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Interaction of influenza virus NS1 protein and the human homologue of Staufen *in vivo* and *in vitro*

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ABSTRACT

A screening for human proteins capable of interacting with influenza virus NS1 has been carried out using the two-hybrid genetic trap in yeast. A cDNA corresponding to the human homologue of *Drosophila melanogaster* Staufen protein (hStaufen) was isolated that fulfilled all genetic controls of the two-hybrid protocol. Using a hStaufen cDNA isolated from a λ human library, the interaction of hStaufen and NS1 proteins was characterised *in vivo* and *in vitro*. Co-transfection of NS1 cDNA and a partial cDNA of hStaufen led to the relocalisation of recombinant hStaufen protein from its normal accumulation site in the cytoplasm to the nuclear location of NS1 protein. NS1 and hStaufen proteins could be co-immunoprecipitated from extracts of co-transfected cells and from mixtures of extracts containing either protein, as well as from extracts of influenza virus-infected cells. Furthermore, both proteins co-localised in the ribosomal and polysomal fractions of influenza virus-infected cells. The interaction was also detected in pull-down experiments using a resin containing purified hStaufen and NS1 protein translated *in vitro*. Deletion mapping of the NS1 gene indicated that a mutant protein containing the N-terminal 81 amino acids is unable to interact with hStaufen, in spite of retaining full RNA-binding capacity. These results are discussed in relation to the possible mechanisms of action of hStaufen and its relevance for influenza virus infection.

INTRODUCTION

The influenza A viruses encode 10 different polypeptides in a genome consisting of eight single-stranded RNA segments of negative polarity (1). The two smallest RNA segments encode two proteins by a differential splicing mechanism (reviewed in 2–4). The influenza virus NS1 protein is the translation product of the collinear mRNA derived from viral RNA segment 8 (5,6). It is the only non-structural protein encoded by influenza A virus and contains around 230 amino acids, although length polymorphism is apparent among different virus strains (7). NS1 is a nuclear protein when expressed from cloned cDNA (8–10) and early in the infection cycle (11), although it is also present in the cytoplasm late in infection (11,12), in association with polysomes (11,13; this report).

The role of NS1 protein during influenza virus infection is not clear. On the one hand, viral temperature-sensitive mutants affected in the NS1 protein show a post-transcriptional defect in late viral gene expression (14) or a reduced viral mRNA synthesis (15). These phenotypes are consistent with an interaction of NS1 protein with the viral polymerase and, indeed, its association with transcription–replication complexes has been described (16). On the other hand, a series of mutant viruses containing progressive deletions from the C-terminus of NS1 protein have been recently prepared by reverse genetics (17). The efficiency of replication of these mutant viruses inversely correlated with the length of the encoded NS1 protein. However, they all were as fit as the wild-type in Vero cells, indicating that under the conditions used NS1 is not essential for virus viability. In other cell types, NS1 could be essential as a virulence factor responsible for counteracting interferon response, as Vero cells are deficient in interferon genes (18). In agreement with this possibility, a mutant influenza virus lacking the expression of NS1 protein replicated efficiently in Vero cells, but very poorly in MDCK cells (19).

It has previously been considered that NS1 expression would increase the efficiency of virus replication as it affects several RNA-related cellular processes: (i) it modulates the splicing of pre-mRNAs (20–22); (ii) it retains poly(A)-containing mRNA in the nucleus, both spliced mRNAs and non-spliceable ones (20,23); (iii) it enhances the translation of viral mRNAs (13,24) by increasing the rate of translation initiation (13); (iv) it inhibits the cleavage of pre-mRNA during its 3′-end processing (25). These effects of NS1 expression in RNA metabolism can be interpreted in terms of the capacity of NS1 to bind several proteins and different types of RNA. Binding of RNA occurs through a domain localised to the N-terminal third of the protein (26–28). NS1 protein can bind: (i) virion panhandle RNA (27,29,30); (ii) poly(A) (23; our unpublished results); (iii) U6 snRNA (31); (iv) the 5′-untranslated region (UTR) of NP mRNA (32). NS1 protein has also been shown to interact with a number of cellular proteins, NS1-I, a protein related to estradiol 17β-dehydrogenase (33), the 30 kDa subunit of CPSF (25) and NS1-BP (34), but the relevance of these interactions for the function of NS1 during virus infection is still an open question. In this report we describe the isolation of the sequence homologue of *Drosophila melanogaster*

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Staufen as a human gene encoding a protein (hStaufen) that interacts with NS1 protein in the two-hybrid genetic trap. hStaufen comprises a family of proteins with capacity to bind dsRNA and tubulin, which can be found associated with polysomes in human cells (35,36). Several protein isoforms are generated by alternative splicing (36), with molecular weights in the range 60–65 kDa (35,36). We show that NS1 and hStaufen proteins interact when co-expressed *in vivo* from cloned cDNAs and during the virus infectious cycle.

