

Regulation of luteinizing hormone receptor mRNA expression by mevalonate kinase – role of the catalytic center in mRNA recognition

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We have shown that hormone-induced downregulation of luteinizing hormone receptor (LHR) in the ovary is post-transcriptionally regulated by an mRNA binding protein. This protein, later identified as mevalonate kinase (MVK), binds to the coding region of LHR mRNA, suppresses its translation, and the resulting ribonucleoprotein complex is targeted for degradation. Mutagenesis and crystallographic studies of rat MVK have established Ser146, Glu193, Asp204 and Lys13 as being crucial for its catalytic function. The present study examined the structural aspects of MVK required for LHR mRNA recognition and translational suppression. Single MVK mutants (S146A, E193Q, D204N and K13A) were overexpressed in 293T cells. Cytosolic fractions were examined for LHR mRNA binding activities by RNA electrophoretic mobility shift analysis. All the single MVK mutants showed decreased LHR mRNA binding activity compared with the wild-type MVK. Double mutants (S146A & E193Q, E193Q & D204N and E193Q & K13A) of MVK also showed a significant decrease in binding to LHR mRNA, suggesting that the residues required for catalytic function are also involved in LHR mRNA recognition. Mutation of the residues outside the catalytic site (D316A and S314A) did not cause any change in LHR mRNA binding activity of MVK when compared with wild-type MVK. To examine the biological effects of these mutants on LHR mRNA expression, a full-length capped rat LHR mRNA was synthesized and translated using a rabbit reticulocyte lysate system in the presence or absence of the MVK mutant proteins. The results showed that mutations of the active site residues of MVK abrogated the inhibitory effect on LHR mRNA translation. Therefore, these data indicate that an intact active site of MVK is required for its binding to rat LHR mRNA and for its translational suppressor function.

The luteinizing hormone receptor (LHR) present on cell membranes of gonadal tissues belongs to the family of leucine-rich repeat-containing G-protein-coupled receptors (LGRs) [1,2]. Interaction of luteinizing hormone (LH) or its placental counterpart, human

chorionic gonadotropin (hCG), with LHR induces Gs-protein-mediated adenylate cyclase activation, which leads to an increase in cellular cAMP levels [1,3,4]. The expression of LHRs varies during the ovarian cycle, and some of these changes in receptor

Abbreviations

GHMP, galactokinase homoserine kinase mevalonate kinase phosphomevalonate kinase; hCG, human chorionic gonadotropin; IRE, iron regulatory element; IRP1, iron regulatory protein 1; LBS, LHR mRNA binding protein site; LGR, leucine-rich repeat-containing G-protein-coupled receptor; LH, luteinizing hormone; LHR, luteinizing hormone receptor; LRBP, LHR mRNA binding protein; MVK, mevalonate kinase.

expression are attributed to post-transcriptional mechanisms [5–9]. It has been shown that the ligand-induced downregulation of LHR is paralleled by a specific, transient disappearance of its mRNA transcripts [7,10]. Further studies on the mechanism of rapid degradation of LHR mRNA led to the identification of a novel RNA binding protein that mediates the post-transcriptional regulation of LHR mRNA in the ovary [10–12]. This protein, initially named LHR mRNA binding protein (LRBP), was later identified to be mevalonate kinase (MVK), a critical enzyme involved in cholesterol biosynthesis [13]. We have shown that MVK impairs LHR mRNA translation *in vitro*, and have established its association with LHR mRNA during ligand-induced LHR downregulation in the ovary [14]. Furthermore, we have shown the direct participation of MVK in ligand-mediated LHR downregulation by demonstrating that the suppression of MVK levels abrogates LHR mRNA downregulation [15]. Thus, a functional role of MVK in LHR mRNA expression has been established unequivocally. These results led us to investigate the structural requirements of MVK for its ability to bind LHR mRNA and also for its inhibitory effect on LHR mRNA translation.

MVK catalyzes the transfer of the γ -phosphate of ATP to mevalonate to form mevalonate 5-phosphate, an intermediate for the formation of isoprene units required for the synthesis of cholesterol and other lipid molecules necessary for the post-translational modifications of proteins [16]. The crystal structure of rat MVK complexed with ATP-Mg²⁺ has been determined at 2.4 Å resolution [17]. Site-directed mutagenesis studies with rat MVK have shown that Ser146, Glu193, Asp204 and Lys13 are residues at the active site of MVK necessary for its catalytic function [17,18]. The active site of MVK has been mapped to the interphase of the two monomer domains of this dimeric protein. As our previous studies have shown that both ATP and mevalonate inhibit the binding of MVK to LHR mRNA, the present investigation examines whether the catalytic site of this multifunctional protein participates in LHR mRNA recognition. Specifically, studies were performed to determine whether the active site of MVK is required for LHR mRNA binding and for its role as a translational suppressor of LHR mRNA. The results showed a substantial decrease in LHR mRNA binding activity when any of the amino acids at the active site were mutated. Furthermore, *in vitro* translation experiments showed decreased LHR mRNA translation inhibition by these MVK mutants when compared with wild-type MVK. Therefore, these data indicate that an intact active site of MVK is required for its binding

to LHR mRNA and for suppression of its translation.

