Similarity between the Association Factor of Ribosomal Subunits and the Protein Stm1p from *Saccharomyces cerevisiae*

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A ribosome association factor (AF) was isolated from the yeast Sacchharomyces cerevisiae. *Partial amino acid sequence of AF was determined from its fragment of 25 kDa isolated by treating AF with 2-(2-nitrophenylsulfenyl)-3-methyl-3'-Bromoindolenine (BNPS-skatole). This sequence has a 86% identity to the product of the single-copy* S. cerevisiae STM1 *gene that is apparently involved in several events like binding to quadruplex and triplex nucleic acids and participating in apoptosis, stability of telomere structures, cell cycle, and ribosomal function. Here we show that AF and Stm1p share some characteristics: both bind to quadruplex and Pu triplex DNA, associates ribosomal subunits, and are thermostable. These observations suggest that these polypeptides belong to a family of proteins that may have roles in the translation process.*

Key words: ribosomal association factor - Stm1p - G4 DNA - ribosomal function - G4p2 - triplex-DNA

The ribosomal subunits, once released at peptide chain termination, may either bind initiation factors that allow them to associate in active 80S complexes which are able to reinitiate another round of synthesis or couple to form inactive 80S monomers which have been suggested that represent non-translating subunits in vivo (Hershey & Merrick 1996). The accumulation of monomer ribosomes takes place when cells are subjected to different metabolically adverse conditions (Jagus et al. 1991, Hinnebusch 1997). These two different situations may occur normally in the eukaryotic cells and may be regulated by the availability of factors that act on the dissociation or association of the ribosomal subunits.

The association of 60S ribosomal subunits to the 40S complex to form the functional 80S initiation complex is an essential step in the initiation of protein synthesis. Two GTPase factors, eIF-5 and eIF-5B, are required for this reaction (Pestova et al. 2000, Majumdar et al. 2002). We have isolated and characterized a thermostable protein of about 43 kDa from *Saccharomyces cerevisiae*, that contrary to eIF5, associates ribosomal subunits to form 80S ribosomes without the need of energy (Herrera et al. 1991). Here we show that the partial amino acid sequence of this ribosomal subunits association factor called association factor (AF) has significant similarity to the one of the protein Stm1p of yeast.

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Stm1p, also called G4p2, was first identified and characterized as a yeast protein that shows a specific binding activity for quadruplex DNA (G4-DNA) (Frantz & Gilbert 1995) and, more recently, for purine motif triple-helical DNA (Pu triplex) (Nelson et al. 2000). The G4 nucleic acid structures are formed under certain ionic in vitro conditions, when DNA or RNA, rich in guanine tracts, associate into four-stranded right-handed helices stabilized by a guanine base tetrad (Williamson 1994, Parkinson et al. 2002). These quadruplex structures might have important cellular roles including telomere function, control of replication and transcription processes (Fukuda et al. 2002, Patel 2002, Siddiqui-Jai et al. 2002), and evidence for their existence in vivo in the nuclei of some protozoans has been presented (Schaffitzel et al. 2001). The formation of Pu triplex is a thermodynamically favored process that originates a duplex DNA with an additional purine-rich DNA strand. The triple helical structures have also been implicated in several cellular mechanisms such as transcription, replication, and recombination (Musso et al. 2000, Rustighi et al. 2002). While there is no direct evidence for the formation of these quadruplexes and triplexes in vivo, cellular proteins, like Stm1p, that specifically recognize them have been described and may be involved in the regulation of the possible biological roles of these structures. Other role that has been suggested for Stm1p is in the control of the apoptosis-like cell death in yeast (Ligr et al. 2001). In addition, a protein from *Candida maltosa* that has significant homology to *S. cerevisiae* Stm1p is a ribosome-associated protein whose release from the ribosomes might allow leaky translation under translation-inhibitory conditions (Takaku et al. 2001). The significance of all these possible functions of Stm1p and its homologues proteins is not yet understood.

In this paper, we show that besides the sequence similarity between AF and Stm1p, both proteins share activities: AF is able to bind quadruplex and Pu triplex DNAs while Stm1p associates ribosomal subunits.

