Mitochondrial HSP70, HSP40, and HSP60 bind to the 3' untranslated region of the *Murine hepatitis virus* genome

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Summary. We have previously shown that mitochondrial-aconitase binds specifically to the 3' terminal 42 nucleotides of the *Murine hepatitis virus* (MHV) RNA along with three additional proteins of 70, 58 and 40 kDa to form a stable RNA-protein complex. Supershift and western blot assays have identified these three proteins as mitochondrial HSP70 (mtHSP70), HSP60, and HSP40. A series of co-immunoprecipitation assays have established that these four MHV RNA binding proteins are associated, even in the absence of MHV RNA. However, the presence of a synthetic RNA containing the sequence bound by these four proteins does increase the amount of co-precipitated protein, in particular the amount of HSP60 which is brought down with antibodies directed against HSP40 and mtHSP70. We have provided evidence for the interaction of these four proteins with the 3' end region of MHV RNA in infected cells by a series of immunoprecipitation RT-PCR assays. We believe it is likely that MHV RNA interacts with m-aconitase prior to its import into mitochondria in cooperation with extra-mitochondrial mtHSP70, HSP60, and HSP40.

Introduction

Murine hepatitis virus (MHV) is a prototypic member of the family *Coronaviridae*, and contains a single-stranded, positive-sense RNA genome approximately 31 kb in length [12]. Viral proteins are translated from six to seven subgenomic mRNAs as well as from the genome. The virus-specific subgenomic and genomic RNAs make up a 3'-coterminal nested-set [16, 40] and contain a leader sequence of approximately 70 nucleotides (nt) at the 5' end [13, 39]. Coronaviruses perform their entire replication program in the cytoplasm of infected cells. Following uncoating, coronaviruses express the largest known replicase polyproteins, which in turn are proteolytically processed to yield a large number of mature proteins

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including RNA-dependent RNA polymerase (RdRp) [4, 34]. The RdRp, perhaps in association with host proteins, directs the synthesis of negative-sense RNAs from the 3' end of the viral genome.

Analysis of the structure of defective interfering (DI) RNAs indicated that approximately 470 nucleotides (nt) at the 5' terminus, and 436 nt at the 3' terminus are required for DI RNA replication in MHV-infected cells [11, 19]. The *cis*-acting signals for the synthesis of negative-strand RNA are contained within the last 55 nucleotides plus the poly (A) tail at the 3' end of the MHV genome [18]. Specific binding of host cellular proteins to two distinct sites within the 3'-UTR of MHV-JHM genomic RNA has been reported [48]. One site, the 3'(+)42 protein binding element [3'(+)42], was mapped within the 3' most 42 nt of the genomic RNA [48], the other site was mapped to nucleotides 166-129 upstream from the 3' end of the viral genome [21]. Cytosolic protein extracts from murine 17Cl-1 cells assayed using a probe containing the 3'(+)42 element formed three RNA-protein complexes, with the slowest migrating and presumably largest complex, complex 1, being the most abundant [28, 48].

Recently, we established that mitochondrial aconitase (m-apo-aconitase) binds specifically to the last 42 nucleotides of the 3' UTR of MHV RNA [28]. However, the interaction of purified m-apo-aconitase by itself was not stable under electrophoretic conditions. Four different proteins with molecular weights of 90 kDa (m-apo-aconitase), 70 kDa, 58 kDa and 40 kDa were required to form a complex with the last 42 nucleotides of the MHV 3' UTR which was stable enough to survive electrophoresis. In this work we have identified the 70 kDa protein bound to the MHV 3' protein binding element as mitochondrial HSP70 (mtHSP70), the 58 kDa protein as HSP60, and the 40 kDa protein as HSP40.

Materials and methods

Virus and cells

Murine 17Cl-1 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ atmosphere [16]. The origin and growth of the JHM strain of MHV (MHV-JHM) virus used in this study have been described previously [16]. Infected cell extracts were prepared from confluent 17Cl-1 monolayers infected with MHV-JHM at a multiplicity of infection (M.O.I.) of 1 pfu/cell.

ATPase activity

The ATPase activity of the post-mitochondrial lysate was measured by determining the amount of free radioactive P_i liberated by [γ -³²P]ATP hydrolysis. The assays were done essentially as previously described [22] except that the partially purified post-mitochondrial lysates (approximately 3 μ g total protein) were incubated at 22 °C with purified m-apo-aconitase (8–10 μ g, graciously supplied by M.C. Kennedy, Gannon University, Erie, PA) and MHV-JHM 3' UTR RNA in ATPase buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 0.1% NP-40, 1 mM DTT, 1 mM ATP containing 2 μ Ci [γ -³²P]ATP plus cocktails of protease and phosphatase inhibitors). At the times indicated, the reaction was stopped by the addition of 100 μ l of acid washed charcoal (Sigma) (7.5% in 50 mM HCl and 5 mM H₃PO₄) to bind free nucleotides. The samples were centrifuged at 13,000 rpm for 5 min, and the radioactivity

contained in the supernatant was determined by liquid scintillation counting. Control reactions were carried out in the absence of purified m-apo-aconitase or by replacing the enzyme with BSA. All assays were performed in triplicate and mean values and standard deviations calculated.