Results

LHR mRNA binding activity of rat MVK mutant proteins

Rat MVK was overexpressed in 293T cells and RNA electrophoretic mobility shift analysis was performed with 10 μ g of cytosolic S100 protein and [³²P]-labeled LHR mRNA binding protein site (LBS) in the presence of ATP and mevalonate at concentrations of 0.05, 0.5 and 1.0 mM. As shown in Fig. 1, ATP and

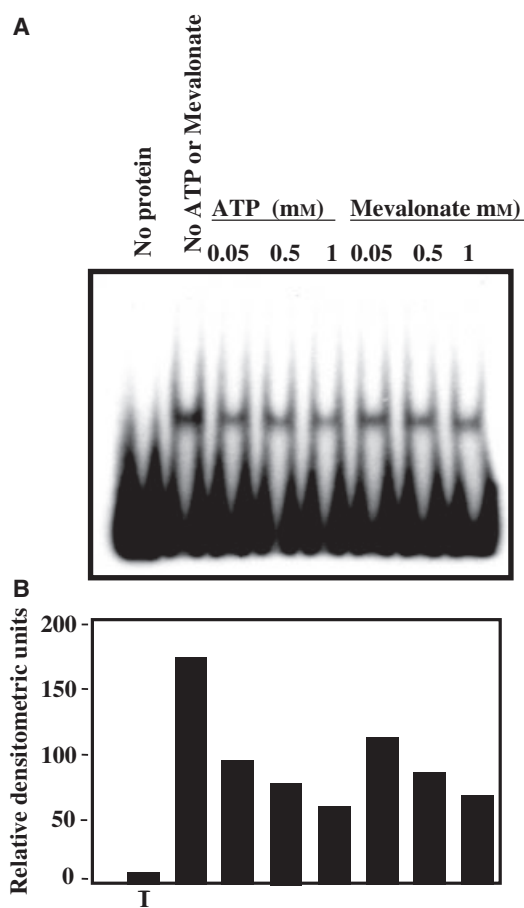


Fig. 1. Effect of ATP and mevalonate on the binding of wild-type MVK to LBS. (A) RNA mobility shift analysis was performed with [³²P]-labeled rat LBS (1.5×10^5 c.p.m.) using 5 μ g of S100 fraction prepared from 293T cells 48 h after transfection with pCMV4-rMVK. ATP and mevalonate were added to the binding reactions in the concentrations indicated. The autoradiogram shown is representative of three independent experiments. (B) The protein bands were quantified by densitometric scanning followed by analysis using NIH IMAGE 1.61 software (mean value \pm SE).

mevalonate caused a decrease in binding of LHR mRNA to MVK. The inhibitory effect of mevalonate was found to be less pronounced when compared with that of ATP. There was a significant decrease at a concentration of ATP or mevalonate of 0.05 mM, but no complete inhibition at the higher concentrations tested. This decrease in binding of MVK to LHR mRNA suggests that the ATP/mevalonate binding region of the protein may be required for RNA binding. Mutational and crystallographic studies by others have proposed that Ser146, Glu193, Asp204 and Lys13 are the important amino acids needed for the catalytic activity of MVK to convert mevalonate to mevalonate 5-phosphate.

To examine whether the catalytic site of MVK is required for RNA binding, we mutated S146 to A, E193 to Q, D204 to N and K13 to A, individually and in combination, to generate the following single and double mutants: S146A, E193Q, D204N and K13A single mutants, and S146A & E193Q, E193Q & D204N and E193Q & K13A double mutants. The mutants were transiently transfected into 293T cells and the cytoplasmic proteins (S100) were prepared 48 h after transfection, as described in Experimental procedures. The expression levels of these mutants and wild-type MVK were examined by western blot analysis using MVK antibody. Figure 2A shows the overexpression of all the single mutants and wild-type MVK, and Fig. 2B shows the overexpression of all the double mutants and wild-type MVK. These data indicate that all the mutants and wild-type MVK were overexpressed in 293T cells with comparable expression levels. These S100 preparations were then used for RNA electrophoretic mobility shift analysis with [³²P]-labeled rat LBS as described previously [19]. Figure 3 shows the RNA binding activity of all the single mutants and wild-type MVK in the S100 fractions, and Fig. 4 shows the binding activity of the double mutants and wild-type MVK in the S100 fractions. As reported before, Figs 3 and 4 show increased LHR mRNA binding activity in the S100 fractions prepared from wild-type MVK compared with the vector alone (mock). All the single and double mutants showed a decrease in RNA binding activity when compared with wild-type MVK. To further establish the role of catalytic amino acids in LHR mRNA binding activity, two amino acid residues outside the active site region, D316 and S314, were mutated to Ala, and these mutants were transfected into 293T cells. The LHR mRNA binding activity of the S100 fractions was then assayed as described in Experimental procedures. The results in Fig. 5 show that there was no change in RNA bind-

