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Heterogeneous nuclear ribonuclear protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA

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Abstract

Alphaviruses are a group of arthropod-borne human and animal pathogens that can cause epidemics of significant public health and economic consequence. Alphavirus RNA synthesis requires four virally encoded nonstructural proteins and probably a number of cellular proteins. Using comparative two-dimensional electrophoresis we were able to identify proteins enriched in cytoplasmic membrane fractions containing viral RNA synthetic complexes following infection with Sindbis virus. Our studies demonstrated the following: (i) the host protein hnRNP K is enriched in cytoplasmic membrane fractions following Sindbis virus infection, (ii) viral nonstructural proteins co-immunoprecipitate with hnRNP K, (iii) nsP2 and hnRNP K co-localize in the cytoplasm of Sindbis virus infected cells, (iv) Sindbis virus subgenomic mRNA, but not genomic RNA co-immunoprecipitates with hnRNP K, (v) viral RNA does not appear to be required for the interaction of hnRNP K with the nonstructural proteins. Potential functions of hnRNP K during virus replication are discussed.

Keywords: hnRNP K; Sindbis virus; Nonstructural proteins; Subgenomic mRNA

Introduction

Alphaviruses are a diverse group of arthropod-borne human and animal pathogens united by a common scheme of replication. There are approximately 30 species of alphaviruses distributed throughout the world (Griffin, 2001). Sindbis virus (SIN) is the type species and one of the best-studied members of the genus. Detailed molecular and genetic approaches have been used to understand the fundamental processes underlying replication, and, due to commonalities in replication scheme, results from such studies have proven largely applicable to other members of the genus (Strauss and Strauss, 1994).

The SIN genome is 11,704 nt in size and is capped at the 5′ end and polyadenylated at the 3′ end thus mimicking a cellular mRNA (Strauss and Strauss, 1994). Following deposition into the host cell cytosol the genome is translated by host cell machinery resulting in the production of two nonstructural polyprotein species P123 and P1234 (Strauss et al., 1983, 1984). P1234, produced as a consequence of translational readthrough of an opal codon at the end of the nsP3 coding sequence, is proteolytically processed to P123 and nsP4 by the nsP2 associated proteinase activity (de Groot et al., 1990; Ding and Schlesinger, 1989; Hardy and Strauss, 1988, 1989; Li and Rice, 1989, 1993). NsP4 possesses RNA-dependent RNA polymerase (RdRp) activity and in complex with P123 recognizes genomic RNA and generates a complementary copy (minus-strand) (Lemm et al., 1994, 1998; Shirako and Strauss, 1994). Further processing of P123 to nsP1 and P23 yields a complex capable of genomic RNA synthesis. Finally complete processing of the nonstructural polyprotein to nsP1, nsP2, nsP3 and nsP4 creates a complex capable of genomic and subgenomic RNA synthesis (Lemm et al., 1994; Li and Stollar, 2007; Shirako and Strauss, 1994). Translation of the subgenomic mRNA in an eIF4F independent fashion gives rise to the structural proteins which assemble with genomic RNA to form new infectious virions (Castello et al., 2006).

In addition to regulation of RNA synthesis by *trans*-acting factors the production of each of the three major alphavirus RNA species requires a specific promoter element. Analysis of alphavirus genome sequences has identified four sequence elements conserved across the genus (CSE); the 5′ untranslated

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region (UTR), a 51 nt CSE, the subgenomic promoter (or junction) region, and the 3′ CSE (Ou et al., 1981, 1982, 1983). The 5′ 44 nt in the untranslated region (UTR) of the genome are predicted to form a stable stem-loop structure just upstream of another stem-loop containing the translational start site which is followed by a second conserved element that is also predicted to form two stem-loop structures (51 nt CSE). The sequence of the 5′ region of the genome is not conserved across the alphavirus genus, but the presence of four stem-loop structures in this region is conserved. Alteration of the 5′ 44 nt of the SIN genome inhibited minus-strand and plus-strand RNA synthesis demonstrating the critical importance of this region in genome replication (Frolov et al., 2001). The junction region at the end the nsP4 coding sequence contains the promoter for subgenomic RNA synthesis (Levis et al., 1990). *In vitro* studies have demonstrated that in the presence of the other nonstructural proteins nsP4 binds to a 24 nucleotide CSE in this region and that this CSE constitutes the core promoter for subgenomic mRNA transcription (Li and Stollar, 2004, 2005). The terminal 19 nt in the 3′ UTR (3′CSE) of the genome and the 3′ terminal polyadenylate tract are critical components of the promoter for minus strand RNA synthesis (Hardy, 2006; Hardy and Rice, 2005; Kuhn et al., 1990; Raju et al., 1999).

While the viral components of the RNA synthetic complex have been well characterized, the host cell proteins required for viral RNA synthesis remain uncharacterized. Genetic analyses have demonstrated that specific changes in the CSEs described above manifest host specific phenotypes (Fayzulin and Frolov, 2004; Gorchakov et al., 2004; Kuhn et al., 1992; Kuhn et al., 1990; Niesters and Strauss, 1990a, 1990b). The implication is that factors in vertebrate cells and mosquito cells interact with viral RNA in different ways resulting in host specific effects of mutations in the viral genome. However, to date the only characterized interaction between a host-protein and viral RNA is that between the mosquito La autoantigen and the 3′ end of the alphavirus minus-strand (Pardigon et al., 1993; Pardigon and Strauss, 1996).

Recent work by Frolova et al. and Cristea et al. has taken a broad approach to the identification of host proteins required for alphavirus replication (Cristea et al., 2006; Frolova et al., 2006). Using subcellular fractionation of infected cells followed by immuno-isolation of nonstructural protein containing complexes these researchers identified host proteins associated with complexes containing viral nsP3. These complexes contain numerous host cell proteins including cytoskeleton proteins, chaperones, heterogeneous nuclear ribonuclear proteins, and ribosomal subunits. A similar, but not identical spectrum of host proteins was obtained for nsP2-containing complexes (Atasheva et al., 2007). Whether subsets of this identified protein pool associate with nsP2 and nsP3 to form multiple, discrete complexes with distinct functions remains unclear. Additionally the involvement of different viral RNA species in the formation of complexes containing host and viral proteins has not been determined.