Antibodies

Mouse monoclonal antibodies directed against mtHSP70, HSP60 and HSP40 were obtained from StressGen Biotechnologies Corp (Victoria, B.C., Canada) and Affinity Bioreagents Inc. (Golden, Colorado). A rabbit antiserum against m-aconitase was provided by Dr. Richard S. Eisenstein, University of Wisconsin, Madison [2]. The monoclonal antibody directed against the MHV nucleocapsid gene, antibody 1-16-1, has been described [15]. Monoclonal antibodies directed against a T7-Tag and anti-SV40 T Ag (Ab-1) were purchased from Novagen (Madison, WI) and Oncogene Science (Cambridge, MA) respectively. A mouse monoclonal antibody directed against phosphotyrosine (PY20) was purchased from BD Transduction Laboratories (Lexington, KY).

Gel mobility shift assay and supershift assay

In vitro transcription reactions were carried out with [α - 32 P]UTP to generate radiolabeled probes [48]. After transcription, the radiolabeled RNAs were purified through Sep-Pak Light C18 cartridges (Waters, Milford, MA). The concentration of radiolabeled RNA was measured spectrophotometrically. Crude cytoplasmic lysates and post-mitochondrial lysates were prepared from 17Cl-1 cells as previously described [28]. RNA-protein binding reactions were performed in a volume of 10 μ l as described previously [48]. Briefly, extracts containing 3–5 μ g of total protein, 2 to 5 ng of one of the 32 P-labeled MHV 3'(+) RNA probes, 10 μ g heparin (Sigma), 500 ng of poly(I)-poly(C) (Sigma) and 6% glycerol were incubated at 22 °C for 20 min in binding buffer (10 mM Tris pH 7.6, 5 mM MgCl₂, 1 mM DTT, 100 mM KCl) and then digested at 22 °C for 20 min with RNase T₁ (Calbiochem). RNA-protein complexes were subjected to nondenaturing polyacrylamide gel electrophoresis, dried, and autoradiographed as described [48].

For supershift assays approximately 5 μ g of protein in the post-mitochondrial lysate was incubated either with 0.5 or 1 μ g of monoclonal antibody, or with buffer (10 mM Tris/HCl, pH 7.4, and 0.05% NP-40) alone in a final volume of 10 μ l at 4 °C for 1 h. After the incubation the mixture was used for the standard RNA binding assay.

Specific RNA affinity purification

A biotinylated synthetic RNA (5'-AGUAAAUGAA UGAAGUUGAUGCAUGGCCAAUUGG AAGA-3') corresponding to nucleotides 42-5 at the 3' end of the MHV genome (counting the first nucleotide upstream from the 3' poly(A) tail as position 1) was purchased from Dharmacon Research (Boulder, CO). Aliquots of biotinylated RNA were cleaved and de-protected as per the manufacturer's guidelines and bound to magnetic streptavidin beads (PerSeptive Biosystems, Farmingham, MA) as described previously [28]. The beads were washed free of unbound RNA with buffer B (10 mM Tris, pH 6.8, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin 0.5 μ g/ml pepstatin) and a preparation of MHV 3'(+)42 RNA binding proteins, partially purified by sequential ion exchange and heparin agarose binding steps [28], was added to the beads in the presence of heparin (Sigma), poly(I)-poly(C) (Sigma), and tRNA, at concentrations of 1 μ g/ μ l, 500 ng/ μ l, and 25 μ g/ μ l, respectively. The binding reaction was incubated for 2 h at 4 °C and washed four times with buffer B. The bound proteins were eluted by boiling the beads in 1 $\times$ SDS loading buffer.

Western blot analysis

Samples (either immunoprecipitates or RNA affinity purified proteins) were added to SDS-lysis buffer preheated to 100 °C and boiled for 5 min. Proteins were separated on either 10% standard or 4–20% gradient (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad) using a semi-dry blotting procedure [43]. Membranes were blocked overnight in TBST (25 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% (v/v) Tween 20), 5% (w/v) dried milk powder, incubated for 1 h with primary antibodies in TBST, 5% (w/v) dried milk powder, washed in TBST, and incubated with a second antibody conjugated to horseradish peroxidase for 1 h. Membranes were developed using an ECL (enhanced chemiluminescence) detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized by exposure to BioMax Light film (Kodak, Rochester, NY).

UV-induced cross-linking assays

UV cross-linking was performed as described previously [48] with the modification that after UV crosslinking and digestion with RNase A samples were immunoprecipitated and the labeled proteins resolved by SDS-PAGE.

Immunoprecipitation

Uninfected and infected 17Cl-1 cells were lysed in 1 ml of ice-cold lysis buffer containing 10 mM Tris-Cl pH 7.6, 50 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 1 mM orthovanadate, 1% NP-40, 50 mM NaF, 10 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM phenylmethylsulphonyl fluoride. Lysates were clarified by centrifugation at 16,000 × g for 10 min. Immunoprecipitation was carried out using Dynabeads Protein A (DynaL Biotech, Lake Success, NY) as per the manufacturer's recommendations. Antibodies were bound to protein A immobilized on the magnetic beads and cross-linked using DMP (dimethyl pimelidate) in 0.2 M triethanolamine pH 9.0. Antibodies cross-linked to the protein A magnetic beads were used to capture the antigens from the 17Cl-1 cell lysates. Immunoprecipitates were washed several times with PBS, extracted in 1× SDS-buffer and then separated by SDS-polyacrylamide gel electrophoresis and analyzed by western blotting.

