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A Lysine Substitution in the ATP-Binding Site of Eucaryotic Initiation Factor 4A Abrogates Nucleotide-Binding Activity

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Eucaryotic initiation factor 4A (eIF-4A) is a member of a family of proteins believed to be involved in the ATP-dependent melting of RNA secondary structure. These proteins contain a derivative of the consensus ATP-binding site AXXGXGKT. To assess the importance of the consensus amino acid sequence in eIF-4A for ATP binding, we mutated the consensus amino-proximal glycine and lysine to isoleucine and asparagine, respectively. The effect of the mutations was examined by UV-induced cross-linking of [α-^{32}P]dATP to eIF-4A. Mutation of the lysine residue (but not of the glycine residue) resulted in the loss of [α-^{32}P]dATP cross-linking to eIF-4A, suggesting that the lysine is an important determinant in ATP binding to eIF-4A.

Eucaryotic initiation factor 4A (eIF-4A) is required for ribosome binding to mRNA during translation initiation (1, 19). eIF-4A can exist in free form or as a subunit in the cap-binding protein complex, eIF-4F. eIF-4F consists of three proteins: eIF-4E, eIF-4A, and p220. It was postulated previously (2) and some evidence has been provided (13) that eIF-4A melts mRNA secondary structure in an ATP-dependent manner. In addition, eIF-4A was shown to exhibit ATPase activity (5). eIF-4A helicase activity is particularly effective when eIF-4A is part of eIF-4F, and this activity requires the participation of another initiation factor, eIF-4B (F. Rozen, I. Edery, and N. Sonenberg, unpublished data). The ability of ATP or dATP to covalently cross-link to eIF-4A upon UV irradiation is consistent with the presence of ATPase and RNA helicase activities in eIF-4A (15).

A large family of at least seven proteins with homology to eIF-4A has recently been described (3, 6–9, 11, 12, 16). Twelve percent of the amino acids are identical in all the proteins of the family (8; J. Schnier, personal communication). On the basis of the reported mRNA helicase activity of eIF-4A (13), it was suggested that eIF-4A-like proteins are involved in ATP-dependent melting or unwinding of double-stranded regions in RNA or DNA. eIF-4A-like proteins contain an ATP-binding consensus sequence, GKT, that is shared by all other ATP-binding proteins and is part of the sequence GXXXXGKT present in many procaryotic and eucaryotic ATP-hydrolyzing proteins, termed the A motif (20). In the eIF-4A-like family of proteins, a related consensus sequence, AXXGXGKT, is present (8). Although the AXXGXGKT sequence in the eIF-4A-like family has been assumed to participate in ATP binding, this has not been directly demonstrated. Figure 1 shows the conservation of the ATP-binding motif in eIF-4A-like proteins (an extended consensus is indicated: AX$^{S}_{T}$G$^{S}_{T}$GKT).

To determine the importance of amino acids in the ATP consensus sequence of eIF-4A for its activity, we mutated the invariable lysine (position 82 according to the full-length protein [11]) to asparagine and the first glycine (position 79) of the consensus sequence to isoleucine. For the studies described in this paper, we used a murine eIF-4A partial cDNA clone that is truncated at its 5′ end (10), encoding a protein which initiates at the second in-frame methionine and which therefore lacks the 16 amino-terminal amino acids (10, 11). This deletion had no measurable effect, compared with that of intact rabbit reticulocyte eIF-4A, on the binding of [α-^{32}P]dATP to eIF-4A as determined by UV-light-induced cross-linking. Mutations were performed in M13mp19, and the wild-type and mutant eIF-4A species were subcloned into a Bluescript vector downstream of a T7 promoter (Stratagene). An oligodeoxynucleotide containing a Shine-Dalgarno sequence was inserted upstream of the coding sequence of eIF-4A. The structure of the constructs (termed pKS/eIF-4A) is shown in Fig. 2A. To express eIF-4A protein from pKS/eIF-4A, *Escherichia coli* HB101 was transformed with these constructs, followed by infection with bacteriophage CE6 that expresses T7 RNA polymerase (17). This procedure resulted in a high production of eIF-4A protein (~0.2 mg of eIF-4A in 5×10^{11} *E. coli* cells). The synthesized, truncated eIF-4A migrated faster than did the eIF-4A purified from rabbit reticulocytes, as expected (Fig. 2B). However, the *E. coli*-expressed eIF-4A was active with respect to its ability to bind dATP (see below).