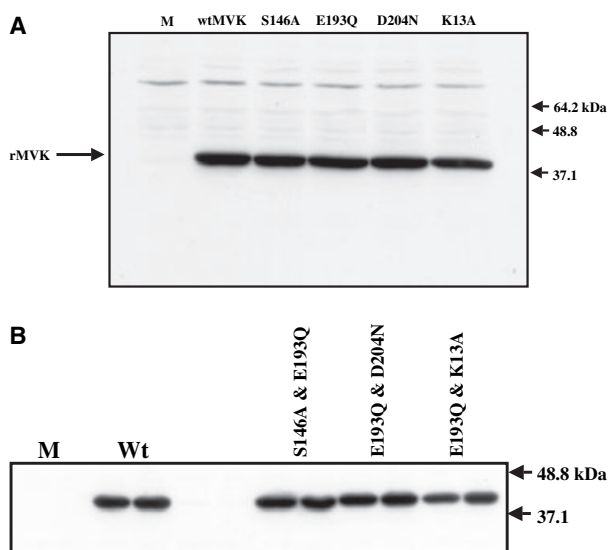


Fig. 2. (A) Overexpression of single mutants of rat MVK. Human embryonic kidney (293T) cells were transiently transfected with pCMV4-rMVK or the four single mutants (S146A, E193Q, D204N and K13A) of MVK in the pCMV4 vector, and cytoplasmic proteins (S100) were prepared 48 h after transfection. Western blot analysis was performed with 30 μ g of S100 fractions from vector alone (M), wild-type MVK (wtMVK) or S146A, E193Q, D204N and K13A mutant MVKs using MVK antibody. (B) Overexpression of double mutants of rat MVK. 293T cells were transiently transfected with pCMV4-rMVK or the three double mutants (S146A & E193Q, E193Q & D204N and E193Q & K13A) of MVK in the pCMV4 vector, and cytoplasmic proteins (S100) were prepared 48 h after transfection. Western blot analysis was performed with 10 μ g of S100 fractions from vector alone (M), wild-type MVK (Wt) or S146A & E193Q, E193Q & D204N and E193Q & K13A mutant MVK proteins using MVK antibody. The blots shown are representative of three experiments with similar results.

ing activity of these mutants when compared with wild-type MVK.

We have shown previously that all the cytidine residues in LBS are required for MVK binding to LHR mRNA, as the mutation of cytidine residues in LBS abolishes its ability to bind to MVK [11]. To verify that the mutations at the active site of MVK did not alter its LHR mRNA sequence specificity for binding, RNA mobility shift analysis was performed using [³²P]-labeled LBS with all the double mutants in the absence and presence of a 10-fold molar excess of wild-type LBS and mutant LBS in which all the cytidine residues were mutated to uridine. The results shown in Fig. 6 indicate that all the mutants compete with wild-type LBS, but not with mutant LBS, similar to that shown previously for wild-type MVK. This indicates that the mutations at the active site do not cause any change in RNA sequence specificity of

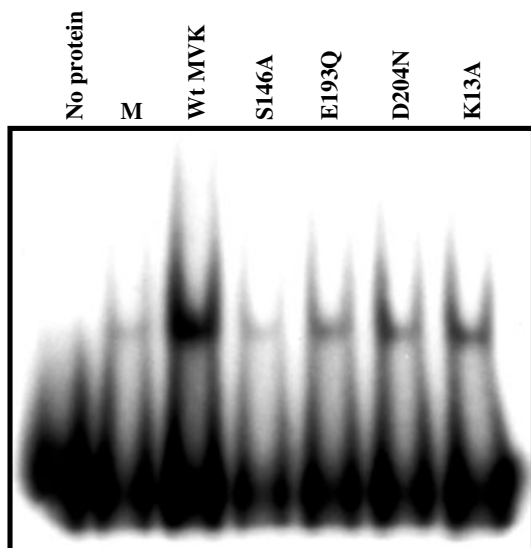


Fig. 3. RNA mobility shift analysis. Gel mobility shift analysis was performed with [32 P]-labeled rat LBS (1.5×10^5 c.p.m.) using no protein or 10 μ g of S100 fractions from 293T cells transfected with empty vector (M), wild-type rat MVK (WtMVK) or the four single mutants of rat MVK (S146A, E193Q, D204N and K13A), as described in Experimental procedures. The autoradiogram is representative of four independent experiments.

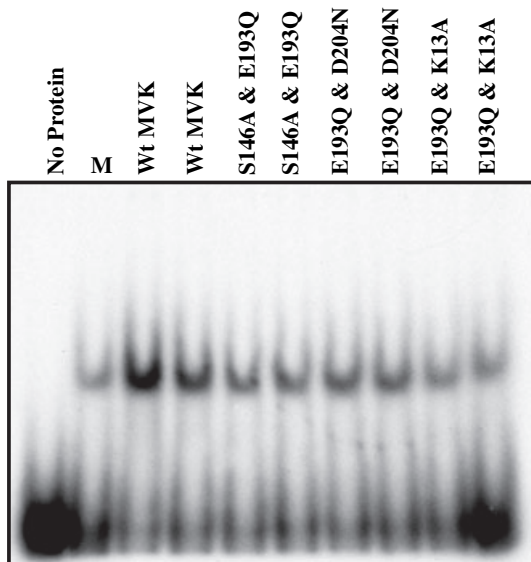


Fig. 4. RNA mobility shift analysis. Gel mobility shift analysis was performed with [32 P]-labeled rat LBS (1.5×10^5 c.p.m.) using no protein or 10 μ g of S100 fractions from 293T cells transfected with empty vector (M) or, in duplicate, wild-type rat MVK (WtMVK) or the three double mutants of rat MVK (S146A & E193Q, E193Q & D204N and E193Q & K13A) as described in Experimental procedures. The autoradiogram shown is representative of three independent experiments.