Immunoprecipitation RT-PCR assays

Fifty microliters of Dynabeads-Protein A suspension per reaction were washed twice with 0.5 ml of RNase-free 0.1 M Na-phosphate (PBS, pH 8.0) and resuspended in 100 µl of PBS. Approximately 15 µg of each monoclonal antibody tested [1-16-1, directed against JHM strain N protein (16), anti-mitochondrial HSP70, anti-HSP40, anti-HSP60, 15 µl of anti-T7-Tag, anti-SV40 T Ag] was added to magnetic beads, and rotated slowly for 30 min at room temperature. The test tubes were then placed in a magnetight separation stand (Novagen) for 2 min and supernatants were removed. The beads were washed twice with 0.5 ml of RNase-free 0.1 M Na-phosphate (pH 8.0) containing 0.1% BSA. Five hundred microliters of binding buffer [10 mM Tris (pH 7.6), 100 mM KCl, 5 mM MgCl₂, and 1 mM DTT] used for RNase protection/gel mobility shift assays was used for the last wash and subsequently discarded. Two hundred micrograms of mock-infected and MHV-JHM infected post-mitochondrial fractions were resuspended in binding buffer and added to the beads. Antigen-antibody binding reactions were rotated for 1 h at room temperature. The immunocomplexes bound to the beads were washed three times with binding buffer.

Proteins and protein-RNA complexes binding to the beads were eluted in 100 µl of 50 mM Tris (pH 8.0), 1% SDS, and 10 mM EDTA at 65 °C for 10 min. The eluted material was treated with 100 µg of proteinase K (10 mg/ml) at 37 °C for 30 min, extracted twice by phenol-chloroform and nucleic acids precipitated by ethanol in the presence of 10 µg

glycogen carrier. RNA was pelleted by centrifugation and used as template for reverse transcription (RT). RT was performed using a MHV-JHM specific primer, spanning nt 340 to 323 at the 3' end of JHM genome (5' GTAGTGCCAGATGGGTTA 3'), and Superscript II (Invitrogen) at 42 °C for 1 h. Synthesized cDNA was used as a template for PCR. The 5' positive sense primer for PCR was the same primer as used in RT reactions. The 3' negative sense primer corresponds to the complementary sequence from nt 1 to 18 at the 3' end of JHM genome (5' GTGATTCTTCCAATTGGC 3'). Amplification was performed at 95 °C for 60 sec, 50 °C for 30 sec, 72 °C for 90 sec for 10 cycles; at 95 °C for 30 sec, 55 °C for 30 sec, 70 °C for 60 sec for the next 10 cycles, at 95 °C for 30 sec, 70 °C for 60 sec for the last 10 cycles; followed by a 15 min extension at 72 °C. Ten nanograms of the plasmid DE 25 was used as a positive control in PCR reactions. RT-PCR was also carried out to detect the abundant cellular GAPDH mRNA. The sequence of the RT primer for the synthesis of GAPDH cDNA is: 5'-GCCAAAAGGGTCATCATCTC-3'. The RT primer also serves as the 5' positive sense primer for PCR. The sequence of the 3' negative sense primer for GAPDH is: 5'-GTAGAGGCAGGGATGATGTTC-3' (primers were provided by Dr. George Davis, TAMUS-HSC). PCR reactions underwent 30 cycles of amplification (each cycle consists of at 95 °C for 45 sec, 62 °C for 45 sec), followed by a 5 min extension at 72 °C. PCR products were resolved by agarose gel electrophoresis.

Results

An ATPase activity present in post-mitochondrial lysate stimulates RNA-protein binding

We have recently demonstrated that m-apo-aconitase is one of the four proteins which bind to the last 42 nucleotides upstream of the poly(A) tail of the MHV genome [3'(+)42 protein binding element] as an RNP complex using gel shift/RNase protection and UV-cross-linking assays. In these assays the addition of a RNase T₁ digestion step prior to electrophoresis increases both the resolution and sensitivity of gel shift assays by digesting unbound RNA and RNA nonspecifically bound to protein, and by decreasing the size and apparent heterogeneity of the RNA-protein complexes [14]. The digested RNA runs as a smear with the dye front at the bottom of the gel, with the majority of the digested probe running out of the gel. Three RNA-protein complexes are detected by this assay. The largest complex, complex 1, is typically the most abundant complex detected, with the two faster migrating complexes being produced in variable amounts [28, 48]. In the course of experiments investigating the binding of m-apo-aconitase to the 3'(+)42 protein binding element, we determined that the addition of ATP and purified m-apo-aconitase to cytoplasmic lysates increased RNA binding activity (Fig. 1A, compare lanes 1 and 4), in gel shift/RNase protection assays. The formation of the largest complex, complex 1, was most dramatically affected (black arrow, Fig. 1A). It should be noted that the lysates used in these experiments contain a large amount of m-aconitase, only a small fraction of which is the apo-enzyme which binds MHV RNA [28]. The addition of ATP to the binding reactions in the absence of added m-apo-aconitase produced only a small increase in RNA binding activity (Fig. 1A, lane 2). When ATP was omitted from the binding reaction (Fig. 1A, lane 3) no increase in RNA binding activity was detected in the presence of m-apo-aconitase.

