-1 generated by fitting the protein sequence of aIF-5A from *Halobacterium* sp. NRC-1 to the structure of aIF-5A from *P. horikoshii OT3*, visualized with Swiss-PdbViewer (58). The backbone and side chain of amino acids that have been mutated in this study (M1–M6, see also Fig. 4A) are indicated.
this substrate apparently initiates at the only CA bond within a single-stranded region at position 32/33 (Fig. 6A), generating a product with the length of 32 nucleotides (marked with an arrow in Fig. 6B), which is further degraded with increasing time. This observation fits well to our finding that only CA bonds within single-stranded regions are accessible for the RNase activity of aIF-5A. However, we could show binding of aIF-5A expressed in *Halobacterium sp. NRC-1* but not in *E. coli* to RNA transcripts longer than the sub6_(IF binding) substrate. These substrates are approximately double in length of the sub6_(IF binding) substrate and can fold into complex structures, but none of them contains a putative binding site for eIF-5A. Among them is the sub1a_(NotI) substrate, which we also used for several *in vitro* degradation assays, sub10_(halo5S), which is an *in vitro* transcript of the 5 S rRNA gene of *Halobacterium sp. NRC-1* (shown in Fig. 7) and sub_11(SK_XbaI), which is known to be bound by eIF-5A (21).

Interestingly, a protein sample of the hypusinated eIF-5A produced exactly the same pattern of degradation products as aIF-5A does when it was incubated with the sub1a_(NotI) substrate, while a sample of the unmodified version of eIF-5A only shows minor RNase activity (Fig. 6C).

**Activity of Halobacterium sp. NRC-1 aIF-5A Depends on Its Oligomeric State**—We have evidence that ribonucleolytic active aIF-5A is in an oligomeric state. First: the protein elutes in two distinct peaks in size exclusion chromatography corresponding to 26–36 and 41–46.5 kDa, respectively, whereas the calculated monomeric mass of the recombinant protein is 15.7 kDa (supplemental Fig. S2A). The peak corresponding to larger protein complexes is only present when protein samples are renatured before gel filtration analysis. Second: native, untagged aIF-5A coelutes with the C-terminal His<sub>7</sub>-tagged aIF-5A when the pull-down assay was performed at low salt conditions (up to 300 mM) and in the presence of 10 mM MgCl<sub>2</sub> (supplemental Fig. S2B), and third: cleavage product formation sharply drops when a minimal protein concentration of about 10–20 ng/μl is reached in *in vitro* RNA cleavage assays (supplemental Fig. S2C).

**FIGURE 5.** RNA cleavage assay. RNA cleavage assay comparing wild type aIF-5A (wt) with mutant forms of aIF-5A (M4 + M6, arginine-lysine at positions 72, 73 (M4) were exchanged to glycine-alanine and arginine-lysine at positions 122, 123 (M6) were exchanged to alanine-glycine; M5, glutamic acid at position 117 was replaced by alanine). Both mutant variants show reduced RNA cleavage activity on sub1a_(NotI) RNA substrate compared with wild-type protein. Incubation was done for 1 h at 42 °C.

**FIGURE 6.** RNA substrate sub6_(IF-binding) is cleaved by aIF-5A and eIF-5A has hypusine-dependent RNase activity. A, secondary structure prediction of substrates sub6_(IF-binding). The putative binding sites for eIF-5A (AAAUGU and UAACCA) are highlighted. B, RNA cleavage activity of His<sub>7</sub>_aIF-5A (100 ng/μl) incubated at 42 °C with RNA substrate sub6_(IF-binding) for the indicated time. Control reactions incubated for 0 or 280 min at 42 °C; M, RNA Decade<sup>TM</sup> Marker. C, RNA degradation assay comparing cleavage activity of aIF-5A—His<sub>7</sub> with hypusinated (hyp +) and nonhypusinated (hyp −) eIF-5A. RNA substrate sub1a_(NotI) was incubated with 11 ng/μl (lane 1), 33 ng/μl (lane 2) and 100 ng/μl (lane 3) of aIF-5A—His<sub>7</sub>, and 9 ng/μl (lane 1), 28 ng/μl (lane 2), and 83 ng/μl (lane 3) of eIF-5A at 42 °C for 2 h. Control reactions; OH −, unspecific hydrolysis of RNA substrate in the presence of 37.5 mM NaOH for 1 h (lane 4) or 2 h (lane 5) at 42 °C; M, RNA Decade<sup>TM</sup> Marker.
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During our biochemical screening for ribonucleases in *Halobacterium*, we observed activity only at salt concentrations below 200 mM NaCl and purified the aIF-5A, which showed endonucleolytic activity on different RNA substrates and has not been reported for its eukaryotic homologue before. The optimal KCl concentration for aIF-5A cleavage activity is around 120 mM KCl. These concentrations are much lower than the intracellular concentrations in Haloarchaea (40). It is known that RNase Z from *Haloferax volcanii*, which is involved in tRNA processing, shows maximal activity at 5 mM MgCl₂ and only 5 mM KCl (46). These data, together with our finding that polyamines are able to influence the ribonucleolytic activity of aIF-5A, suggest that additional, yet unknown cellular factors guarantee the RNA-enzyme interaction or the RNase activity at the normal intracellular salt concentration of Haloarchaea in vivo.