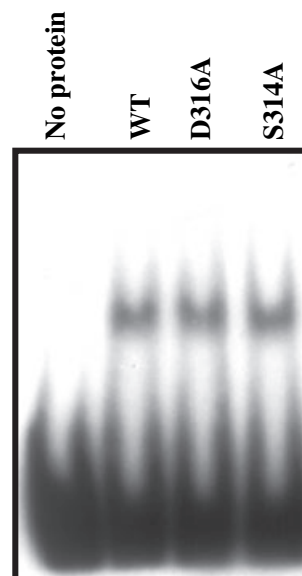


Fig. 5. RNA mobility shift assay. Gel mobility shift analysis was performed with [32 P]-labeled rat LBS (1.5×10^5 c.p.m.) using no protein or 10 μ g of S100 fractions from 293T cells transfected with wild-type rat MVK (WT) or the two mutants of rat MVK outside the active site (D316A and S314A), as described in Experimental procedures. The autoradiogram shown is representative of three independent experiments.

MVK. The results therefore clearly show that all four amino acids at the active site of the enzyme required for catalysis also participate in binding to LHR mRNA.

Effect of MVK mutant proteins on the translation of LHR mRNA *in vitro*

Our previous studies have shown that binding of MVK to LHR mRNA suppresses the translation of mRNA *in vitro* [14]. As the mutation of any of the four amino acids at the active site of MVK decreases its binding to LHR mRNA, we examined whether these mutants were able to reverse the inhibitory effect of wild-type MVK on LHR mRNA translation. For this purpose, we employed the *in vitro* translation assay using the rabbit reticulocyte lysate system and subsequent immunoprecipitation of the resulting FLAG-tagged rat LHR [14]. First, the ability of the overexpressed wild-type MVK to suppress LHR mRNA translation was examined as a positive control. *In vitro* translation reactions were performed with full-length capped 3'-FLAG-tagged LHR mRNA in the presence and absence of different concentrations of S100 fraction prepared from cells overexpressing wild-type MVK. The resulting translation products were

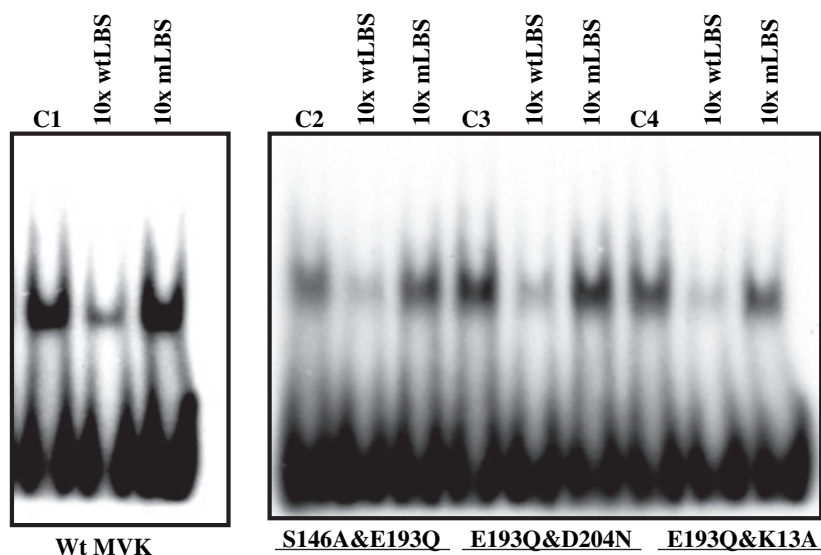


Fig. 6. RNA mobility shift analysis. Competition with wild-type (wtLBS) and mutated (mLBS) LBS. RNA mobility shift analysis was performed with 5 μ g of S100 fractions prepared from 293T cells transfected with wild-type rat MVK (WtMVK) or the three double mutants of rat MVK (S146A & E193Q, E193Q & D204N and E193Q & K13A). Unlabeled wtLBS and mLBS (all C \rightarrow U) were added in the binding reaction in molar excess, as described in Experimental procedures. C1, C2, C3 and C4 represent control reactions without unlabeled wtLBS or mLBS. The autoradiogram is representative of three independent experiments.

immunoprecipitated with FLAG antibody, separated by SDS-PAGE, and then subjected to autoradiography as described previously [14]. The results (Fig. 7A,B) showed that the addition of the S100 fraction from the overexpressed wild-type MVK caused a concentration-dependent decrease in the translation of LHR mRNA, similar to that seen with the purified LRBP preparations [14]. The translation reactions were then performed in the presence of S100 fractions prepared from cells overexpressing each of the mutant proteins of MVK. The results in Fig. 8A,B indicate that all the single and double mutant MVKs showed no substantial inhibition of the translation of LHR mRNA when compared with wild-type MVK. The data therefore indicate that the amino acids S146, E193, D204 and K13, present at the active site of the enzyme and required for the catalytic activity of MVK, are also essential for its binding to LHR mRNA and for the suppression of LHR mRNA translation.

Discussion

We have unraveled a novel post-transcriptional mechanism of LHR regulation in the ovary that involves the LRBP MVK as a critical *trans*-acting factor [13,15,19]. We have shown that LRBP binds to the coding region of LHR mRNA and causes translation inhibition and subsequent mRNA decay *in vitro* [10,11,14,19].