In this study we have taken an unbiased approach to analyze the components of cellular fractions containing viral RNA synthetic complexes. Rather than targeting viral proteins we analyzed proteins whose abundance changed in cytoplasmic membrane fractions following SIN infection. By this method we identified heterogeneous nuclear ribonuclear protein K (hnRNP K) as enriched in cytoplasmic membrane fractions from infected cells. Analysis of interacting partners revealed that nsP1, nsP2 and nsP3 co-immunoprecipitated with hnRNP K and hnRNP K co-localized with nsP2 in infected cells. Additionally subgenomic mRNA, but not genomic RNA co-precipitated with hnRNP K suggesting that hnRNP K is associated with the SIN transcriptase complex. Finally siRNA induced knockdown of hnRNP K infection correlated with a reduction in the number of cells in which reporter gene expression from a viral subgenomic promoter was detected indicating hnRNP K has a role, as yet undetermined, during SIN infection.

Results

Identification of hnRNP K in membrane fractions of infected cells

In order to obtain subcellular fractions active for SIN RNA synthesis HeLa cells were infected with SIN expressing GFP (Fig. 1A) at a multiplicity of 100 pfu/cell. Cells were harvested into hypotonic buffer 6 h post-infection and homogenized. Nuclei were removed by low speed centrifugation and the resulting supernatant was centrifuged at high speed to pellet

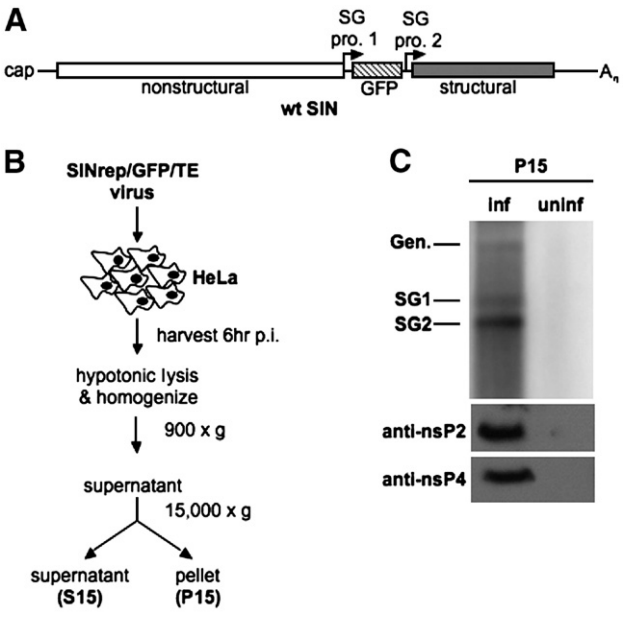

Fig. 1. Isolation and analysis of cytoplasmic membrane fractions from SIN infected and uninfected HeLa cells. (A) Structure of the wt SIN genome capable of GFP expression. (B) Schematic representation of the cytoplasmic membrane isolation procedure described in Materials and methods. (C) RNA synthetic activity of the isolated membrane fractions and Western blot analysis. Fractions were incubated with template in the presence of [α-^{32}P]-CTP, unlabeled ATP, GTP, and UTP, and actinomycin D. Denatured products were separated on a 1.5% agarose-phosphate gel and visualized by phosphorimaging. P15 fractions from SIN infected and uninfected cells were analyzed by Western blot using antisera to nsP2 (upper panel) and nsP4 (lower panel).

cytoplasmic membranes (P15, Fig. 1B). HeLa cells rather than BHK-21 cells were used initially to facilitate the proteomic analyses that were consequently performed on the isolated membrane fractions. The SIN expressing GFP was used to monitor the percentage of HeLa cells that were infected. Reports vary as to the susceptibility and permissivity of HeLa cells to SIN infection. By flow cytometry we observed approximately 50% of cells to be infected under the conditions used (data not shown).

P15 fractions were tested for SIN RNA synthetic activity (Fig. 1C). No RNA synthetic activity was observed in P15 fractions isolated from uninfected cells. However [^{32}P]-αCTP labeled products were observed from P15 fractions from infected cells. Three products corresponding to the genomic RNA, a subgenomic RNA encoding the reporter gene GFP (SG1), and a subgenomic RNA encoding the viral structural proteins (SG2) were detected. Western blot analysis demonstrated fractions from infected cells contained the viral proteins nsP2 and nsP4, essential components of the viral RNA synthetic complex.

To further understand the composition of the viral RNA synthetic complex and identify host proteins that were enriched or lost from cytoplasmic membranes in infected cells we performed two-dimensional difference in gel electrophoresis (2D-DIGE) of P15 fractions from infected and uninfected cells. Proteins in each fraction were labeled with a specific fluorophore (Cy5 uninfected, Cy3 infected, Fig. 2). Equal quantities of total protein were mixed and separated in two dimensions using isoelectric focusing followed by SDS-PAGE. Proteins labeled with Cy5 and Cy3 were detected at the appropriate wavelengths using a Typhoon imaging system (Amersham) and the images were overlaid. A number of proteins were present in one sample but not the other. The gel was stained and spots corresponding to the differentially present proteins were isolated and analyzed by liquid chromatography-tandem mass spectrometry following tryptic digest. Proteins were identified by MASCOT analysis of the peptide spectrum. Of the proteins identified by this method most were stress response or cytoskeletal proteins, however two spots from the infected sample were identified as heterogeneous nuclear ribonucleoprotein K (hnRNP K, spots are circled in the highlight box in Fig. 2). HnRNP K is a poly(C)-binding protein involved in a number of processes that regulate mammalian gene expression (Bomsztyk et al., 2004). The two observed forms of hnRNP K separated in the first dimension but migrated together in the second, and are thought to differ in their phosphorylation state. Interestingly, while a number of members of the hnRNP family of proteins have been identified as associating with virus nonstructural protein containing complexes in other studies, hnRNP K was not one of them (Cristea et al., 2006; Frolova et al., 2006).