MATERIALS AND METHODS

Biological materials

The COS-1 cell line (37) was kindly provided by Y. Gluzman and the HeLa cell line was purchased from the ATCC. They were cultured as described previously (38). The vaccinia recombinant virus vTF7-3 (39) was a gift of B. Moss. *Saccharomyces cerevisiae* strain HF7c (*MAT**a**, his3, GAL1-HIS3, GAL4-lacZ, trp1, leu2*) was obtained from Clontech and used for two-hybrid screening. Plasmids pGBT9 (called BD in this study as it encodes the GAL4 DNA-binding domain) and pGAD424 (called AD, from activation domain) were obtained from Clontech, as were plasmids pVA3 (GAL4), pTD1(p53) and pCL1(Tag), used as internal controls. The *Escherichia coli* MH4 (leu$^-$) strain was obtained from M. Serrano. Plasmids pG4NS1 and pRSHisNS1, as well as plasmids expressing deleted forms of NS1 protein, have been described (27). In plasmid pSVa232NS1 (20), NS1 protein is expressed from a SV40 promoter. Plasmids pRSTL and pRHST were described previously (35). The cDNA sequence of hStaufen encoding the dsRNA-binding domains (476 C-terminal amino acids) was fused in-frame to the pCMV vector to generate plasmid pCMV-HST. The pRSET expression vectors were obtained from Invitrogen and plasmid pArII, containing the rDNA from *Artemia salina*, was provided by J. Renard. The monoclonal antibody specific for the T7 tag present in the pRSET vectors was purchased from Novagen. The antisera specific for NS1 protein have been described (27), as well as the antiserum specific for hStaufen protein (35). The serum specific for PKR was a gift from M. Esteban.

Transfection

For transfection of T7 promoter-containing plasmids, cultures of COS-1 cells growing in 35 mm dishes were infected with vTF7-3 virus at a multiplicity of infection of 5–10 p.f.u./cell. After virus adsorption for 1 h at 37°C, the cultures were washed with DMEM and transfected as follows. DNAs were diluted to 100 µl of DMEM and, in a separate tube, cationic liposomes (2 µl/µg DNA) were diluted to 100 µl in DMEM. The contents of both tubes were mixed, kept at room temperature for 15 min and added to the culture plates containing 1 ml of DMEM. Cationic liposomes were prepared as described (40). Transfection of plasmids for expression under RNA polymerase II promoters was carried out as indicated above, except that the vaccinia virus infection was omitted.

Two-hybrid screening

Plasmid pBD-NS1 contained the NS1 cDNA from plasmid pSVa232NS1 (20) cloned into vector pGBT9. It was used to screen a human kidney cDNA fusion library cloned into pGAD vector. Using 10 mM 3-aminotriazole, the recombinant plasmid pBD-NS1 alone did not induce growth in histidine-free medium. The procedures for library amplification, yeast cell transformation, screening for growth in the absence of histidine and β-GAL activity were those recommended in the Matchmaker protocol (Clontech). Rescue of positive pGAD plasmids was done by transformation into *E.coli* MH4 (leu$^-$) cells and selection on M9 plates lacking leucine. cDNA clones corresponding to the thSTL insert were obtained from a HeLa cDNA library constructed in λgt10 (Clontech), using standard procedures (41). Sequencing was carried out in a Perkin-Elmer 373 automatic sequencer using specific oligonucleotide primers.

Protein analyses

The purification of His-HST protein, which contains the C-terminal 476 amino acids of hStaufen protein fused to a His tag and a T7 antigenic tag, was carried out by chromatography on Ni^{2+}–NTA resin as described previously (35). The RNA present in the purified protein was eliminated by washing with 2 M KCl and the resin was used for pull-down experiments as described below.

For immunoprecipitation, immune matrices were prepared by incubation of protein G–Sepharose with either anti-NS1 or unrelated rat sera. These matrices were incubated with soluble extracts prepared in TNE/1% NP-40 buffer from influenza virus-infected or mock-infected HeLa cells. After washing in TNE/1% NP-40 buffer, the bound material was eluted by boiling in Laemmli sample buffer and processed by western blot as described previously (35). The sera used were: rabbit anti-NS1 serum (1:300 dilution); rabbit anti-STL serum (1:800 dilution); rabbit anti-PKR serum (1:500 dilution).