The aim of the present study was to identify the region/amino acid residues of MVK required for LHR mRNA recognition. MVK is a member of the galactokinase homoserine kinase mevalonate kinase phosphomevalonate kinase (GHMP) kinase superfamily of enzymes that are known to have a left-handed β - α - β fold, which is found in other RNA/DNA binding proteins [20]. Mutagenesis and crystallographic studies of rat MVK by others have shown that amino acids Ser146, Glu193, Asp204 and Lys13 are involved in the phosphorylation of mevalonate to mevalonate 5-phosphate [17,18]. As shown in the crystal structure of rat MVK bound to ATP [17], depicted in Fig. 9A, the active site of the enzyme is located at the interface of the N- and C-terminal domains of the monomer. The adenosine base of ATP is bound at the base of the left-handed β - α - β fold, where it interacts with a number of residues in the β - α - β motif. A new crystal structure of *Leishmania major* MVK complexed to mevalonate [21] has allowed us to show that the remainder of the active site is formed by two antiparallel β -sheets linked by a short α -helix, spanning residues Gly12 to Leu33 (shown in magenta in Fig. 9A). This motif (containing the conserved 'motif 1' [20]) serves as the base of the binding site for mevalonate and, at the same time, contributes important catalytic residues, such as Lys13 (Fig. 9A). The segment β 7-loop- α 5 is the ATP binding loop with Lys13 in β 2, Ser146 in α 5,

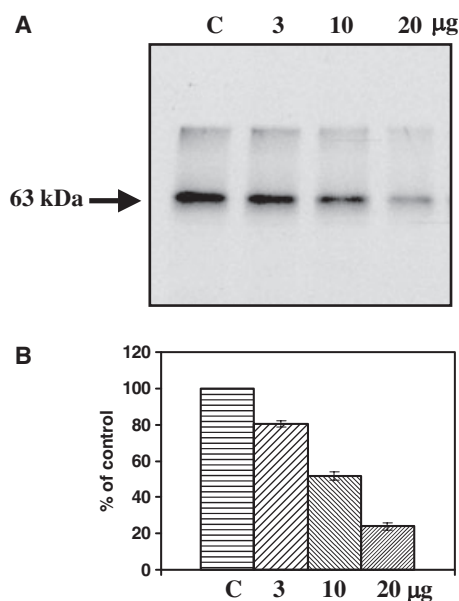


Fig. 7. *In vitro* translation of rat LHR mRNA. Effect of overexpressed wild-type rat MVK. (A) FLAG-tagged rat LHR mRNA (200 ng) was *in vitro* translated using 0.6 mBq of [³⁵S]methionine in the presence of increasing concentrations of S100 protein (3–20 µg) from pCMV4-rMVK-transfected 293T cells overexpressing wild-type rat MVK. The translated LHR proteins were immunoprecipitated and SDS-PAGE was performed and processed to develop the autoradiogram. The control (C) experiment was performed in the absence of S100 protein. The autoradiogram is representative of three independent experiments. (B) The protein bands were quantified by densitometric scanning followed by analysis using NIH IMAGE 1.61 software, and graphed as a percentage of the control (mean value ± SE).

Glu193 in $\alpha 6$ and Asp204 in $\alpha 7$. During catalysis, Asp functions as a general base to abstract the proton from the hydroxyl group of mevalonate [17]. According to the proposed mechanism, the C5 hydroxyl of mevalonate is in close proximity to both Asp204 and the γ -phosphate of ATP within 4 Å [17]. This is suitable for donating its proton to Asp and for accepting the phosphoryl group from ATP. The side chains of Glu193 and Ser146 help to stabilize the transition state of the γ -phosphate group of ATP by magnesium ion. Lys13 is also found to be within 3–4 Å of the C5 hydroxyl group of mevalonic acid. This basic residue helps to lower the pKa value of the C5 hydroxyl group and stabilizes the deprotonated C5 alkoxide group. Thus, Asp204 and Lys13 are very crucial residues for catalysis that are conserved in other members of the GHMP family.

To examine the requirement of these amino acids at the active site of MVK for LHR mRNA binding, based on our earlier studies, we mutated Ser146 to Ala, Glu193 to Gln, Asp204 to Asn and Lys13 to Ala.