Enrichment of hnRNP K in the cytoplasmic membrane fraction during virus infection was confirmed by Western blot analysis of subcellular fractions from infected and uninfected HeLa and BHK-21 cells (Fig. 3). Levels of hnRNP K in the nuclear fraction from infected cells are reduced compared to mock infected, whereas infection results in an increase in hnRNP K in P15 (cytoplasmic membrane) fractions. These

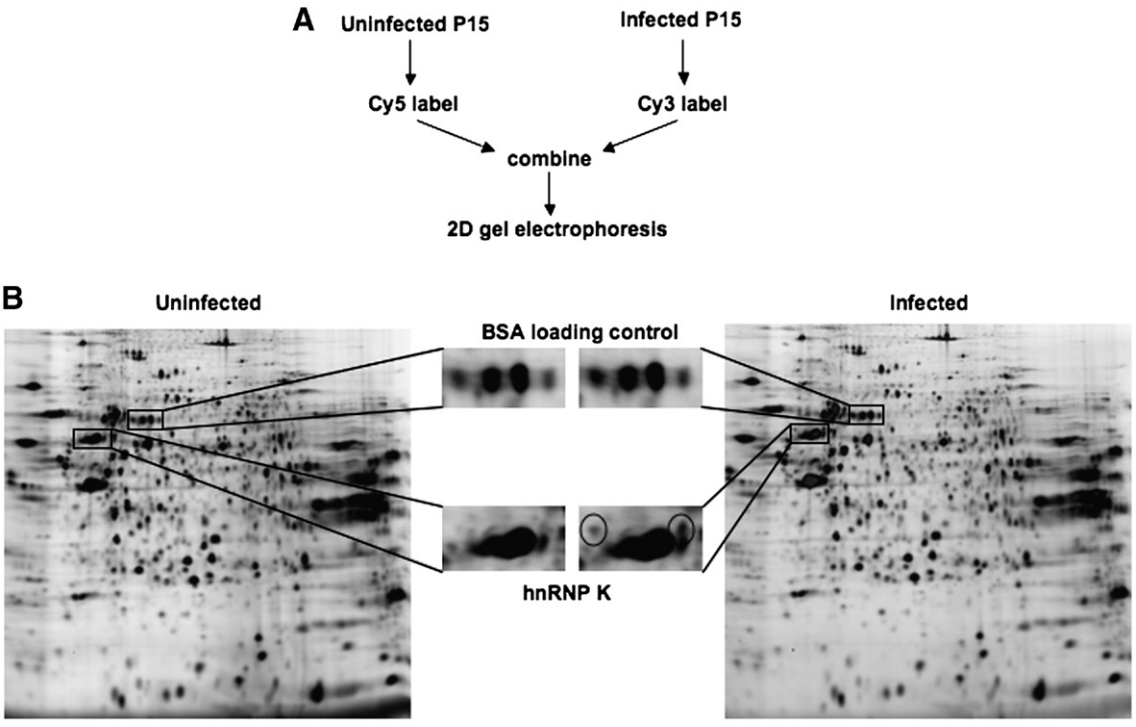

Fig. 2. Two-dimensional difference in-gel electrophoretic (2D-DIGE) analysis of infected and uninfected membrane fractions. (A) Schematic representation of 2D-DIGE procedure outlined in Materials and methods. (B) Detection of Cy5 labeled proteins (uninfected) and Cy3 labeled proteins (infected) in a single gel. Highlight boxes show the BSA loading control for each channel, and the protein spots in the infected sample identified as hnRNP K by liquid chromatography tandem mass spectrometry.

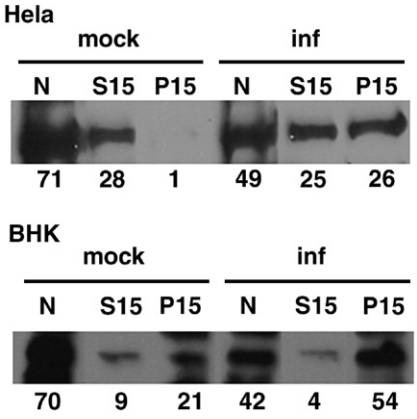

Fig. 3. Re-localization of hnRNP K in infected cells. Nuclear (N), soluble cytoplasmic (S15), and cytoplasmic membrane (P15) fractions from mock infected and SIN infected HeLa and BHK-21 cells were analyzed for the presence of hnRNP K by Western blot using rabbit polyclonal antiserum against human hnRNP K. Protein was quantitated using ImageJ software (NIH) and the percentage of hnRNP K in each fraction is shown below the relevant lane.

data indicate that hnRNP K is being relocalized from the nucleus to the cytoplasm or retained in the cytoplasm during SIN infection. No increase in P15 hnRNP K levels was found in VSV infected cells (data not shown).

hnRNP K interacts with components of the viral RNA synthetic machinery

The data from subcellular fractionation indicated that hnRNP K relocalized to cytoplasmic membranes during SIN infection and suggested that it may be interacting with the membrane bound SIN RNA synthetic complex. To examine this possibility we performed immunoprecipitation assays. Lysates were generated from infected (6 h post-infection) and uninfected BHK-21 cells in which proteins had been labeled using [^{35}S]-methionine from 4-6 h post-infection. Nuclei were removed by centrifugation and proteins in the supernatants were immunoprecipitated using mouse monoclonal anti-hnRNP K (Fig. 4A, lanes 1 and 2) or rabbit polyclonal anti-nsP2 serum (Fig. 4A, lane 3). Immunoprecipitiation of hnRNP K from infected cell lysates led to the co-precipitation of nsP1, nsP2, and nsP3. The nsP3 protein band may be comprised of phosphorylated nsP3 (nsP3b) and/or unphosphorylated (nsP3a) (Li et al., 1990). The protein labeled with an asterisk consistently co-precipitated with hnRNP K and nsP2, but its identity is currently unknown. We were unable to detect nsP4, however this was probably due to the low abundance of this protein during virus infection.