MATERIALS AND METHODS

Preparation of ribosomes and high-salt ribosomal wash - The strain A364A of *S. cerevisiae* was grown to late-log phase in media as previously described (Hartwell 1967). Cells were homogeneized according to (Herrera et al. 1991) and polysomes, prepared following the method previously reported (Gallis et al. 1975), were treated with 0.5 M KCl to obtain the ribosomal pellet and the high salt ribosomal wash (Herrera et al. 1991).

Purification and assay for AF activity - The ribosome association factor AF was purified and assayed for ribosomal subunits association activity according to the methodology described by Herrera et al. (1991). The Stm1p was also assayed for association activity.

Peptide sequencing - Purified AF (5-10 µg) was treated with 2-(2-nitrophenylsulfenyl)-3-methyl-3'-Bromoindolenine (BNPS-skatole) as described in (Fontana 1972). The generated peptides were fractionated on a 15% SDS-PAGE gel, electrotransferred to a PVDF membrane and visualized by staining with Amido Black (Aebersold 1989). Peptides were excised and sequenced. The sequence of a 25 kDa fragment was obtained.

Purification of Stm1p or G4p2 - E. coli strain BL21DE was transformed with the plasmid pGEXG4p2 bearing the gene for the yeast protein G4p2 fused to glutathione S-transferase. A bacterially expressed G4p2 was obtained after proteolytic clevage of the glutathione S-transferase domain with factor Xa. These procedures were performed as described by Frantz and Gilbert (1995).

High temperature treatment of Stm1p - Five µg of purified Stm1p suspended in 35 µl of 20 mM K_2HPO_4 pH 7.2, 100 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM $PhMeSO_2F$, 1.0 mM iodoacetic and 25% glycerol was heated at 90ºC for 15 min and then centrifuged at 30, 000 x g for 10 min to eliminate precipitated proteins. The supernatant (3 µg) was immediately subjected to a PAGE.

Polyacrylamide gel electrophoresis - The proteins were analyzed by 10% SDS/PAGE according to Laemmli (1970) in the presence of 4 M urea. After electrophoresis the gels were stained with Coomasie Blue.

Protein concentration determination - Protein concentration was determined as described (Lowry et al. 1981).

Preparation of G4-DNA and triplex DNA - G4-DNA structures were prepared under conditions specified previously (Frantz & Gilbert 1995). Preparation of Pu triplex DNA was performed following the procedure described in (Nelson et al. 2000).

Electrophoretic mobility shift assays (EMSA) and competition experiments - G4 DNA or Pu triplex DNA were 5'-labeled with [γ^{32}P] with T4 polynucleotide kinase and precipitated twice with ethanol. Labeled G4 DNA (33,000 cpm; 8 fmol) or labeled Pu triplex DNA (22,000 cpm; 4 fmol) was incubated with Stm1p or AF in a final volume of 10 µl at room temperature for 10 min according to Frantz and Gilbert (1995). The reactions for G4 DNA.AF complex formation were loaded on 7.5% polyacrylamide gel and electrophoresed at room temperature in TBE buffer (50 mM Tris borate, 1 mM EDTA, pH 8.2) at 30 mA for 2 h. The reactions for Pu triplex DNA.AF complex formation were run in similar conditions but in the presence of 0.5 TBE buffer. The gels were dried and exposed for several hours to Imaging screen and analyzed in a Molecular Imager ® FX (Bio Rad, Hercules, CA).

Competition experiments were carried out as above with saturating amounts of G4 DNA probe. Ten-fold serial dilutions of competitor nucleic acids were premixed with AF and incubated following (Frantz & Gilbert 1995). Relative mass number of competitor to probe ranged from 0.1 to 1000 and were determined by absorbance at 260 nm. Single-stranded competitors were fully denatured by treatment with 100 mM NaOH at 100°C just prior to use.