Fig. 1. An ATPase activity is associated with crude cytoplasmic extract having MHV RNA binding activity. **A** RNase protection/gel mobility shift assays were carried out as described in Materials and methods. Reactions were performed in the presence of crude lysate alone (1) or were supplemented by the addition of 1 mM ATP (2 and 4), 10 μ g m-apo-aconitase (3, 4, 5), or with 1 mM ATP γ S (5). The reaction mixtures were UV-crosslinked prior to gel electrophoresis as described previously for assays with purified m-apo-aconitase [28]. Irrelevant lanes between 4 and 5 were removed from the figure using Photoshop. The black arrow indicates the position of RNA-protein complex 1. **B** Replicate RNA-protein binding reactions were supplemented with 1 mM ATP, 2 μ Ci of [γ - 32 P]ATP, and 2 μ g of m-apo-aconitase (open markers) or with BSA (filled markers). Reactions were incubated at 22 $^{\circ}$ C and at various times samples were assayed for ATPase activity as described in Materials and methods. All assays were performed in triplicate and mean values and standard deviations are shown

These effects were consistently observed in multiple experiments. Substituting BSA for m-apo-aconitase in these binding reactions resulted in no increase in RNA binding activity (data not shown). These finding led us to generate a working hypothesis that the lysate might possess an ATPase activity and that ATP hydrolysis generated energy for a conformational change in m-apo-aconitase which resulted in more efficient RNA binding. We therefore analyzed RNA binding activity in the presence of ATP- γ S, a non-cleavable analogue of ATP. The substitution of ATP- γ S for ATP did not stimulate RNA binding activity (Fig. 1A, lane 5).

To further investigate the role of ATP in the RNA binding reaction, we measured the ability of RNA binding reactions containing m-apo-aconitase, ATP, and cytoplasmic lysate to hydrolyze $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, as described in Materials and Methods. As shown in Fig. 1B, ATP was hydrolyzed in the complete binding reaction. Only minor amounts of ATPase activity were observed when purified m-apo-aconitase was omitted from the reaction or when it was replaced with BSA. Similar results were obtained in multiple experiments. Thus stimulation of RNA binding activity and ATPase activity were both dependent on the presence of cytoplasmic lysate, m-apo-aconitase, and ATP. ATPase activity was strictly dependent on the presence of MgCl_2 in the reaction buffer, since no activity was detected in the absence of MgCl_2 or in the presence of 20 mM EDTA (not shown). Thus cytoplasmic lysate exhibits a cation dependent ATPase activity characteristic of members of the HSP70 family of chaperones [27].

*mtHSP70, HSP60 and HSP40 proteins bind to the last
42 nucleotides of the MHV 3' UTR*

We have previously shown by northwestern blotting and affinity chromatography that in addition to m-apo-aconitase, three other proteins with molecular masses of approximately 70, 58, and 40 kDa bind to RNA corresponding to the last 42 nucleotides of the MHV genome [28]. The presence of an ATPase activity in cytoplasmic lysates which was stimulated by the addition of m-apo-aconitase and RNA containing the 3'(+)42 protein binding element, plus the association of HSP70 family members with proteins destined to be imported into mitochondria [10], suggested to us that the 70 kDa protein might be a member of the HSP70 family. To investigate this possibility we performed a "supershift" assay with antibodies to mtHSP70 and HSP/HSC70. These two monoclonal antibodies to related HSP70 family members do not cross-react. The antibody specific for mtHSP70 produced a supershifted RNP complex (Fig. 2A, lane 4, gray arrow). Although the amount of the supershifted RNA-protein complex was relatively small, it was quite specific; antibody to the closely related HSP/HSC70 protein failed to produce a supershifted band (Fig. 2A, lane 3) as did cytoplasmic lysate in the absence of antibody (Fig. 2A, lane 2), although both contained RNA-protein complex 1 (indicated by the black arrow). The relatively small fraction of RNA-protein complexes which were supershifted suggests that an excess of mtHSP70 was present in the binding reaction over that which bound to MHV RNA in complex 1. Alternatively, complex 1 might be formed by a heterogeneous population of proteins and only those containing mtHSP70 are supershifted, or the antibody used had a relatively low affinity for mtHSP70. Labeled RNA incubated with antibody to mtHSP70 in the absence of cytoplasmic lysate did not produce any RNA-protein complexes, only small RNase T₁ digestion products were detected (Fig. 2A, lane 1). Similar experiments were attempted with an antisera to mitochondrial aconitase (kindly provided by Dr. Richard S. Eisenstein, University of Wisconsin, Madison) but were unsuccessful due to large amounts of ribonuclease present in this rabbit polyclonal antisera (data not shown). Identical

Fig. 2. mtHSP70, HSP40 and HSP60 bind to MHV 3'(+)-42 RNA. **A** Post-mitochondrial lysates were incubated with either no antibody (2), anti-mtHSP70 antibody (4), or anti-HSP70 antibody (3) for 60 min on ice. RNase protection/gel mobility shift assays with MHV RNA were then performed under standard conditions. 1 is a sample in which the assay was performed with ^{32}P labeled MHV RNA in absence of lysate but in the presence of anti-mtHSP70 antibody. The black arrow indicates the position of the RNA-protein complex formed in the absence of antibody. The gray arrow indicates the position of the supershifted band. **B** Post-mitochondrial lysates were incubated with either no antibody (1), or with anti-phosphotyrosine antibody (3). RNase protection/gel mobility shift assays with MHV RNA were then performed under standard conditions. 2 is a sample in which the assay was performed with ^{32}P labeled MHV RNA in absence of lysate but in the presence of anti-phosphotyrosine antibody. The black arrow indicates the position of the RNA-protein complex formed in the absence of antibody. The gray arrow indicates the position of the supershifted band. **C** Post-mitochondrial lysates were incubated with either no antibody (1), with anti-HSP40 antibody (3), or with anti-HSP60 antibody (4). RNase protection/gel mobility shift assays with MHV RNA were then performed under standard conditions. 2 is a sample in which the assay was performed with ^{32}P labeled MHV RNA in absence of lysate but in the presence of monoclonal antibody. The black arrow indicates the position of the RNA-protein complex formed in the absence of antibody. The gray arrow indicates the position of the supershifted band