It has been previously reported that hnRNP K is differentially phosphorylated (reviewed in (Bomsztyk et al., 2004), and the observation of two forms of hnRNP K separating in the first dimension in Fig. 2 suggested that they may be a result of differential phosphorylation. Immunoprecipitation of proteins from cells labeled with [^{32}P]-orthophosphate demonstrated that a subset of hnRNP K was phosphorylated in both infected and uninfected cells. It also appears that the phosphorylated form(s) of hnRNP K are those interacting with the viral nonstructural proteins. Additionally a protein corresponding to a phosphorylated form of nsP3 (nsP3b) was seen to co-precipitate with both hnRNP K and nsP2 (Fig. 4B, lanes 1 and 3). While we were unable to readily observe [^{35}S]-labeled hnRNP K co-precipitating with nsP2 using anti-nsP2 serum (Fig. 4A, lane 3), we were able to observe co-precipitation of [^{32}P]-labeled hnRNP K with nsP2 (Fig. 4B, lane 3). This result provided reciprocal confirmation of the interaction between nsP2 and hnRNP K, and suggested that phosphorylated hnRNP K may be the predominant form within the nsP-containing complex.

hnRNP K co-localizes with nsP2 in SIN infected cells

Co-immunoprecipitation experiments demonstrated that hnRNP K interacted with nonstructural protein complexes. Immunofluorescent microscopy was performed to confirm that hnRNP K and nsP2 co-localized during viral infection. Infected cells were fixed 6 h post-infection and stained with both mouse monoclonal anti-hnRNP K serum and rabbit polyclonal anti-nsP2 serum. Cells were washed and treated with FITC conjugated anti-mouse and TRITC conjugated anti-rabbit secondary antibodies. Comparison of the infected and uninfected cells showed hnRNP K formed punctate foci in the cytoplasm of infected cells (Fig. 5, panels A and D). Immunofluorescence also showed that nsP2 has a similar distribution (panel B), and when the images were merged colocalization of the two proteins was apparent (panel C). In combination with immunoprecipitation these data strongly suggest that hnRNP K is interacting with viral components of the viral RNA synthetic machinery. While the immunofluorescence analysis was not intended to be truly quantitative there does appear to be a decrease in the amount of hnRNP K in the nucleus of infected cells (compare panel A and D of Fig. 5). It is also interesting to note that the pattern of hnRNP

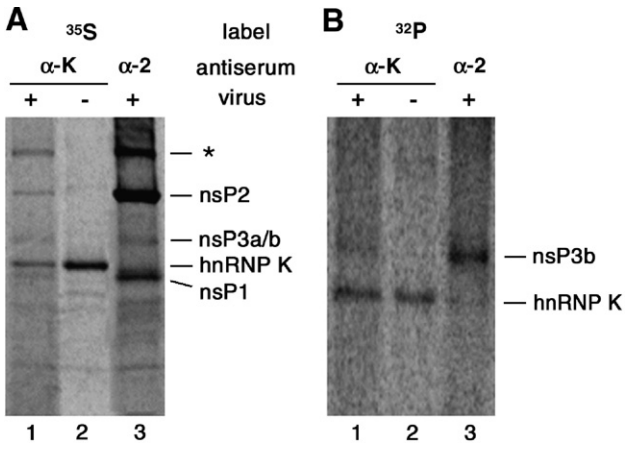

Fig. 4. Co-immunoprecipitation of viral nonstructural proteins with hnRNP K. BHK-21 cells were infected with a multiplicity of 10 pfu/cell and labeled with (A) [^{35}S]-methionine and cysteine or (B) [^{32}P]-orthophosphate for 2 h at 4 h post-infection. Cytoplasmic extracts were prepared, and proteins were immunprecipitated with hnRNP K-specific monoclonal antibody (α-K) or nsP2-specific polyclonal antiserum (α-2). Proteins from uninfected and infected cells were analyzed by SDS-PAGE and visualized by phosphorimagery.

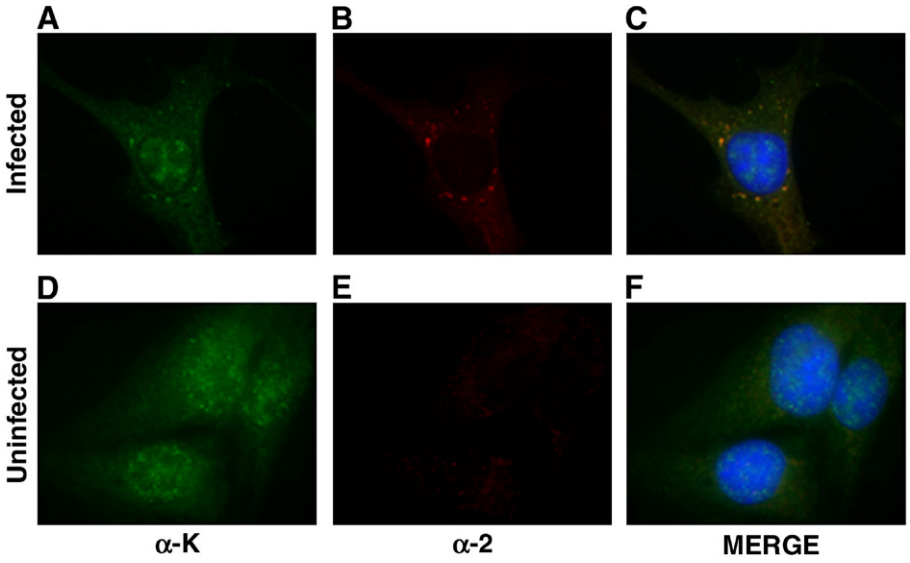

Fig. 5. Co-localization of hnRNP K with nsP2 in SIN infected cells. SIN infected and uninfected BHK-21 cells were permeabilized with methanol 6 h post-infection and dual stained with mouse anti-hnRNP K monoclonal antibody (α-K), and rabbit anti-nsP2 polyclonal serum (α-2). Cells were treated with goat anti-mouse FITC (green) and goat anti-rabbit TRITC (red) secondary antibodies. Cell nuclei were stained with DAPI (blue).