RESULTS

Earlier report from our laboratory suggested that AF was related to the elongation factor EF-1α (Herrera et al. 1991). However, using purified antiserum against EF-1α, it was determined that AF does not cross react with the antibody to EF-1α (data not shown). Therefore, we wanted to determine the gene responsible for AF activity though we obtained aminoacid sequence information from the purified protein. Partial amino acid sequence of AF was obtained from its fragment of 25 kDa isolated by treating AF with BNPS-skatole as described in experimental procedures. The sequence, in the single letter amino acid code is WGDDKKELSAEKEAQADAXXXIAQDAAE, where X indicates no positive identification of the amino acid residue at these positions. A BLASTP search of the Yeast Proteoma Data Base at the National Center for Biotechnology Information identified one protein, the product of the *STM1* gene, with 86% identity to AF. The Stm1 protein was originally reported to bind quadruplex nucleic acids (Frantz & Gilbert 1995) and has been shown to bind triplex DNA (Nelson et al. 2000).

In view of the fact that the result presented imply that AF is similar to Stm1p, we determined whether AF is able to bind G4 nucleic acids. Fig. 1 shows using mobility shift assays with an excess of parallel G4 DNA oligomer probe that different amounts of AF form complexes with G4 DNA (B, lanes 8-12) and the complexes are detectable even at the lower AF concentration used. As controls Fig.1 also shows the complexes of parallel G4 DNA with variable amounts of protein Stm1p (A, lanes 3 to 7) fused to glutathione S-transferase (GST- Stm1p) as described in Material and Methods. The glutathione S-transferase domain neither interferes with the formation of the complex nor forms a complex by itself (data not shown). In addition, Fig. 1 shows that the structure of G4 DNA (C, lane 1) which is denatured by treatment with NaOH at high temperature (D, lane 2) is required for both proteins to form the complex (lanes 13 and 14). This implies that AF, similar to Stm1p, binds specifically to a G4 DNA structure.

To ascertain the affinity of AF to different DNAs, a series of competition experiments were carried out. Fig. 2 shows that AF has high affinity for G4 DNA as increasing concentration of competitor G4 DNA inhibits the formation of AF.G4 DNA radioactive complex up to 85-90% with 1000 relative mass competitor over the probe. In addition, AF shows practically no affinity for single-stranded DNA and DNA oligomer probe which are not participating in quartet or triplex formation.

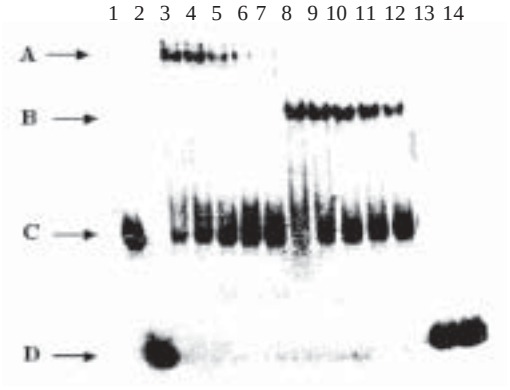

Fig. 1: binding of GST-Stm1p and association factor (AF) to G4 DNA. Different amounts of purified AF or GST-Stm1p were incubated with [γ-^{32}P]G4 DNA (8 fmol; 33,000 cpm) and complex formation was analyzed by EMSA according to experimental procedures. A: complex G4 DNA.GST-Stm1p formed with radioactive G4 DNA plus GST-Stm1p at 6.5 pmol (lane 3); 3.25 pmol (lane 4); 1.63 pmol (lane 5); 0.82 pmol (lane 6); 0.41 pmol (lane 7); B: complex G4 DNA.AF formed with radioactive G4 DNA plus AF at 0.93 pmol (lane 8); 0.41 pmol (lane 9); 0.23 pmol (lane 10); 0.12 pmol (lane 11); 0.06 pmol (lane 12); C: G4 DNA (lane 1); D: denatured G4 DNA alone (lane 2) or with 0.93 pmol AF (lane 13) or with 6.5 pmol GST-Stm1p (lane 14).