experiments using a labeled RNA containing an 11 nucleotide mutation known to interfere with formation of the RNA-protein complex [47], as expected, failed to contain either the shifted or supershifted complexes (not shown).

mtHSP70 undergoes tyrosine phosphorylation under stress conditions [6]. It has been reported previously that infection with MHV-3 induces the tyrosine phosphorylation of cellular proteins [23]; thus it seemed logical to investigate the phosphorylation status of the mtHSP70 proteins in the RNP complex. Pre-incubation of the cytoplasmic lysate with anti-phosphotyrosine antibodies led to the presence of two supershifted RNP complexes with a corresponding decrease in the intensity of the unshifted RNP complex (Fig. 2B, lane 3). These data suggest that at least one of the proteins binding to the MHV 3'(+)-42 protein binding element is tyrosine phosphorylated, possibly mtHSP70.

HSP40 potentiates the function of the HSP70 family of protein both *in vitro* and *in vivo* [3]. This suggested to us the possibility that the 40 kDa MHV 3'(+)-42 binding protein could be HSP40. To investigate this hypothesis we performed a "supershift" assay with an antibody to HSP40. As shown in Fig. 2C, pre-incubation of crude post-mitochondrial lysate with anti-HSP40 antibody prior to our standard RNase protection/gel mobility shift assay resulted in the presence of two additional slowly migrating protected RNP complexes (lane 3) compared to our standard assay (lane 1). The result of the supershift assay suggested that the 40 kDa protein we had observed upon UV-crosslinking was HSP40. The finding that two of three RNA binding proteins which we had identified were HSP family members suggested to us that the 58 kDa protein might be HSP60. A supershift assay with anti-HSP60 produced a supershifted band, as shown in Fig. 2C, lane 4, confirming our suspicion that the 58 kDa protein was HSP60. The finding that antibodies to HSP40 and HSP60 only supershifted a relatively small portion of RNA-protein complex 1 suggests that for these two chaperones, only a small fraction of these molecules participated in the formation of MHV RNA-protein complexes. Alternatively, complex 1 might be formed by a heterogeneous population of proteins and only those containing HSP40 or HSP60 are supershifted, or the antibodies used had relatively low affinities.

To confirm the results of our gel shift analyses, we employed two additional sets of immunologic assays. Previously we showed that four proteins with molecular weights of 90 kDa (m-apo-aconitase), 70 kDa, 58 kDa and 40 kDa bound to an RNA affinity reagent corresponding to the MHV 3'(+)-42 protein binding element and were eluted with SDS-PAGE sample buffer [28]. MHV 3'(+)-42 RNA binding proteins were affinity purified in the presence of tRNA, heparin and poly(I)-poly(C) to block non-specific binding to the affinity matrix as described in Materials and Methods, resolved by SDS-PAGE and electrophoretically transferred to membranes. Immunoblot analysis of the material eluted from the specific RNA affinity matrix with anti-mtHSP70 (Fig. 3A, lane 1) or anti-HSP40 (lane 2) antibodies demonstrated immunoreactive species with molecular masses of approximately 70 kDa and 40 kDa, respectively. These experiments confirmed the identity of the 70 kDa protein as mtHSP70 and the 40 kDa protein as HSP40.

The second immunologic assay we employed was an immunoprecipitation assay after transferring label from a ³²P-labeled RNA probe containing the MHV 3'(+)-42 protein binding element to its cognate protein binding partners by UV-crosslinking. Immunoprecipitation with anti-HSP40 pulled down four proteins

Fig. 3. MHV 3'(+)-42 binding proteins are immunoreactive with anti-mtHSP70, anti-HSP40, and anti-HSP60 antibodies. **A** Proteins recognizing the MHV 3'(+)-42 protein binding element were partially purified and then subjected to affinity purification with a biotinylated RNA immobilized on magnetic beads as described in Materials and methods. Bound proteins were eluted by boiling the beads in 1× SDS-PAGE loading buffer, resolved by SDS-PAGE (4–20% gradient gel), and transferred to a nitrocellulose membrane. The membrane was probed with either anti-mtHSP70 antibody (1) or with anti-HSP40 antibody (2). The positions of molecular weight markers are indicated. **B** Post-mitochondrial lysates were incubated with 5 ng of ³²P-labeled MHV 3'(+)-42 RNA for 20 min at 22 °C and digested with limiting amounts of RNase T1 as described previously [48]. The RNA-protein complexes formed were irradiated with UV light for 30 min. The samples were digested with RNase A (20 µg/µl) and directly immunoprecipitated with anti-mtHSP70 (1) or irrelevant antibody (2) anti-HSP40 (3) or anti-HSP60 (4) and the immune-complexes were analyzed by SDS-PAGE (4–20% gradient gel) and autoradiography. 4 was overexposed to detect the signals from anti-HSP60 immunoprecipitation. The positions of the molecular weight markers are indicated to the right of the figure. The positions of immunoprecipitated proteins are indicated by arrows on the left side of the figure

with apparent molecular weights of approximately 40, 58, 70, and 90 kDa (Fig. 3B, lane 3). A parallel immunoprecipitation reaction with anti-mtHSP70 antibody produced the identical result (Fig. 2B, lane 1). An irrelevant control polyclonal rabbit antibody (Fig. 3B, lane 2) failed to precipitate any labeled proteins. A control immunoprecipitation reaction with a monoclonal antibody to the HSP/HSC70