K staining in the nucleus consistently appeared more condensed in infected cells, suggesting a rearrangement of the protein in the nucleus as well as the cytoplasm following SIN infection.

hnRNP K interacts with the SIN subgenomic mRNA

Given the interaction and colocalization of hnRNP K with viral nonstructural proteins we wanted to determine the role of viral RNA in the formation of the protein complex. BHK-21 cells were infected with SIN (Toto1101) using a multiplicity of 10 pfu/cell. RNA in infected cells was labeled with [^{3}H]-uridine in the presence of actinomycin D. Cells were lysed and protein-RNA complexes were immunoprecipitated with antiserum to hnRNP K, nsP2 or six-histidines (his). RNA was isolated from immunoprecipitated complexes by phenol extraction. Fig. 6A shows an agarose-phosphate gel of RNA isolated from the immunoprecipitations. As expected no RNA was precipitated with the anti-his antiserum (Fig. 6A, lanes 5 and 6), however immunoprecipitation of hnRNP K co-precipitated SIN subgenomic mRNA (Fig. 6A, lane 2), but not genomic RNA. In contrast immunoprecipitation of nsP2 co-precipitated SIN genomic and subgenomic RNA (Fig. 6A, lane 4). This demonstrated that hnRNP K is a component of the ribonuclear protein complex containing the SIN subgenomic mRNA, and given the observed interaction with nsP1, nsP2 and nsP3, may interact with the viral transcriptase complex.

To determine if the interaction of hnRNP K with the viral nonstructural proteins was direct or mediated through the subgenomic mRNA immunoprecipitations of hnRNP K-containing complexes were performed following exposure of the cell lysates to RNase T1. Data in Fig. 6B demonstrates that

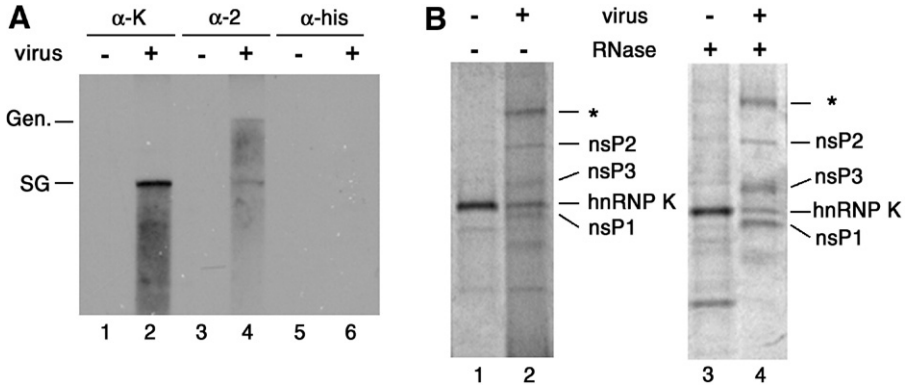

Fig. 6. Interaction of hnRNP K with the vRNP containing SIN subgenomic mRNA. (A) [^{3}H]-uridine labeled RNA from uninfected cells and SIN infected was immunoprecipitated using anti-hnRNP K monoclonal antibody (α-K), anti-nsP2 polyclonal antibody (α-2), or anti-his polyclonal serum (α-his). RNA was isolated from immunoprecipitated complexes by phenol/chloroform extraction. Ethanol precipitated RNA was separated by agarose-phosphate gel electrophoresis and visualized by fluorography. (B) [^{35}S]-labeled cytoplasmic extracts from infected and uninfected cells were mock treated (lanes 1 and 2), or incubated for 1 h at 30 °C in the presence of 10 u/μl RNase T1 (lanes 3 and 4). Proteins were then immunoprecipitated with hnRNP K specific monoclonal antibody, analyzed by SDS-PAGE and visualized by phosphorimagery.

digestion of RNA does not prevent co-precipitation of the nonstructural proteins with hnRNP K indicating that the interaction with nonstructural proteins was direct and not mediated through the subgenomic mRNA.

hnRNP K functions during SIN replication

HeLa cells were transfected with siRNA directed against human hnRNP K or firefly luciferase (negative control). Cells were treated for 72 h and the level of hnRNP K expression was determined by Western blot analysis. Cells treated with hnRNP K specific siRNA showed approximately a 50% decrease in hnRNP K expression compared to cells treated with a control luciferase siRNA (Fig. 7A).

Cells treated with siRNA for 72 h were infected with SIN engineered to express GFP from a second subgenomic mRNA. Knock-down of hnRNP K expression consistently resulted in a decrease in the number of GFP positive cells as measured by flow cytometry 6 h post-infection when compared to infection of cells treated with control siRNA to firefly lucifrase (Fig. 7B). This experiment was performed using triplicate samples on two separate occasions. Analysis of this decrease in GFP positive cells showed it to be significant (*T*-test p-value <0.05) indicating that reduction of hnRNP K levels does affect SIN infection although the specific requirement for hnRNP K during virus replication is not yet understood. Given the results presented above that suggest an interaction between hnRNP K and the transcriptase complex, the decrease in GFP positive cells may be a consequence of disrupting subgenomic mRNA transcription or translation.

