Mitochondrial Aconitase Binds to the 3'-UTR of Mouse Hepatitis Virus RNA

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1. INTRODUCTION

Cis-acting signals located in the 3'-UTR of the viral genome were first identified by deletion analyses of MHV defective interfering (DI)-RNAs (Kim, Jeong, and Makino, 1993; Lin and Lai, 1993). The *cis*-acting signals for the synthesis of minus-strand RNA are contained within the last 55 nucleotides (nt) plus the poly (A) tail (Lin, Liao, and Lai, 1994). Our lab has previously shown that host cell proteins specifically bind to two distinct sites within the MHV 3'UTR (Yu and Leibowitz, 1995b). In the current work, we show that the RNA-protein (RNP) complex formed within the last 42 nt of the genomic RNA contains four proteins of molecular mass 90, 70, 58 and 40 kDa and identify the 90 kDa protein as mitochondrial aconitase.

2. MATERIALS AND METHODS

2.1 RNase protection/gel mobility shift and UV cross-linking assays

Cytoplasmic extracts were prepared from Dounce homogenized 17Cl-1 cells by a modification of a previously described method (Yu and Leibowitz,

The Nidoviruses (Coronaviruses and Arteriviruses).
Edited by Ehud Lavi *et al.*, Kluwer Academic/Plenum Publishers, 2001.

1995b). RNase protection/gel mobility shift and UV cross-linking assays were performed as described (Yu and Leibowitz, 1995b). Unlysed cells and nuclei were removed by centrifugation at 750g for 10 min. The supernatant was centrifuged at 10,000g for 30 min at 4°C and this supernatant was stored as the post-mitochondrial fraction.

2.2 Purification of RNA binding proteins

Proteins which interacted with the last 42 nt of the MHV genome [3'(+)42] were purified from cytoplasmic lysates by sequential batch fractionation over High Q and High S ion exchange matrices (Bio-Rad) and a heparin agarose matrix (Sigma). At each step samples were assayed for RNA binding activity and analyzed by SDS-PAGE.

A biotinylated synthetic RNA corresponding to nt 42–5 at the 3' end of the MHV genome [position 1 is the first nt upstream of the 3' poly(A) tail] was purchased from Dharmacon Research. The RNA solution was adjusted to 100 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT and bound to 1 mg of BioMag Streptavidin beads (PerSeptive Biosystems). The eluate from the heparin agarose matrix was added to the beads for 2 h at 4°C. After four washes proteins were eluted with 2 M KCl or by boiling in SDS-PAGE loading buffer.

2.3 Peptide sequencing

The partially purified 90 kDa protein was resolved from other proteins by SDS-PAGE, located by staining, and was cut out from the gel. The gel slice was digested with trypsin and the resulting tryptic peptides purified by HPLC and analyzed by MALDI mass spectrometry. Two peptides were subjected to sequential Edman degradation.

3. RESULTS

3.1 Partial purification of the MHV-JHM 3' (+) RNA binding proteins

Cytosolic extracts from murine 17Cl-1 cells were assayed with a ^{32}P-labeled transcript corresponding to nt 16-84 upstream from the 3' end of the MHV-JHM genome. Three RNA-protein complexes formed, with the

slowest migrating complex, complex 1, being the most abundant (Figure 1A). The specificity of the RNA-protein binding was confirmed by competition experiments (not shown). Molecular masses of cytoplasmic proteins that bind to the MHV-JHM 3'(+)42 RNA were estimated by UV-induced cross-linking assays. Four proteins of 90, 70, 58 and 40 kDa were consistently detected (Fig. 1B). The 90 kDa protein was the most prominent. *In situ* cross-linking (not shown) revealed that complex 1 contained the same four protein species.