(cytoplasmic) protein also failed to precipitate any labeled proteins (not shown). Immunoprecipitation of UV-cross linked RNP complexes with anti-HSP60 also pulled down all four proteins, although the signals were weak even after 7 days of exposure (Fig. 3B, lane 4) as opposed to a one day exposure for the other immunoprecipitation experiments. The immunoprecipitation reactions were all performed after treating the RNA-protein complex with RNase. The finding that each of these antibodies pulled down the same four proteins after extensive RNase A digestion suggests that these four proteins bound to the MHV 3'(+)-42 containing RNA as a complex rather than as four proteins binding to different regions on this RNA. This data is consistent with our previous glutaraldehyde crosslinking experiments which suggested that there were protein:protein interactions amongst all four proteins (90, 70, 58 and 40 kDa) which made up RNP complex 1 [28].

*The effect of RNA on complex formation by m-aconitase,
mtHSP70, HSP60 and HSP40*

A multi-protein RNP complex can form either by binding of a pre-formed multi-protein complex to an RNA, or multiple proteins may recognize the RNA separately, and only establish protein:protein contacts after they have bound to the RNA. In order to clarify the situation we immunoprecipitated RNP complexes formed in UV-crosslinking reactions using unlabeled RNA containing the MHV 3'(+)-42 protein binding element. These immunoprecipitates were compared to immunoprecipitates prepared in parallel from partially purified cytoplasmic lysate in the absence of RNA. Both sets of immunoprecipitates were subjected to immunoblot analysis. When we used anti-mtHSP70 or anti-HSP60 to pull down the RNP complex or lysate in the absence of added RNA, and blotted the immunoprecipitated material separated by SDS-PAGE with anti-HSP40, HSP40 could be detected in both the cases, whereas samples immunoprecipitated with non-specific antibody did not contain HSP40 (Fig. 4A). However, the signal for HSP40 in immunoprecipitates with anti-HSP60 was considerably augmented by the addition of RNA. When the immunoprecipitation experiments were carried out with anti-mtHSP70, anti-HSP60, or anti-HSP40, and the immunoprecipitated materials were blotted and probed with anti-m-aconitase, m-aconitase was detected irrespective of the presence of RNA, but was not detected in immunoprecipitates with non-specific antibody (Fig. 4B). However, the signals for m-aconitase were greatly increased by the presence of RNA in the immunoprecipitation reactions with anti-HSP40 and anti-HSP60. Thus the association of HSP40 with HSP60 and with m-aconitase is stimulated by the presence of MHV (+)-42 containing RNA. These data suggest that these proteins are recruited to an RNA-protein complex.

*Complexes of HSP40, mtHSP70, and HSP60 with MHV RNA
are present in MHV infected cells*

To search for the presence of specific MHV RNA-protein complexes in cells rather than formed after the in vitro addition of synthetic RNA probes we performed a series of immunoprecipitation-RT-PCR assays. MHV RNA-protein complexes

Fig. 4. Co-immunoprecipitation of HSPs is increased by the addition of RNA. Partially purified lysates were incubated with, indicated by (+), or without, indicated by (-), cold MHV 3'(+)/42 RNA (5 ng) for 20 min at 22 °C and digested with RNase. The RNA-protein complexes formed were UV-crosslinked, digested with RNase A (20 µg/µl), and directly immunoprecipitated with irrelevant antibody as well as various specific antibodies, and the immune-complexes were separated by SDS-PAGE (4–20% gradient gel) and transferred to nitrocellulose membranes. **A** RNA-protein complexes formed were immunoprecipitated either with anti-mtHSP70, anti-HSP60, or unrelated antibodies. The membranes were then probed with an anti-HSP40 antibody. **B** RNA-protein complexes formed were immunoprecipitated either with anti-mtHSP70, anti-HSP40, anti-HSP60, or unrelated antibodies. The membranes were then probed with anti-m-aconitase antibodies

Fig. 5. Mitochondrial HSP70, HSP60 and HSP40 specifically bind to MHV-JHM RNA. RNA-protein complexes were immunoprecipitated from mock infected and MHV-JHM infected 17Cl-1 cell post-mitochondrial lysates with various monoclonal antibodies bound to protein A bound to magnetic beads and amplified by RT-PCR. **A** RT-PCR was performed with primers directed against the MHV-JHM genome 3' untranslated region. PCR products were resolved by electrophoresis in a 1% agarose gel. 1, 100 bp DNA ladder; 2, positive control for PCR using DIssE cDNA as a template; 3, no cDNA negative control for PCR. PCR products obtained from immunoprecipitates of MHV-JHM infected post-mitochondrial lysates with monoclonal antibodies directed against SV40 T Ag (4), T7 Tag (5), MHV-JHM nucleocapsid (7), mtHSP70 (8), HSP60 (9), and HSP40 (10). 6 contains the PCR reaction from the immunoprecipitate of mock infected post-mitochondrial lysate with anti-MHV-JHM nucleocapsid antibody. **B** 1 contains a 100 bp DNA ladder. 2 and 3 are positive controls for PCR with DIssE cDNA and GAPDH cDNA templates, respectively. 4 and 5 are analyses of samples immunoprecipitated with anti-mtHSP70; 6 and 7 are analyses of samples immunoprecipitated with anti-HSP 60; 8 and 9 are analyses of samples immunoprecipitated with anti-HSP 40. RT-PCR was performed with primers directed against the MHV-JHM genome 3' untranslated region (4, 6, 8) or with primers for the abundant mRNA for GAPDH (5, 7, 9). PCR products were resolved by electrophoresis in a 1.5% agarose gel