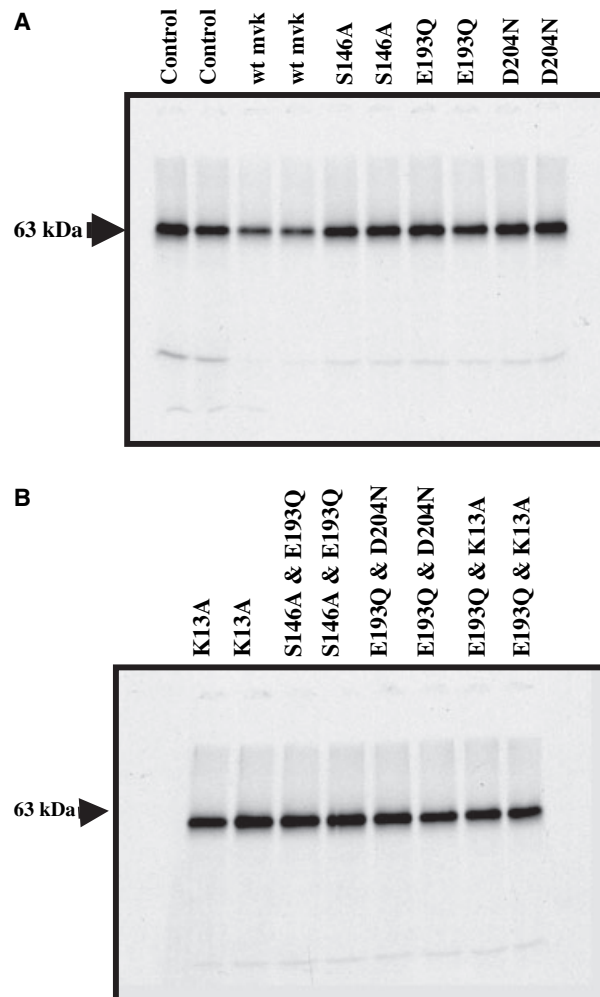


Fig. 8. *In vitro* translation of rat LHR mRNA. Effect of mutant rat MVK proteins. FLAG-tagged rat LHR mRNA (200 ng) was *in vitro* translated using 0.6 mBq of [³⁵S]methionine in the presence of 10 µg of S100 protein from 293T cells transfected with wild-type MVK (wtmvk) and all the single (A) and double (B) mutants of rat MVK. All were performed in duplicate. The translated LHR proteins were immunoprecipitated and SDS-PAGE was performed and processed to develop the autoradiogram. Controls were performed with no S100 proteins. The autoradiogram is representative of three independent experiments.

As illustrated in Fig. 3, RNA electrophoretic mobility shift analysis using rat LBS as probe showed significant decreases in the binding activities of all the single mutants of MVK proteins overexpressed in 293T cells when compared with wild-type MVK, indicating that these amino acids are also essential for LHR mRNA binding. This was further confirmed by RNA gel shift analysis, with double mutants of MVK proteins showing decreased LHR mRNA binding activity (Fig. 4). The role of these amino acids in LHR mRNA binding

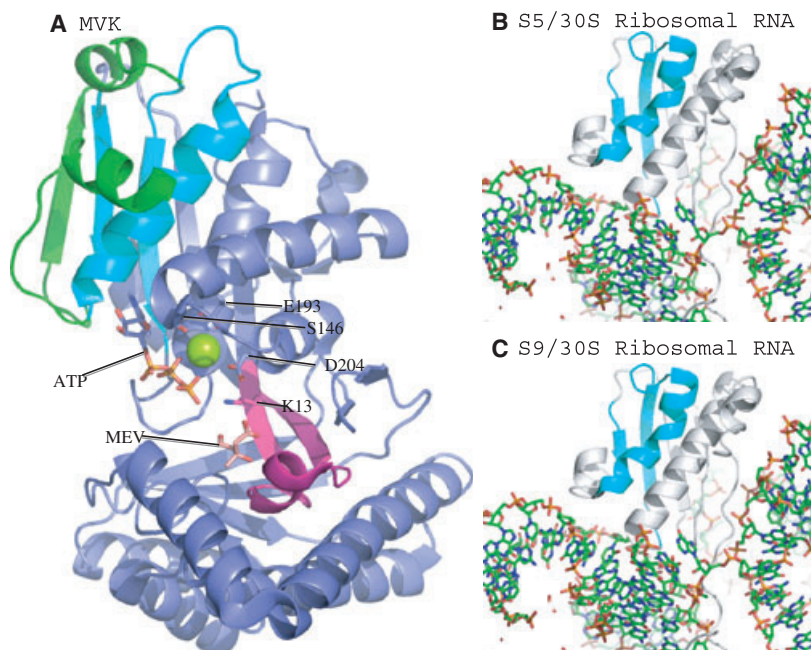


Fig. 9. (A) Structure of rat MVK (MVK) bound to ATP-Mg²⁺ [17] with a model of mevalonate based on the crystal structure of mevalonate bound to *Leishmania major* MVK [21]. Residues comprising the left-handed β - α - β motif are colored cyan. The β - α - β motif is disrupted by a 50 residue insert that is colored green. ATP and mevalonate are shown in stick representation. Residues forming the base of the mevalonate binding pocket, Gly12 to Leu33, are colored magenta. The side chains of Asp204 interacting with Lys33 are indicated, as are the side chains of Glu193 and Ser146 interacting with the Mg²⁺ ion (green sphere). The model was constructed by superimposing the crystal structure of *L. major* MVK bound to mevalonate onto the structure of rat MVK bound to ATP-Mg²⁺. Mevalonate from the *L. major* structure was then extracted onto the rat MVK complex. (B, C) Structures of two other left-handed β - α - β motifs interacting with RNA are shown for S5 and S9 ribosomal RNA proteins both bound to the 30S ribosome [33].

activity was further established by mutations outside the catalytic region. These mutants did not produce any change in the RNA binding activity of MVK (Fig. 5). As a decrease in the RNA binding activity of MVK was observed when the amino acids at the active site were mutated, we examined the LHR mRNA translational suppressor function of these mutated MVK proteins using a rabbit reticulocyte lysate system. A substantial reversal of translational suppression of LHR mRNA by all the MVK mutant proteins was observed (Fig. 8A,B). This indicates that these amino acids at the catalytic site of MVK are crucial for its function as a translational suppressor of LHR mRNA.