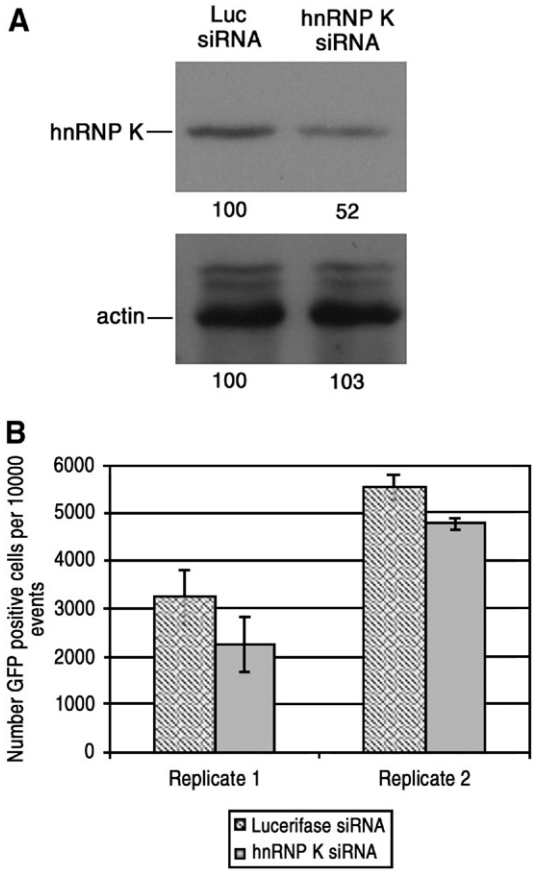

Fig. 7. Reduction of hnRNP K expression in HeLa cells by siRNA treatment and the effect on SIN infection. (A) Cells were treated for 72 h with siRNA as described in Materials and methods. Following treatment cells were harvested and hnRNP K expression analyzed by Western blot using a monoclonal antibody to hnRNP K. Protein was quantitated using ImageJ software (NIH). Actin was used as a loading control. (B) Cells were infected with SIN expressing GFP from a subgenomic promoter following 72 h of treatment with siRNA targeted against hnRNP K or firefly luciferase (negative control). Six hours post-infection cells were trypsinized and the number of GFP-positive cells was determined by flow cytometry. This experiment was performed twice (replicates 1 and 2) using triplicate samples for each experiment. Results of flow cytometry are presented graphically.

Discussion

Identifying host factors that associate with viral molecules to regulate the processes required for virus replication remains a major goal of modern virological study. Recent studies have attempted to identify host factors associated with the SIN RNA synthetic complex by isolating nonstructural protein-containing complexes from infected cells and identifying the components, both viral and cellular (Atasheva et al., 2007; Cristea et al., 2006; Frolova et al., 2006). In this study we have taken an unbiased approach to identify proteins found in cellular fractions that contain viral nonstructural proteins and possess active viral RNA synthetic complexes. Using 2D-DIGE we were able to identify hnRNP K as being enriched in cytoplasmic membrane fractions of infected cells. This process proved useful for the identification of proteins enriched in specific cellular fractions and avoids the possibility of falsely identifying host factors as a consequence of interactions with a common partner of targeted viral proteins. However, the procedure we employed will also miss a number of proteins required by the virus if their abundance in the cellular fractions examined does not change following infection.

HnRNP K is a multifunctional poly(C) binding protein involved at every level of eukaryotic gene expression (reviewed in (Bomsztyk et al., 2004). HnRNP K interacts with C-rich tracts of DNA and RNA regulating transcription and translation. As with other poly(C)-binding proteins, hnRNP K possesses three K-homology (KH) domains that bind nucleic acid. In addition to these domains hnRNP K possesses a K-interaction (KI) domain that is responsible for many of the known hnRNP K interactions. Immunofluorescence data demonstrated that hnRNP K re-localized in cells following SIN infection and co-localized with components of the viral RNA synthetic machinery (Figs. 3 and 5). Immunoprecipitation analyses indicate that hnRNP K interacts with SIN nonstructural proteins and the viral subgenomic mRNA (Figs. 4 and 6). Decreasing the expression of hnRNP K correlated with a decrease in reporter gene expressed from a viral subgenomic mRNA. Precisely what role hnRNP K plays during viral replication is unclear, however these data suggest that it may be a component of the viral transcriptase complex.

The specific interaction of hnRNP K with the vRNP containing subgenomic mRNA implies a role in the regulation of

structural protein versus nonstructural protein expression. Subgenomic mRNA is produced in approximately four-fold molar excess over viral genomic RNA during the single-cell replication cycle (Strauss and Strauss, 1994). The direct interaction of hnRNP K with the nonstructural proteins may lead to upregulation of the transcriptase activity facilitating more efficient transcription of the subgenomic mRNA. We do not currently know if hnRNP K functions in this manner during SIN infection, however it is interesting to note that hnRNP K associates with numerous host-kinases and also possesses a kinase activity itself, playing a significant role in numerous signal transduction pathways (Ostareck-Lederer et al., 2002; Ostrowski et al., 2000; Weng et al., 1994). NsP3 is the only alphavirus protein that is phosphorylated, and mutations in this protein that reduce phosphorylation have a significant effect on viral RNA synthesis (De et al., 2003; LaStarza et al., 1994; Li et al., 1990; Vihinen et al., 2001). While many of the mutations result in decreased minus-strand RNA synthesis at least one mutation in the conserved N-terminal domain of nsP3 specifically caused a decrease in subgenomic mRNA synthesis (LaStarza et al., 1994). As nsP3 does not possess a kinase activity and phosphorylation and dephosphorylation are well-characterized means of regulating protein function it is interesting to speculate that hnRNP K or an associated kinase may play a role in regulating transcriptase activity through phosphorylation of nsP3.