RNA-binding proteins were purified from cytoplasmic lysate as described in Materials and Methods and assayed by gel mobility shift/RNase protection (Fig. 1A), UV cross-linking, and SDS-PAGE (Fig. 1B). Virtually all of the RNA binding activity was in the High Q matrix flow through fraction. This fraction was bound to High S matrix and RNA binding activity was then eluted with 150 mM KCl. This fraction was subjected to non-specific affinity chromatography with heparin-agarose. This purification scheme enriched for RNA-binding proteins. A 90 kDa protein present in material purified by this method was well resolved from other proteins and strongly labeled by UV cross-linking (Fig. 1B). Two-dimensional gel electrophoresis of this material indicated that this 90 kDa band was a single protein (not shown).

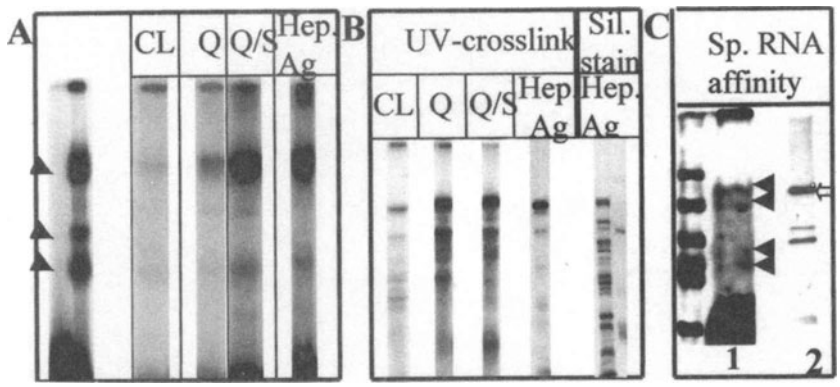

Figure 1. Panel A. A standard RNase protection gel mobility shift assay in on the left. The enrichment of binding activity during purification from crude lysate (CL) and from matrices as described in the text. Panel B. UV cross-linking patterns obtained during purification. Panel C. Affinity purified material visualised by silver staining (lane 1) and by Western Blot with anti-m-aconitase antibody (lane 2). Molecular wt. markers are in the left lane.

3.2 Identification of the 90 kDa MHV-JHM 3' (+) RNA binding protein

A large scale preparation of heparin agarose affinity matrix purified

material was reduced, carboxymethylated and subjected to SDS-PAGE. The 90 kDa band was excised from the gel, digested with trypsin, fractionated by HPLC and analyzed by MALDI-mass spectrometry. The three best fits with the MS data were bovine, swine, and human mitochondrial aconitases (m-aconitase) with 63%, 54%, and 54% masses matched respectively. Two peptides were sequenced and yielded the sequences IVYGHLDDPANQEIER and LTIQGLK. A BLAST search of the Swiss-Prot database revealed 100% sequence identity with residues 69-83 and 724-730 of human m-aconitase.

Proteins binding to the MHV 3' (+)42 protein binding element were further purified by affinity chromatography. Ninety, 70, 58, and 40 kDa proteins were eluted from the specific RNA affinity matrix (Fig. 2A). The 90 kDa protein co-electrophoresed with purified bovine m-aconitase and was immunoreactive with an anti-m-aconitase antibody (Fig. 1C). The 90 kDa protein contained in RNA-protein complex 1 isolated by native gel electrophoresis was also recognized by this antibody (not shown). These results strongly support the identification of the 90 kDa protein in RNA-protein complex 1 as m-aconitase.

RNase protection/gel mobility shift reactions were carried out with purified bovine mitochondrial holo- and apo-aconitase but failed to detect binding to the MHV 3'(+)42 RNA. When a UV cross- linking step was added prior to electrophoresis, only the mitochondrial apo-aconitase formed a complex. This complex co-electrophoresed with complex 2 formed with cytoplasmic lysate (Fig. 2A). Specificity of binding of apo-aconitase to MHV 3'(+)42 RNA was verified by competition (Fig. 2A).