A number of metabolic enzymes have been characterized as RNA regulatory proteins [22]. One of the well-characterized enzymes performing two entirely different functions is the cytosolic protein aconitase [23,24]. Aconitase has been identified as iron regulatory protein 1 (IRP1), which binds to the iron regulatory elements (IREs) present in the 3'-untranslated regions of mRNAs [23–26]. Recently, its crystal structure as a cytosolic aconitase [27] and its complex with frog ferritin IRE-RNA [28] have been solved. These

studies found extensive overlap between the enzyme active site and RNA binding site of IRP1. Many of the amino acids at the active site of aconitase were found to serve both catalytic and RNA binding functions, thus showing the functional plasticity of these amino acids. In its IRP1 form, domains 3 and 4 undergo a substantial shift in their relative positions to the central core formed by domains 1 and 2, to open up a hydrophilic cavity for IRE between the core and domain 3. This shows the conformational flexibility of this protein to perform an entirely different function. Similarly MVK acts as a dual function protein, possessing both catalytic function and LHR mRNA binding activity. This RNA binding activity of MVK leads to translational suppression and triggers LHR mRNA degradation *in vitro* [14,19]. This is in agreement with other studies, in which it has been reported that translational arrest/inhibition or aberrant termination can lead to the degradation of eukaryotic mRNA [29–32].

In the present study, we have demonstrated that the amino acids Ser146, Glu193, Asp204 and Lys13 at the active site of MVK, required for catalysis, are also required for LHR mRNA binding. This dual function

nature is very similar to the functional flexibility of some of the amino acids identified at the active site of the cytosolic enzyme aconitase. These data therefore indicate that an intact active site of MVK is required for its binding to rat LHR mRNA and for its translational suppressor function. Although β - α - β motifs are found in a number of known RNA binding proteins, the exact details of the mode of interaction between RNA and this motif still remain somewhat unclear. Inspection of two other left-handed β - α - β motif-containing proteins cocrystallized with RNA as part of the 30S ribosome particle (shown in Fig. 9B,C) has failed to reveal a conserved mode of interaction between the protein motif and RNA [33]. The diverse locations of RNA relative to the motif make any proposed structural argument about the mode of RNA binding speculative at this point, but we believe that our data suggest that some kind of structural linkage between substrate and RNA binding is a reasonable hypothesis. Two structural scenarios that explain the present mutagenesis data are as follows: (a) the RNA binding site overlaps with the site of ATP binding and may exploit common side chain interactions; or (b) the conformations of residues involved in RNA binding may be directly or indirectly coupled to the binding of ATP and mevalonate.

Once LHR mRNA becomes an untranslatable mRNA-protein complex by binding to MVK, it may be recruited to the RNA degradation pathway with the help of other interacting partners of MVK that are associated with translation and/or mRNA decay machinery.

Experimental procedures

Chemicals

The QuickChange 11 XL Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA, USA). [α - 32 P]UTP was obtained from Perkin Elmer Life Sciences (Boston, MA, USA) and Redivue L-[35 S]methionine (*in vitro* translation grade) was purchased from Amersham Biosciences (Arlington Heights, NJ, USA). mMessage mMachinE T7 Ultra and MAXIscript Kits were products of Ambion (Austin, TX, USA). EDTA-free protease inhibitor mixture tablets and Quick spin columns (G-50 Sephadex) for radiolabeled RNA purification were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). RNasin and Flexi Rabbit Reticulocyte Lysate System were purchased from Promega (Madison, WI, USA). Centriplus YM-10, Centricon YM-10 and Microcon YM-10 were products of Millipore Corporation (Bedford, MA, USA). Anti-FLAG M2-Agarose affinity gel was purchased from Sigma (St Louis, MO, USA). Bicinchoninic acid reagent

was from Pierce (Rockford, IL, USA). Enlightning (rapid autoradiography enhancer) reagent was a product of NEN Life Science Products, Inc. (Boston, MA, USA). DL-Mevalonic acid lactone and ATP (Mg salt) were purchased from Sigma. Mevalonic acid lactone was converted to potassium mevalonate by incubation with a 5% molar excess of KOH at 38 °C for 1 h, adjusted to pH 7.8 and stored at -20 °C.

Construction of MVK cDNA mutants

The mutants of rat wild-type MVK in pCMV4 vector were prepared using the QuickChange 11 XL Site-Directed Mutagenesis Kit from Stratagene. The mutagenic sense (S) primers used were as follows: S146A, 5'-GCGGGCTTGGGCTCCGCTGCAGCCTACTCGGTG-3'; E193Q, 5'-GCCTACGAGGGCAGAGAGTATCCATGGG-3'; D204N, 5'-CCTCTGGCGTGAACAATCCGTCAGCACC-3'; K13A, 5'-GTGTCTGCTCCAGGGCAGTCATTCTCCATGG-3'; D316A, 5'-CACGCCTCCCTGGCCAGCTCTGTCTAG-3'; S314A, 5'-GTGGGCCACGCCGCCCTGGACCAGCTG-3'. The single mutants were then subsequently employed for the synthesis of S146A & E193Q, E193Q & D204N and E193Q & K13A double mutants using the appropriate mutagenic primers as shown above. The mutations were verified at the DNA Sequencing Core at the University of Michigan Medical School.