In addition to the role of hnRNP K in cellular signaling cascades it also known to regulate translation by direct binding to untranslated regions (UTR) of some mRNAs (Evans et al., 2003; Habelhah et al., 2001; Ostareck et al., 2001; Ostareck et al., 1997). Binding of hnRNP K to the 3′ UTR of specific mRNAs in combination with PCBP-1 and/or PCBP2 silences translation (Ostareck et al., 2001; Ostareck et al., 1997). This silencing can be relieved by the phosphorylation of hnRNP K (Ostareck-Lederer et al., 2002). A similar silencing effect has be reported for hnRNP K and PCBP1/2 when they are bound to HPV-16 mRNA encoding the L2 structural protein (Collier et al., 1998).

A contrasting role of hnRNP K in translational regulation has been reported for c-*myc* mRNA. Once again in combination with PCBP1 or 2, hnRNP K binds to the 5′UTR of the mRNA and enhances cap independent translation of c-*myc* (Evans et al., 2003). Recent reports have demonstrated that the subgenomic mRNA of SIN can be translated in an eIF4F independent fashion (Castello et al., 2006). Proteolysis of eIF4G preventing its interaction with eIF4E failed to inhibit translation of the SIN subgenomic mRNA while blocking translation of SIN genomic and cellular mRNA. These observations may relate to the work presented in this manuscript; we have identified a protein that is known to promote cap-independent translation, and shown that it specifically interacts with the viral subgenomic mRNA that is translated in a cap-independent fashion, but not the viral genomic RNA that is translated in a cap-dependent manner.

HnRNP K is predominantly a nuclear protein. During SIN infection it appears that a subset of hnRNP K relocalizes to cytoplasmic membranes in association with viral molecules. Precisely what interactions lead to the recruitment of hnRNP K to the vRNP containing the subgenomic mRNA remain to be elucidated, however it is known that phosphorylation of hnRNP K through the mitogen activated protein-kinase (MAPK) pathway leads to cytoplasmic accumulation of hnRNP K (Habelhah et al., 2001). The MAPK is induced during SIN infection and may in part be responsible for the relocalization of hnRNP K (Nakatsue et al., 1998). Given that hnRNP K contains multiple protein and nucleic acid interaction domains it is possible that simultaneous interaction of this protein with both are responsible for relocalization and formation of complexes important in the viral replication cycle. We are continuing to investigate the interactions required for co-opting hnRNP K into complexes containing viral molecules.

Materials and methods

Cells and viruses

BHK-21 and HeLa cells were obtained from American Type Culture Collection, Rockville, Maryland. These cells were grown in Alpha MEM (BHK-21) or DMEM (HeLa) (Invitrogen) supplemented with 10% fetal bovine serum and vitamins. SIN was generated by transfection of BHK-21 cells with infectious RNA in vitro transcribed from pToto1101 (Rice et al., 1987). SIN expressing GFP from a second subgenomic promoter was generated by transfection of BHK-21 cells with infectious RNA transcribed in vitro from pwtSIN (the kind gift of Ilya Frolov, construction described in Frolova et al., 2002).

Cell fractionation and RNA synthesis assay

The protocol for the generation of cytoplasmic membrane fractions has been previously described. Briefly 1×10^8 HeLa cells were infected with a m.o.i. of 100 with SIN (optimal multiplicity previously determined by titration). Cells were harvested into a hypotonic buffer 6 h post-infection and homogenized. Nuclei were removed by low speed centrifugation (900×*g*). Post-nuclear homogenates were centrifuged at 15,000×*g* and pellets (P15) were resuspended in 500 μl hypotonic buffer plus 15% glycerol and stored in 100 μl aliquots at −80 °C.

P15 fractions were checked by Western blot for SIN protein content, and for RNA synthetic activity. Standard reaction mixtures for RNA synthetic activity assays contained 50 mM Tris–HCl (pH 7.8); 50 mM KCl; 3.5 mM $MgCl_2$; 10 mM dithiothreitol; 10 μg actinomycin-D per ml; 1 mM ATP, GTP, UTP; 40 mM CTP; 1 mCi [α-^{32}P]-CTP per ml (800 Ci/mmol, Perkin Elmer); 800 units RNasin per ml; 15 μg total protein from P15, P100 or S100; H_2O to total volume of 50 μl. Reactions were incubated at 30 °C for 60 min at which point 5 units of alkaline phosphatase was added and incubation continued for 20 min. Reactions were terminated by the addition of SDS to 2.5% and proteinase K to 100 μg/ml. RNA was isolated by phenol/chloroform extraction and ethanol precipitated. RNAs were denatured with

glyoxal, separated by electrophoresis, and visualized using a phosphorimager.

2-D difference in-gel electrophoresis and protein identification

2-D gel experiments were performed as previously described with minor modification (Gong et al., 2004). Briefly, 250 μg of P15 cytoplasmic fraction from infected and uninfected HeLa cells was resuspended in lysis buffer (7M urea, 2 CHAPS, 2 M thiourea, and 10 mM DTT) and 1 μg of BSA was added to each sample as internal control. Infected and uninfected samples were separately labeled with CyDye (Amersham Biosciences) combined and isoelectric focused on an 18 cm non-linear pH 3–10 Immobiline DryStrip (Amersham Biosciences) for 50–70 kVh on a IPHphor II unit. IEF gels were washed as described and loaded on 10–15% SDS-PAGE gels and run at constant 15–25 mAmp per gel at 4 °C.

Proteins in the gel were detected using a Typhoon 9200 imager at wavelengths corresponding to the Cy3 and Cy5 excitation wavelengths producing two separate images. These images were normalized to the BSA control spots with IPLab Spectrum (Signal Analysis Corporation) and made into 2 frame-loop movie with QuickTime (Apple, Inc.). Spot detection was performed visually.

After scanning, gels were rinsed in sterile water and stained with Colloidal Blue (Invitrogen) for 30 min and destained for up to 3 days. Protein spots were hand cut from the gel with a sterile scalpel. Spots were stored at −80 °C.