Previously, we described that some dehydrogenases including archaeal enzymes are able to catalyze RNA cleavage, mostly at labile CA bonds and resembling the cleavage pattern of RNase A. Preferential cleavage adjacent to A nucleotides was also reported for bacterial RNA interferases, which are part of toxin-antitoxin systems, like ChpBK and Kid from *E. coli* or MazF from *E. coli* or *B. subtilis* (47–51). The cleavage mechanism of the Kid toxin resembles that of the acid base catalysis of RNases A and T1 (49). It is likely that this is a widespread mechanism among RNA-cleaving proteins that also accounts for the aIF-5A activity. The RNA cleavage reaction by dehydrogenases requires RNA binding by the Rossman fold, which is also binding the mono- or dinucleotides during the dehydrogenase reaction (33). Our analyses revealed that the cleavage pattern of aIF-5A is similar to that of some dehydrogenases (Fig. 2, B and C), but some differences were detected: cleavage products that accumulate during the incubation with dehydrogenases are further processed during the incubation with aIF-5A (band #1, #2, and #4 in Fig. 2C). RNase A on the other hand lacks a conserved RNA binding domain and degrades the sub5a_(cut) substrate to much smaller products than aIF-5A does. This finding and the absence of a Rossman fold in aIF-5A suggest a mode of RNA binding that is different from that of dehydrogenases and RNase A, but finally result in a similar pattern of RNA cleavage products. Crystal structures of three archaeal homologues of translation initiation factor 5A from *Halobacterium* have been solved (37, 52, 53). Archaeal IF-5A contains two domains connected by a short hinge (Fig. 4B). The C-terminal domain has 10–12% sequence identity and a striking structural similarity to CspA (37) (Fig. 8), the major cold shock protein of *E. coli*, which is known to be an RNA chaperone (38). CspA consists of a five-stranded β-barrel structure and binds RNA without apparent sequence specificity. A fold present in CspA and the C-terminal domain of aIF-5A was classified as oligomer binding (OB) fold, often found in sugar- and nucleotide-binding proteins (39). It is possible that the in vitro RNA binding capacity of aIF-5A is mediated by this fold. An SH3-like barrel motif covers almost the entire N-terminal part containing the hypusine residue and a KOW motif (36). SH3-like motifs preferentially recognize and bind to canonical PXG motifs (X denotes any amino acid) (54), thereby mediating protein-protein interactions. Recently, several SH3-like motifs have been found to bind to non-PXXP sequences, and a direct SH3-SH3 interaction is involved in control of IB1 (islet-brain) homodimerization (55). KOW domains are defined as a new class of nucleic acid binding folds and are found in ribosomal proteins and the bacterial transcription antitermination protein NusG (36). These structural features strongly suggest that aIF-5A is able to interact both with RNA (KOW, OB) and proteins (SH3), which is supported by our finding that aIF-5A is forming homooligomers (supplemental Fig. S2, A–C). It is possible that the natural RNA target is bound by one or both domains of IF-5A in vivo, and hypusination strengthens or even locks this binding. This may be the reason why RNA binding could only be shown for the hypusinated protein in vitro because the binding of the unmodified protein would only be transient and not stable enough to be seen in gel shift experiments.

A single serine to proline change at position 149 within the C-terminal CspA-like domain of yeast eIF-5A resulted in

**FIGURE 7. Hypusine-dependent RNA binding of haloarchaeal IF-5A.** His-tagged haloarchaeal aIF-5A isolated from *Halobacterium* sp. NRC-1 (Halo) but not from *E. coli* (E. coli) binds to sub10_(halo5A) RNA substrate in electrophoretic mobility shift assays. Indicated amounts of recombinant protein were preincubated with substrate for 40 min on ice before the mixtures were separated on a native 5% polyacrylamide gel containing 5% glycerol and 0.5 x Tris borate/EDTA.