To determine the ability of *E. coli*-expressed eIF-4A to bind ATP, we used UV-light-induced cross-linking of [α-^{32}P]dATP to the protein. We partially purified the *E. coli*-expressed eIF-4A, since cross-linking was inefficient in a crude extract. Fractions enriched in eIF-4A were obtained by gel filtration on Sephadex G-100 in 50 mM Tris hydrochloride (pH 7.5)–100 mM KCl–0.2 mM EDTA–1 mM dithiothreitol. This was followed by DEAE-cellulose chromatography in the same buffer with the factor eluting between 230 and 260 mM KCl (data not shown). Figure 3A shows a Western blot (immunoblot) of the partially purified wild-type and mutant forms of eIF-4A. Mutations in eIF-4A had no effect on the amounts of protein produced in *E. coli*, indicating similar stabilities for mutant and wild-type eIF-4A.

Cross-linking reaction mixtures (25 μl) containing wild-type or mutant eIF-4A in 64 mM KCl–30 mM Tris hydrochloride (pH 7.5)–6% glycerol–0.2 mM EDTA–5 mM magnesium acetate–30 μCi of [α-^{32}P]dATP (3,000 Ci/mmol) were irradiated with a 15-W General Electric G15T8 germicidal lamp from a distance of 3 cm for 20 min at 4°C. Unlabeled dATP was then added to a final concentration of 3 mM and incubated with 2 μl of mouse ascites fluid containing eIF-4A

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eIF4AI	(mouse)	QAQSGTGKTA
eIF4AII	(mouse)	QAQSGTGKTA
TiF1/TiF2	(yeast)	QAQSGTGKTG
PL10	(mouse-sperm.)	CAQTGSGKTA
p68	(mammals,amphibian)	VAQTGSGKTL
Vasa	(drosophila)	CAQTGSGKTA
MSS116	(yeast-mitochondria)	RAKTGTGKTF
SrmB	(E.coli)	SAPTGTGKTA
	consensus:	A $^{S}_{T}$G$^{S}_{T}$GKT
eIF4A(G→I)		I
eIF4A(K→N)		N

FIG. 1. Conservation of the ATP-binding motif in eIF-4A-like proteins. Amino-acid sequences were from the work of Linder et al. (8). The sequence of SrmB was modified from that originally published (12; J. Schnier, personal communication). Eight eIF-4A-like proteins have been aligned to demonstrate the ATP-binding domain common to all proteins. Positions of amino acids that form the ATP-binding consensus sequence (see below) are boxed. The two mutations are indicated below the consensus. Mutagenesis was performed on a *Bam*HI-Asp-718 eIF-4A fragment (10) subcloned into M13mp19. The mutagenic oligodeoxynucleotide primer used for generating the Lys→Asn mutation was 5′-GTAGCTGTTATC CCAGTCC-3′, where the underlined nucleotide represents the mismatch. The mutagenic oligodeoxynucleotide primer used for generating the Gly→Ile mutation was 5′-GTTTTCCCAGTTATA GACTGG-3′. Mutagenesis was performed as described by Zoller and Smith (21), and the sequence of mutants was verified by dideoxy sequencing (14).

monoclonal antibody at 4°C for 2 h. Immunoprecipitates were collected by centrifugation, washed, and boiled for 5 min in electrophoresis sample buffer. This was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, as described by Sarkar et al. (15). By this technique, eIF-4A from rabbit reticulocyte has been shown to be specifically cross-linked to [α-^{32}P]ATP or dATP (15). Recent experiments have also demonstrated that dATP is as efficient as ATP in serving as the energy source for the unwinding of an RNA duplex by eIF-4A and eIF-4B (F. Rozen, I. Edery, and N. Sonenberg, unpublished results).