were immunoprecipitated from post-mitochondrial lysates prepared from mock infected and MHV-JHM infected 17Cl-1 cells with various monoclonal antibodies and amplified by RT-PCR with primers directed against the 3' untranslated region of the MHV genome. A monoclonal antibody to the MHV nucleocapsid (N) protein, a protein which is known to bind to MHV RNAs, was used as a positive control. Antibodies to mtHSP70, HSP40, and HSP60 all co-precipitated MHV RNA which could be amplified by RT-PCR (Fig. 5A, lanes 8–10). The assay appeared to be specific in that monoclonal antibodies to irrelevant antigens (lanes 4 and 5) did not bring down complexes containing MHV RNA. To further investigate the specificity of this assay we performed a second set of immunoprecipitation

reactions and attempted to detect mRNA for the highly expressed house keeping gene GAPDH as well as for MHV specific RNAs (Fig. 5B). Antibodies to mtHSP70, HSP60, and HSP40 captured MHV RNA (lanes 4, 6, 8), but failed to precipitate GAPDH mRNA (lanes 5, 7, 9). The relative amount of MHV RNA captured by the three anti-HSP antibodies in this experiment correlates well with the immunoprecipitation UV-crosslinking experiment shown in Fig. 3B. Thus we have now demonstrated that mtHSP70, HSP60, and HSP40 bind to MHV RNAs present in infected cells.

Discussion

The untranslated regions of most positive stranded RNA viruses interact with host factors during their replication. We have previously shown that m-aconitase binds specifically to the last 42 nucleotides of the 3' UTR of MHV RNA along with three other proteins with molecular weights of approximately 70 kDa, 58 kDa and 40 kDa to form an RNP complex [28]. In this study, we have used supershift assays and western blot assays of RNA affinity purified protein to identify the three additional components of this RNA-protein complex as mtHSP70, HSP60, and HSP40. We have also demonstrated this four protein – RNA complex in post-mitochondrial extracts of MHV infected murine cells by immunoprecipitation of RNA-protein complexes followed by RT-PCR assays.

Both m-aconitase and mtHSP70 are nuclear encoded, possess mitochondrial targeting sequences, and are thought to translocate into mitochondria shortly after synthesis. mtHSP70 plays an important role in importation of proteins into the mitochondrial matrix [10]. However mtHSP70 has been documented to be present in extra-mitochondrial locations by biochemical fractionation, immunofluorescent labeling and confocal microscopy, and immunoelectron microscopy [32, 35, 38]. This protein is also known as a glucose response protein (GRP75); a senescence-related gene product, mortalin; and as a peptide binding protein, PBP74. This compendium of names reflects the different intracellular compartments and functions which have been described for this protein in addition to its role in refolding and import of mitochondrial proteins. Mammalian HSP40s have a predominantly cytoplasmic distribution in the cell [31]. Although the functional HSP60 complex is predominantly localized to mitochondria in eukaryotes [8], detailed immunoelectron microscopy studies in a wide variety of cells and tissues show that 15–20% of HSP60 immunoreactivity is present at discrete extra-mitochondrial sites, including unidentified cytoplasmic vesicles [8, 37, 38] and secretory granules [45]. MHV replicates in the cytoplasm, making it unlikely that MHV interacts with these proteins inside mitochondria. We believe that it is most likely that MHV RNA interacts with m-aconitase prior to its import into mitochondria in cooperation with extra-mitochondrial mtHSP70, HSP60, and HSP40. The alternative scenario which would allow this interaction to occur, requires export of at least m-aconitase from mitochondria.

HSP40, HSP60, and mtHSP70 are all molecular chaperones. The folding of most newly synthesized proteins in the cell requires the interaction of a variety

of protein cofactors known as molecular chaperones. These molecules have been identified in most cellular compartments of different organisms, and recognize and bind to nascent polypeptide chains and partially folded intermediates of proteins, preventing their aggregation and misfolding [reviewed in [5]]. There are several families of chaperones; those most involved in protein folding are the 40-kDa heat shock protein (HSP40; DnaJ), 60-kDa heat shock protein (HSP60; GroEL), and 70-kDa heat shock protein (HSP70; DnaK) families. HSP70 family members are regulated by HSP40 (DnaJ or its homologs). These essential and ubiquitous partner proteins make up the DnaJ family, with HSP40 (hdj-1) [30] and Hsdj (hdj-2) [29] being the best studied human homologs. They function to increase ATP turnover, facilitate chaperone action, and promote a more stable interaction with protein substrates [17, 25]. The ATPase activity we detected in partially purified lysates during RNA binding reactions is consistent with the known properties of HSP70's ATPase activity. It also suggests the possibility that mtHSP70 is interacting with a DnaJ domain containing partner, such as HSP40 during RNA binding reactions.