**FIGURE 8. Structural comparison of CspA from *E. coli* (gray) with the C-terminal domain of aIF-5A from *Halobacterium* sp. NRC-1 (white).** Structures were visualized and superimposed with Swiss-PdbViewer (58).
mRNA stabilization in vivo and a temperature-sensitive (ts) phenotype (19). The prolonged mRNA half-lives of this ts_eIF-5A mutant and our observed ribonucleolytic activity of aIF-5A support the assumption that translation initiation factor 5A plays a role in RNA degradation. RNA binding by eIF-5A, which has been shown to be sequence-specific by SELEX experiments, requires hypusination of a single lysine residue in the N-terminal domain (21). We assume that hypusination of aIF-5A occurs in Halobacterium sp. NRC-1, because hypusination of aIF-5A was reported to be a general feature of the archaeal domain of life (56). Halobacterium has a homologue of the key enzyme (deoxyhypusyn synthase) necessary to catalyze this modification and is arrested in its cell cycle by the hypusination inhibitor N-1-guanyl-1,7-diaminoheptane (57). Hypusination of aIF-5A seems to be necessary for its RNA binding as it is for eIF-5A because only the protein isolated from Halobacterium sp. NRC-1 binds to RNA in electrophoretic mobility shift assays (Fig. 7). It is not required for RNA cleavage activity in our model substrates in vitro. This may be an indication for stabilization of an RNA-protein complex by hypusination. A weak initial association of eIF-5A to RNA could by stabilized or fixed by the hypusinated residue. In the case of the unmodified protein, the complex with RNA could dissociate before the RNA cleavage reaction could take place and only the hypusinated protein would be able to cleave RNA. This is the case for the eukaryotic protein, shown in Fig. 6C. Because of adaptation to extreme saline conditions in the haloarchaeal cytoplasm, aIF-5A could be able to associate with RNA long enough for the RNA cleavage reaction in vitro without stabilizing the RNA-protein complex by hypusination. This may be the reason why both variants of aIF-5A from Halobacterium sp. NRC-1 show the same ribonucleolytic activity in vitro but only the complex of hypusinated protein with RNA is stable enough to persist during electrophoresis. We cannot exclude the possibility that hypusination may influence cleavage activity on natural substrates of aIF-5A, which remain to be determined.

To learn more about the domains of aIF-5A required for RNA cleavage, we tested aIF-5A variants in vitro. Neither the N-terminal nor the C-terminal domain alone were able to cleave RNA, indicating that both domains are involved in RNA binding and/or catalysis. The structural model of aIF-5A (Fig. 4B) implies that RNA may be bound to a cleft at the interface of the two domains. Furthermore we replaced several charged amino acids of aIF-5A by uncharged amino acids. As seen in Fig. 8, most of these amino acids are localized around the central cleft and are therefore candidates to participate in acid base catalysis. Neither His28 nor the positively charged amino acids at positions 53–54 affect RNA cleavage by aIF-5A. RNA cleavage activity was however affected when (i) Arg1 was exchanged, which is located in the N-terminal domain, (ii) amino acids at positions 72/73, which are located in the hinge region, were exchanged, or (iii) amino acids at positions 117 or 122/123 of the C-terminal domain were exchanged. From the structural model (Fig. 8) it is likely that an RNA molecule running through the central cleft is in contact with all these amino acids. This finding is also in agreement with the observation that none of the two aIF-5A domains by itself is able to cleave RNA. The fact that the exchange of the single Glu117 resulted in strong reduction of RNA cleavage suggests that it is rather involved in catalysis than in RNA binding. The mutant still shows some cleavage activity, which may be explained by the presence of additional Glu residues in close proximity. It is conceivable that an acid base catalysis is not carried out by a single acidic and a single basic residue but that other amino acids, which are localized close by are also involved in this process.

We also have to take into account that aIF-5A most probably forms oligomers, which opens the possibility that amino acids from different monomers that are in a certain position in the oligomer can contribute to catalysis. Crystallographic studies revealed that aIF-5A from Methanococcus jannaschii is present as a dimer in a certain kind of crystals. These dimers are connected through intermolecular hydrogen bond interactions of β3-strands of the N-terminal domains of each molecule (52).

 Whereas our results demonstrate for the first time an RNA cleavage activity of an archaeal IF-5A, its biological functions are still unknown. Specificity of the RNA cleavage activity, which seems to be mostly determined by structural features of the substrate and the incomplete degradation of RNA substrates by aIF-5A compared with R Nas A, suggest that its biological purpose may not be the undirected breakdown of any RNA but of a regulatory kind. The finding that aIF-5A has the structural capability to interact with proteins and forms homooligomers is a hint that its sequence-unspecific ribonucleolytic activity may be directed in vivo by cellular factors of still unknown nature. Futures studies will be aimed to identify the natural substrates of aIF-5A and potential interaction partners to get more insight into its functions.

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