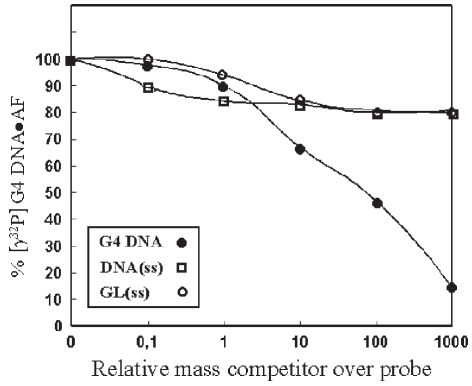

Fig. 2: binding specificity of purified association factor (AF) for G4 DNA. Complexes of purified AF (0.93 pmol) with [γ-^{32}P] G4 DNA (8 fmol, 33,000 cpm) or with serial dilutions of unlabeled competitor nucleic acids were resolved by EMSA. Bound probe was visualized by autoradiography and quantitated as described in experimental procedures. Competitor nucleic acids are indicated. G4 DNA: unlabeled tetrameric parallel structure; DNA(ss): single-stranded salmon sperm DNA; GL (ss): single-stranded GL oligomer

We also verified whether AF binds to Pu triplex DNA. For this, AF was incubated with labeled Pu triplex as described in experimental procedures. EMSA was performed to analyze the formation of the AF.Pu triplex DNA complex. As shown in Fig. 3, a significant amount of the complex AF.Pu triplex DNA is formed (A, lane 2) indicating that AF recognizes an intact purine motif triplex.

Since AF is able to bind G4 DNA, it was determined whether Stm1p has any activity for joining ribosomal subunits. The results are represented in Fig. 4. *S. cerevisiae* dissociated ribosomes sediment mainly as 40S and 60S subunits (Fig. 4A). Stm1p was tested for its ability to join ribosomal subunits. Fig. 4B shows that, in the presence of the protein, the majority of the subunits remains associated, sedimenting as 80S monomers. This result is similar to the one obtained with AF as control (Fig. 4C).

AF is a thermostable factor, therefore, we wanted to see if Stm1p was also resistant to the temperature. G4p2 was purified and then analyzed by SDS gel electrophoresis according to experimental procedures. Fig. 5B shows the presence of only one protein band of 32 kDa in the Stm1p preparation. Stm1p was incubated at 90ºC for 15 min. Fig. 5C shows that the band of Stm1p stains with the same intensity indicating that this polypeptide is thermostable. As a control, Fig. 5A shows the thermostable band of 43-kDa of preheated AF.

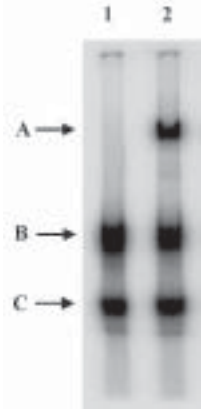

Fig. 3: binding of association factor (AF) to [γ-^{32}P] Pu triplex DNA. Purified AF (0.93 pmol) was incubated with [γ-^{32}P] Pu triplex DNA (4 fmol, 22,000 cpm) and complex formation was analyzed by EMSA according to experimental procedures. A: complex Pu triplex DNA.AF (lane 2); B: unbound Pu triplex DNA (lane 1 and 2); C: unbound duplex DNA (lane 1 and 2)

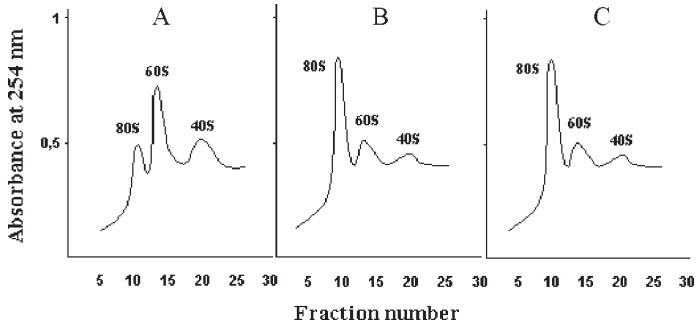

Fig. 4: association of ribosomal subunits by Stm1p. Equal amounts of dissociated ribosomes (1.2 A_{260} units) were incubated at 3 mM Mg^{2+} as described in experimental procedures, with (A) none protein, (B) 6.23 pmol Stm1p, and (C) 6.23 pmol association factor. At the end of the incubations, the samples were layered over 10-32% linear sucrose gradients as described.