In vitro transcription

The cDNA used to generate rat LBS was chemically synthesized and contained the T7 RNA polymerase promoter sequence at the 5' end. [α - 32 P]-labeled LBS was *in vitro* transcribed from cDNA template using an Ambion *in vitro* transcription kit. The full-length capped and FLAG-tagged rat LHR mRNA was synthesized using an mMessage mMachinE T7 Ultra Kit. The wild-type and mutant rat LBSs were synthesized using the MAXIscript Kit. The radiolabeled LHR mRNA was prepared using 2.2 M Bq of [α - 32 P]UTP in the reaction mixture. After transcription, the RNAs were treated with RNase-free DNase I and extracted with nuclease-free water-saturated phenol-chloroform-isoamyl alcohol (50 : 49 : 1). Unincorporated nucleotides were removed using Quick spin columns (G-50 Sephadex). RNA was precipitated with an equal volume of isopropyl alcohol at -20 °C. The precipitated RNA was washed three times with 75% ethanol, air-dried and dissolved in nuclease-free water. Both radiolabeled and unlabeled RNAs were quantified spectrophotometrically at 260 nm.

In vitro translation

In vitro translation reactions (reaction volume, 25 μ L) were performed using the Flexi Rabbit Reticulocyte Lysate

System, as described by the manufacturer. Proteins synthesized *in vitro* were labeled with [³⁵S]methionine and separated by 10% SDS-PAGE (BioRad mini gel) according to the method of Laemmli. The gel was fixed in 40% methanol (v/v) and 10% acetic acid (v/v) for 20 min, and then incubated in Enlightning reagent for another 30 min. The gel was then dried under vacuum for 20 min at 80 °C and exposed to X-ray film for autoradiography.

Immunoprecipitation

FLAG-tagged *in vitro*-translated rat LHR was immunoprecipitated using anti-FLAG M2-Agarose affinity gel; 25 µL of the *in vitro*-translated reaction mixture was diluted to 500 µL with dilution buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100). Anti-FLAG M2-Agarose affinity gel was washed three times with wash buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl), added to the diluted translation reaction mixture (40 µL gel suspension per 500 µL diluted translation reaction mixture) and incubated overnight in an end-over-end shaker at 4 °C. The sample was centrifuged for 5 s at 10 600 *g* at room temperature and the supernatant was removed. The beads were washed three times with wash buffer and 30 µL of 2× SDS-PAGE sample buffer was added. The beads with sample buffer were heated at 65 °C for 20 min, centrifuged at 10 600 *g* for 5–10 s and the supernatant was collected. The supernatant was then applied to 10% SDS-PAGE.

RNA electrophoretic mobility shift analysis

RNA electrophoretic mobility shift analysis was performed as described previously [10]. Briefly, 10 µg of cytosolic S100 protein sample was incubated with $(1-2) \times 10^5$ c.p.m. of [α -³²P]UTP-labeled rat LBS. Binding reactions were carried out in buffer A (10 mM Hepes, pH 7.9, 0.5 mM MgCl₂, 50 µM EDTA, 5 mM dithiothreitol and 10% glycerol) (pH 7.5) containing 50 mM KCl and protease inhibitor mixture. Incubations were performed in the presence of 5 µg of tRNA and 40 U of RNasin (Promega) at 30 °C for 30 min. Unprotected radiolabeled RNA was degraded by the addition of 25 U of RNase T1 at 37 °C for 30 min. Samples were then incubated with heparin (final concentration, 5 mg·mL⁻¹) on ice for 10 min to reduce nonspecific binding. The RNA-protein complexes were resolved by 5% native polyacrylamide (70 : 1) gel electrophoresis at 4 °C. The gel was then dried and exposed to Kodak X-Omat AR film for visualization by autoradiography.

Preparation of cytosolic proteins (S100 fraction)

Transiently transfected 293T cells were detached from culture dishes with NaCl/P_i-EDTA and pelleted using 500 *g* for 5 min. The cell pellets were homogenized at 4 °C

in buffer A containing 50 mM KCl and EDTA-free protease inhibitor mixture. Homogenates were centrifuged at 105 000 *g* for 90 min at 4 °C, the supernatants (S100) were collected and total protein was quantified using a bicinchoninic acid Protein Assay Kit (Pierce).

Overexpression of rat MVK in 293T cells

Human embryonic kidney cells (293T cells) were transiently transfected with rat MVK cDNA cloned into pCMV4 vector using Fugene 6 reagent, as described by the manufacturer (Roche Molecular Biochemicals). Cells were collected 48 h post-transfection, and the cytosolic proteins (S100) were prepared as described previously [13]. The S100 fractions were analyzed for MVK by western blot analysis.

Western blot analysis

Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane using 25 mM Tris buffer containing 192 mM glycine and 20% methanol (pH 8.3) for 1 h at 4 °C. Rat MVK was detected using a rabbit polyclonal anti-N-terminal rat MVK IgG preparation (40 µg·mL⁻¹), followed by a polyclonal donkey anti-rabbit IgG conjugated to horseradish peroxidase (1 : 10 000) as a second antibody. The presence of immune complexes was detected by chemiluminescence using an ECL kit (Amersham Biosciences).

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