Figure 3B shows the sodium dodecyl sulfate-polyacrylamide gel analysis of dATP cross-linking to wild-type eIF-4A and the two mutants after immunoprecipitation with an anti-eIF-4A monoclonal antibody. Two concentrations of eIF-4A were used to ensure that the extent of cross-linking was proportional to eIF-4A concentration. The Coomassie blue stain of the gel showed equal amounts of immunoprecipitated wild-type and mutant forms of eIF-4A (data not shown). The substitution of asparagine for lysine vastly reduced the extent of dATP cross-linking to eIF-4A (compare Fig. 3B, lanes 3 and 4 with lanes 1 and 2). However, mutation of the first glycine to isoleucine in the sequence GXGKT had no effect on the cross-linking. We conclude from these results that the lysine is a critical amino acid for ATP binding but that glycine is not required.

The inability of the Lys→Asn mutant to bind dATP is consistent with its conservation in most ATP-binding proteins (19), suggesting that Lys-82 is a critical amino acid in the ATP-binding domain. X-ray crystallography of adenylate kinase demonstrated that the GXXXXGKT sequence interacts with the phosphate groups of ATP and that, more specifically, the lysine is in proximity to the α-phosphate of ATP (4). The lack of effect of the Gly-79 mutation on dATP binding suggests that this amino acid is not directly involved in the binding of ATP, although it may be involved in its

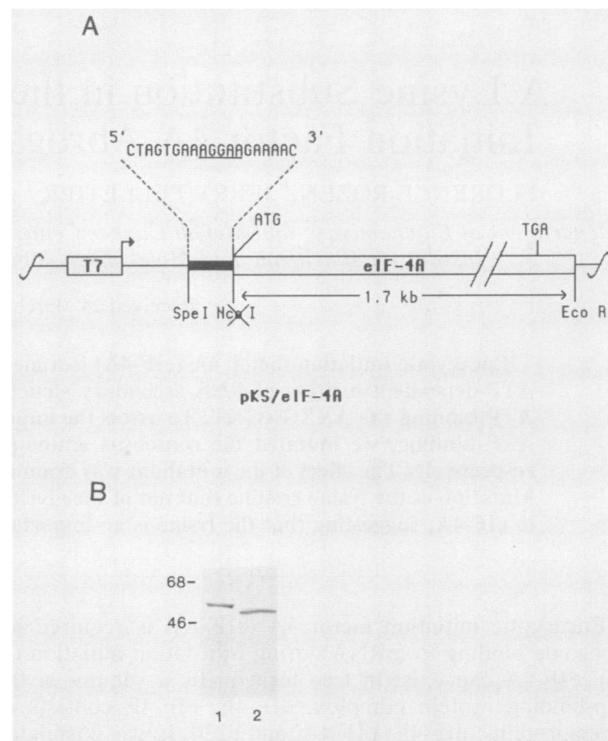

FIG. 2. Murine eIF-4A expression in *E. coli*. (A) The 1.7-kilobase *Bam*HI-*Eco*RI DNA fragment of eIF-4A, beginning at the second ATG in the reading frame and containing most of the 3′ noncoding region (10), was subcloned into the KS(+) form of the Bluescript vector (Promega Biotec). To provide a bacterial ribosome-binding site, an 18-mer oligodeoxynucleotide containing the Shine-Dalgarno sequence (underlined) was subcloned immediately upstream of the initiator AUG. The resulting plasmid was termed pKS/eIF-4A. (B) High-level expression and partial purification of eIF-4A was achieved as described in the text. eIF-4A was precipitated with 70% ammonium sulfate, and a sample of this preparation was analyzed on a sodium dodecyl sulfate-polyacrylamide gel (10% polyacrylamide) and immunoblotted (18) with anti-eIF-4A monoclonal antibody (2). Lane 1, Immunoblot of rabbit reticulocyte eIF-4A (100 ng); lane 2, immunoblot of *E. coli*-expressed eIF-4A (equivalent to 100 ng of eIF-4A as determined by immunoblotting).