For identification, protein spots were destained and digested with using a Trypsin IGD Kit (Sigma) following manufacturer's directions. Dried spots were resuspended in 10 ul HPLC grade water and analyzed on a ThermoFinnigan (San Jose, CA) LCQ Deca XP ion-trap mass spectrometer that recorded mass spectra and data-dependent tandem mass spectra of the peptide ions. MS/MS spectra were searched against protein sequences for *Homo sapiens* using MASCOT analysis for peptide identification (Perkins et al., 1999). Peptide identification results were parsed using the Protein Results Parser program written in-house. Protein identification was viewed as confirmed only when a peptide obtained a score at or above the identity or extensive homology score (95% confidence) reported by MASCOT.

2D-DIGE and protein identification was performed on three separate occasions incorporating dye-swap controls to ensure specific fluorophores were not leading to aberrant identifications. On all three occasions hnRNP K was identified as described in the Results.

Immunofluorescence

BHK-21 and HeLa cells grown on glass cover slips were infected with Sindbis virus in a minimal volume of PBS for 1 h at an m.o.i. of 10 or 100, respectively. At 6 h post-infection, cells were rinsed with TBS, fixed with ice cold methanol, and permeabilized in methanol overnight at −20 °C. Cells were rinsed three times with TBS and incubated for 1 h in a dilution of primary antibodies in 1% BSA in TBS. Cells were rinsed three times in TBS and incubated for 30 min in a dilution of secondary antibody in 1% BSA in TBS. DAPI was added to 0.1 μg/ml and incubated in the dark for 20 min. Cells were rinsed and mounted on glass slides with Vectashield media (Vector Labs). Antibodies and dilutions: polyclonal rabbit-anti-hnRNP K (Santa Cruz sc-25373): 1:50; monoclonal mouse-anti-hnRNP K (Santa Cruz sc-28380) 1:50; polyclonal rabbit anti-nsp2 serum 1:750; goat anti-rabbit TRTIC (Jackson ImmunoResearch Laboratories, Inc 111-025-003) 1:250; goat anti-mouse FITC (Jackson ImmunoResearch Laboratories, Inc 115-095-003) 1:250. Cells were visualized on an Applied Precision Deltavision Nikon Eclipse TE200 with Softworx v2.50 (Figs. 3 and 5).

Protein labeling and immunoprecipitation

Approximately 2×10^6 BHK-21 cells were infected with SIN at an m.o.i. of 10 pfu/cell. Cells were incubated for 3 h. at 37 °C. Medium was removed and replaced with MEM lacking methionine and cysteine or MEM lacking phosphate (Invitrogen). Cells were incubated for a further 30 min at 37 °C. Medium was removed and replaced with methionine and cysteine depleted, or phosphate depleted medium supplemented with [^{35}S]-methionine and cysteine (Express protein labeling mix, Perkin Elmer) or [^{32}P]-orthophosphate (Perkin Elmer) to a final concentration of 50 μCi/ml. Cells were incubated for 2 h at 37 °C then harvested using a mild detergent buffer (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 1% NP40, 0.4% sodium deoxycholate). Nuclei were removed by centrifugation at 13,000×*g* for 1 min. SDS was added to the supernatants to a final concentration of 0.1%. Proteins were immunoprecipitated from material equivalent to 5×10^5 cells using anti-nsP2 rabbit polyclonal antiserum or anti-hnRNP K monoclonal antibody (Santa Cruz Biotechnology), and Affi-Prep® Protein A support (Bio-Rad). Immunoprecipitated proteins were analyzed by SDS-PAGE in 10% polyacrylamide gels under reducing conditions and detected by phosphorimaging.

Immunoprecipitation of viral RNA

Approximately 2×10^6 BHK-21 cells were infected with SIN at an m.o.i. of 10 pfu/cell. Cells were incubated for 3 h. at 37 °C. Medium was removed and replaced with medium containing 5 μg/ml actinomycin D. Cells were incubated for a further 30 min and medium was replaced with medium containing 30 μCi/ml [^{3}H]-uridine (Amersham) and 5 μg/ml actinomycin D. Cells were incubated at 37 °C for a further 2 h and harvested in a mild detergent buffer as described above. Immunprecipitations were performed as described above with the appropriate anti-serum. Following the final wash protein A beads were boiled in 200 μl of SDS containing buffer (10 mM Tris–HCl, pH 7.4, 2 mM EDTA, 0.5% SDS). RNA was phenol/chloroform extracted from supernatants, ethanol precipitated and separated by agarose-phosphate gel electrophoresis following glyoxal denaturation. RNA was visualized by fluorography.

siRNA knockdown

Prior to knockdown HeLa cells were seeded in 6 well plates in antibiotic free media. 60 pmol of siRNA targeting hnRNP K (sc-38282, Santa Cruz Biotechnology) or a control luciferase targeting siRNA (Dharmacon) were transfected with siRNA Transfection Reagent (Santa Cruz Biotechnology) in 1 ml Opti-MEM (Invitrogen) following manufacturer's directions. 5 to 6 h post-transfection 1 ml of growth media with 2× serum and antibiotics was added and after 18–24 h growth media was replaced with fresh media. Cells were either harvested following 72 h treatment for analysis by Western blot, or infected with SIN expressing GFP. Levels of infection were determined by FACS analysis 6 h post-infection using a FACSCalibur analyzer with CellQuest Pro software (Becton Dickinson).

Western blot analysis

Samples from P15 and S15 fractions from 2.5×10^5 cells were separated by SDS-PAGE (8% polyacrylamide), transferred to a polyvinyldifluoride (PVDF) membrane that was blocked with 5% milk in 25 mM Tris–HCl, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20 (pH 7.4). Blots were probed with anti-nsP2 rabbit polyclonal antiserum, anti-nsP4 rabbit polyclonal antiserum, or anti-hnRNP K polyclonal antiserum (Santa Cruz Biotechnology). Proteins were detected using an anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich).

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