Gel supershift assays with anti-phosphotyrosine antibodies (Fig. 2B) demonstrate that at least one of the proteins in the complex formed with MHV 3'(+)-42 containing RNAs and m-aconitase, mtHSP70, HSP60, and HSP40 is tyrosine phosphorylated. Although m-aconitase, HSP40, and mtHSP70 contain predicted potential tyrosine phosphorylation sites, only mtHSP70 has been experimentally demonstrated to undergo tyrosine phosphorylation [6, 26]. Thus mtHSP70 is the protein most likely to be responsible for the supershift we observed with anti-phosphotyrosine antibody. Immunoprecipitation/western blot assays with anti-mtHSP70 and anti-phosphotyrosine antibodies indicate that a portion of the mtHSP70 is tyrosine phosphorylated, at least in mouse fibroblasts which contain MHV 3'(+)-42 RNA binding activity (Nanda and Leibowitz, unpublished). The phosphorylation/dephosphorylation status of many RNA binding proteins plays a major role in terms of their interaction with RNA. Enzymatic treatment of crude lysates with alkaline phosphatases greatly inhibits their RNA binding activity (Nanda and Leibowitz, unpublished). This marked decrease in RNA binding activity after dephosphorylation of the lysate argues in favor of an important role for mtHSP70 tyrosine phosphorylation in the formation of the RNP complex.

Previously published Northwestern blot data [28] suggest that all four of the proteins are individually able to interact with MHV 3'(+)-42 containing RNA. A series of co-immunoprecipitation assays (Fig. 4) have established that these four proteins are associated, even in the absence of RNA. However, the data clearly indicated that the presence of RNA enhanced the association of HSP60 with HSP40 and with m-aconitase. The addition of RNA also enhanced the association of m-aconitase with HSP40, but did not affect the association of mtHSP70 with HSP40 or m-aconitase. One possible interpretation of these results is that those associations which we observed to be RNA independent are due to the known associations of these proteins with each other (mtHSP70 with m-aconitase, HSP70s with HSP40), whereas the remainder are dependent on RNA and the formation of an RNA-protein complex. Our data do not distinguish between two possibilities, formation of this four protein RNP complex from the separate

proteins as opposed to RNA binding to a pre-existing four protein complex. In either case our results clearly indicate that these four proteins form a complex with MHV-JHM 3'(+)-42 containing RNA.

We have established here that m-aconitase, mtHSP70, HSP60 and HSP40 interact with each other as well as with MHV RNA. The nature of the interaction between HSP40 and HSP70 is complex. The interaction between the J domain and the HSP70 ATPase domain is well established. An interaction between HSP40 and the peptide-binding domain of HSP70 also occurs *in vitro*. Other HSP70 family members have been shown to bind to RNA substrates and may guide the appropriate folding of these RNAs and affect subsequent regulatory processes such as mRNA stability and/or translation [7, 33, 49]. Thus it is quite possible that HSP40 potentiates the activity of mtHSP70 and HSP60, and thus stabilizes the interaction of m-aconitase with the RNA. It is quite interesting that Tomita and colleagues have recently reported that a yeast HSP40 family member, Ydj1p, is involved in forming brome mosaic virus replication complexes active in negative-strand RNA synthesis, and suggested that a chaperone system involving Ydj1p participates in viral replicase folding or assembly into the active replication complex [41].

It has recently been demonstrated that GroEL, the *E. coli* Hsp60 homolog, can associate with lipid membranes while remaining functional as a protein folding chaperone [42]. Replicating MHV RNA has been localized to early endosomal vesicles [44]. It is possible that the association of HSP60 with the viral RNA may contribute to its intracellular localization. This could occur if the interaction of HSP60 (likely in association with the other three proteins which make up the RNA-protein complex we have identified) with the RNA assists the intracellular translocation of RNA-protein complexes [24]. GroEL has also been suggested to be part of a protein complex that protects bacterial transcripts from RNase E-mediated degradation [5, 36].

Molecular chaperones rarely function alone; rather they function together in complex pathways [9]. In mammalian cells, components of the mitochondria, namely GRP75 (mtHSP70) and HSP58, a homolog of bacterial groEL, were found to associate with each other and transiently interact with newly synthesized mitochondrial proteins [27]. Only a few partnerships between various HSP40s and HSP70s have been elucidated. The most well-studied chaperone partnership is one found in *E. coli* between DnaK, a chaperone of the HSP70 class, and DnaJ, an HSP40. Both genetic and biochemical evidence indicates a functional partnership between these two chaperones [1, 20, 46]. However, a chaperone complex containing mtHSP70, HSP60 and HSP40 along with m-aconitase has not been reported previously. We have provided evidence for the existence of such a complex and for its interaction with the 3' end region of MHV RNA. The precise functional significance of this interaction for MHV replication is still unclear, although our previous work is consistent with its having an enhancing effect on viral RNA stability [28]. It is clear that a single HSP70 protein may interact with more than one HSP40 family member [31]. Our knowledge of the interactions between the various chaperones themselves, as well as with newly synthesized

proteins or other chaperone target proteins is incomplete. It will be interesting to see how modulating the expression of these HSPs affects MHV replication.

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