hydrolysis. While glycine is not as strongly conserved as lysine in other ATP-binding proteins, it is present in all eight eIF-4A-like proteins. Therefore, it might function in ATP utilization in eIF-4A-like proteins. We could not measure hydrolysis of ATP in our assay because contaminating ATPases were present in the *E. coli*-expressed eIF-4A preparation.

The results presented here, although informative, do not prove that Lys-82 is directly involved in ATP binding. The possibility that an amino acid substitution can cause a conformational change in the molecule, resulting in an impaired ATP-binding activity, cannot be excluded. In any event, the ability to inactivate ATP binding of eIF-4A by a single amino acid substitution should prove useful in understanding the mechanism of function of eIF-4A and eIF-4A-like proteins. For example, it can be used as a dominant mutation in which the mutated protein might interfere with the function of the normal protein, resulting in a characteristic phenotype. In addition, mutagenesis of the other conserved domains found in the eIF-4A-like proteins should help to elucidate their importance and function in mRNA binding and unwinding.

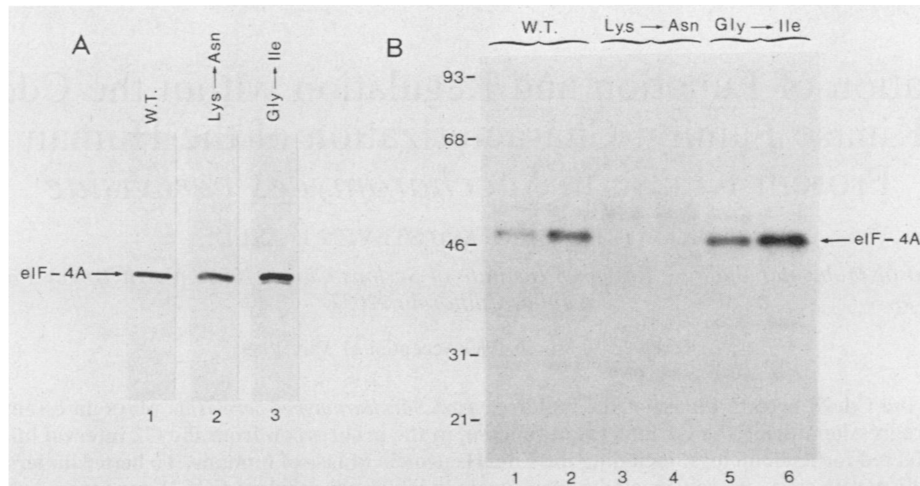

FIG. 3. Immunoblotting and UV-induced cross-linking of [α-^{32}P]dATP to wild-type and mutant forms of eIF-4A. (A) High-level expression and partial purification of eIF-4A was achieved as described in the legend to Fig. 2B. Wild-type and mutant forms of eIF-4A were analyzed on a sodium dodecyl sulfate-polyacrylamide gel (10% polyacrylamide) and immunoblotted (18) with anti-eIF-4A monoclonal antibody (2). Lane 1, 100 ng of wild-type eIF-4A; lane 2, 100 ng of Lys→Asn mutant; lane 3, 100 ng of Gly→Ile mutant. (B) Reaction mixtures were UV irradiated and immunoprecipitated as described in the text. Lanes 1 and 2, ≈0.5 and 1 μg, respectively, of wild-type eIF-4A; lanes 3 and 4, ≈0.5 and 1 μg of Lys→Asn mutant; lanes 5 and 6, ≈0.5 and 1 μg of Gly→Ile mutant.

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