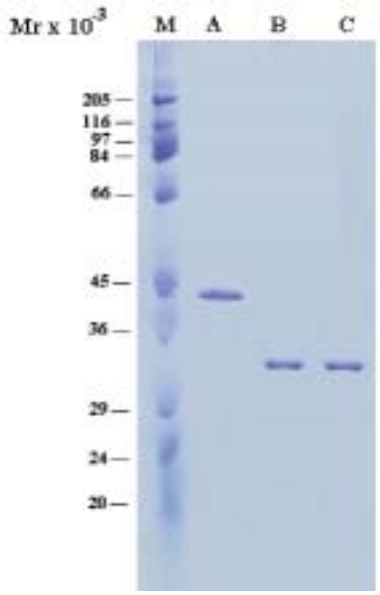

Fig. 5: thermostability of Stm1p. Stm1p (3 µg) was subjected to electrophoresis on a 10% polyacrylamide gel with SDS according to experimental procedures. Lanes - M: molecular mass markers; A: preheated association factor (2 µg); lane B, Stm1p without treatment; C: Stm1p preheated at 90°C for 15 min.

DISCUSSION

Given that the yeast *S. cerevisiae* has its genome completely sequenced (Goffeau et al. 1996), we demonstrated that a peptide from AF has a sequence 86% identical to sequences within the protein encoded by the single-copy *S. cerevisiae STM1* gene. Also, it was recognized that AF binds specifically to quadruplex and Pu triplex DNA similar to Stm1p. The protein Stm1p associates ribosomal subunits as AF does. Although this indicates that the structural similarity between these two proteins is enough to generate a sharing of their activities, these proteins have still a significant difference in their relative molecular weights. AF gene encodes a 43-kDa protein and *STM1* gene encodes a 32-kDa protein.

A piece of information that will be very important to understand the nature of the dissimilarity between these two proteins is the determination of the complete sequence of AF. This will allow computer-assisted alignment of the protein sequence of Stm1p with AF sequences that will show which region of the AF is absent in the Stm1p. This difference of about 11 kDa could not represent simply post-translational modifications of the AF protein. This point is supported by the fact that AF is not a glycoprotein (data not shown). The variation in molecular mass could be explained by several alternatives: a) Stm1p could result from proteolytic cleavage of the full-length AF protein. This is a common post-translational modification; b) Stm1p could be originated by alternative initiation of translation of the AF message. This mechanism has been reported for other proteins (Han & Zhang 2002, Prats & Prats 2002); c) the two proteins could be synthesized from mRNA isoforms generated by alternative splicing. Several related proteins are produced using this post-transcriptional control (Auboeuf et al. 2002, Waltzer et al. 2002) which is known to be related to changes in intracellular pH, cell cycle or tissue specificity (Vallano et al. 1999, Dahme et al. 2002, Xu et al. 2002). To clarify this last possibility oligonucleotide primers designed to the Stm1p sequence could be used to screen a yeast cDNA library. If two cDNA clones are found, one should encode AF and the other Stmp1. Sequence analysis of these clones will reveal the difference between the isoforms.

At this point, we should ask one important question: are the biological functions of these proteins related? Our data strongly suggest that AF acts in the protein synthesis process whereas the information obtained in the literature about the function of Stm1p is very diverse. One of the activities reported for Stm1p is related to the protein synthesis process. That is: *STM1* acts as a multicopy suppressor of temperature-sensitive mutants which are defective in ribosome synthesis by affecting several early steps in the rRNA processing pathway (Tabb et al. 2001). Therefore, this data suggest that Stm1p may have an effect on the ribosomal function, vital for the mRNA translation, which may be a functional link between AF and Stm1p.

Another unsolved aspect is whether a concerted action between the two activities (quadruplex and Pu triplex DNA binding and association of ribosomal subunits) present in both proteins exists or not, when these polypeptides are fulfilling their biological functions. Additional experiments are necessary to clarify these issues.

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