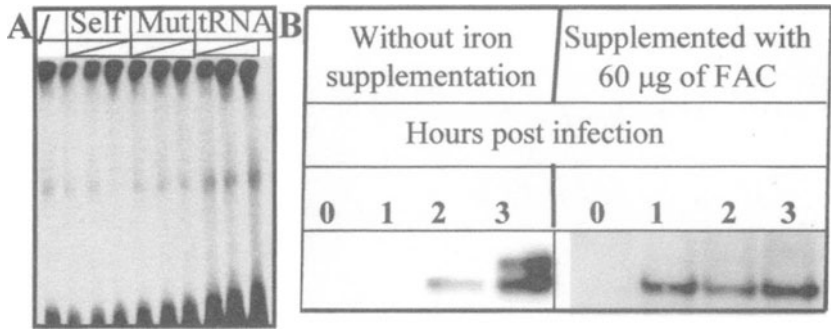

Figure 2. Panel A. Specific binding of m-apo-aconitase in the presence of competitor RNAs. B. Western Blot analysis of N protein levels in control and FAC treated cells.

3.3 Iron supplementation increases the level m-aconitase and N protein

The 5' UTR of m-aconitase contains an IRE which links cellular iron status and m-aconitase expression (Kim *et al.*, 1996). To seek functional effects of the interaction of m-aconitase with the MHV 3'(+)42 protein binding element, we examined the effect of ferric ammonium citrate (FAC) supplementation on virus replication. The ability of MHV to form plaques was unchanged by FAC treatment of cells. Immunoblots showed that iron supplementation increased N protein accumulation at 1-3 h p.i. compared to untreated cultures (Fig. 2B).

4. DISCUSSION

Two protein binding elements have been mapped within the MHV 3 (+) UTR and are implicated in viral replication (Liu, Yu, and Leibowitz, 1997; Yu and Leibowitz, 1995a; Yu and Leibowitz, 1995b). In this work we have demonstrated that there are at least four protein components in the RNP complex formed at the 3' most *cis*-element in the MHV-JHM 3' UTR; m-aconitase is one component of the complex.

Mammalian cells have two aconitases encoded by separate nuclear genes: the mitochondrial enzyme and cytoplasmic aconitase, better known as iron regulatory protein (IRP). The IRP has 30% amino acid identity with m-aconitase (Kennedy *et al.*, 1992) and ~56% overall sequence similarity. Activity of the enzymes depend on the presence of an iron sulfur [4Fe-4S] cluster in the catalytic center. IRP is a conditional cytoplasmic mRNA-binding protein which interacts with iron-responsive elements (IREs) located in the 5' UTR of ferritin mRNA and the 3' UTR of transferrin receptor (TfR) mRNA and coordinates post-transcriptional regulation of cellular iron metabolism (Klausner, Rouault, and Harford, 1993). Disassembly of the IRP iron-sulfur cluster upon iron starvation yields the RNA-binding form of the IRP. We have demonstrated that only the m-aconitase apoprotein binds to the MHV 3'(+)42 RNA, and that this binding is specific. M-aconitase, like its IRP homolog, is a bifunctional protein whose dynamic [4Fe-4S] cluster determines if it functions as an enzyme or as a RNA binding protein.

It is surprising that m-aconitase, a mitochondrial matrix protein, binds to a viral RNA that replicates in the cytoplasm. RNA binding activity did not partition with the majority of m-aconitase detected in mitochondrial lysates (not shown). We believe that m-aconitase interacts with MHV RNA prior to importation into mitochondria. Alternatively, many mitochondrial proteins

have been demonstrated to be present outside of mitochondria under certain conditions (Soltys and Gupta, 1999).

Iron supplementation increased expression of N protein as early as one h p.i. with a corresponding change in MHV-specific mRNA levels (not shown). The effect on viral protein synthesis parallels a modest increase in m-aconitase level in iron treated cells. We believe that m-aconitase binding to the 3' UTR increases the expression of viral proteins, similar to the role of IRP in regulating TfR (Klausner, Rouault, and Harford, 1993).

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