To carry out pull-down experiments, NS1 protein was translated by coupled transcription–translation *in vitro* using the TNT system (Promega) and [^{35}S]Met-Cys. The translation mixture was centrifuged for 2 h at 100 000 *g* to recover the post-ribosomal supernatant. Alternatively, wild-type or mutant NS1 proteins were labelled *in vivo* with [^{35}S]Met-Cys after infection/transfection of COS cells with either pG4NS1 or mutant plasmids pG4NS1Δ1-81, pG4NS1Δ82-238, pG4NS1Δ114-238 or pG4NS1Δ171-238. Aliquots of labelled NS1 proteins were incubated with Ni^{2+}–NTA resin containing purified His-HST protein in TNE/0.5% NP-40 buffer for 1 h at room temperature. After washing five times with the same buffer, the retained material was recovered by boiling in Laemmli sample buffer and analysed by polyacrylamide gel electrophoresis and autoradiography. In some experiments, labelled NS1 protein was treated with RNase (20 µg/ml) for 30 min at 4°C. Under these conditions, a labelled riboprobe added to the NS1 protein preparation was completely degraded.

Immunofluorescence was performed as described previously (35) using the following sera: rat anti-NS1 serum (1:400 dilution); rabbit anti-STL serum (1:200 dilution); Texas Red-labelled goat anti-rabbit IgG serum (1:200 dilution); FITC-labelled goat anti-rat IgG serum (1:200 dilution). Images were obtained in a Zeiss axiovert 100 fluorescence microscope equipped with a high resolution CCD camera. Images were coloured and overlayed using the Adobe Photoshop program.

Isolation of polysomes was carried out as described previously (35,42). In brief, the cytoplasmic fraction was obtained by cell lysis in isotonic buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.5) containing 0.5% NP-40, centrifuged for 10 min at 10 000 *g* and 4°C and finally centrifuged on a 7–47% sucrose

gradient in isotonic buffer, for 2 h at 40 000 r.p.m. and 2°C in a SW41 rotor. As a negative control, polysomes were disrupted by incubation of the cell cultures with puromycin (100 µg/ml) for 1 h before harvesting the cells.

RESULTS

Two-hybrid screening with NS1 protein as bait

In view of the multiple alterations detected in cells expressing NS1 cDNAs (13,20,21), we carried out a two-hybrid screen using NS1 protein as bait to identify cellular proteins that interact with NS1 and may mediate these effects. Yeast cells harbouring plasmid pBD-NS1, which expresses NS1 protein fused to the GAL4 DNA-binding domain, were transformed with a human kidney cDNA fusion library, constructed in plasmid pGAD, and selected for growth in the absence of histidine. Among the colonies rescued only a few were positive in the β-GAL *in situ* assay and these were further tested after isolation of the plasmids and retransformation. One of the clones (clone C) was confirmed as positive and fulfilled all the controls recommended in the two-hybrid interaction protocol (data not shown). Clone C was analysed by restriction assay and partial sequencing. It contained an insert of ~1 kb (thSTL) that was used to screen a standard human cDNA library. Several λ clones were identified and the cDNA inserts were sequenced. The compiled sequence corresponds to the short transcript of the hStaufen gene (36), contains a 496 amino acid open reading frame and a long 3′-UTR. The predicted protein sequence is homologous to the C-terminal half of the *D.melanogaster* Staufen protein, but it shows much smaller homology to other members of the Staufen family of dsRNA-binding proteins (35,36).

Interaction of NS1 and hStaufen proteins *in vitro*

The interaction of NS1 protein and the thSTL fragment of hStaufen protein (amino acids 92–417), isolated in the two-hybrid screen, was confirmed *in vitro* by co-immunoprecipitation experiments. The His-thSTL and NS1 proteins were individually expressed or co-expressed by transfection of pRSTL and pG4NS1 into vaccinia vTF7-3 virus-infected COS cells. Soluble extracts were prepared and immunoprecipitated with a NS1-specific antiserum. The presence of either NS1 or thSTL proteins in the immunoprecipitates was ascertained by western blot using NS1- and STL-specific sera. The results are presented in Figure 1. It is clear that the thSTL protein was co-immunoprecipitated together with NS1 in the NS1-specific immunoprecipitates, both when the proteins were co-expressed in the same culture (Fig. 1, lane NS1–STL) and when extracts of cells expressing either protein separately were mixed *in vitro* (Fig. 1, lane NS1+STL). Similar results were obtained if anti-STL serum was used for the immunoprecipitation step (data not shown). Under the conditions used, it could be estimated that 50% of the thSTL protein present in the extract was associated with NS1 protein. The implication of RNA in the binding *in vitro* was tested by RNase treatment of the cell extracts. When the extracts from cells co-expressing NS1 and thSTL proteins were treated with RNase prior to the immunoprecipitation step, the co-immunoprecipitation of thSTL protein was indistinguishable from that presented in Figure 1, indicating that NS1–thSTL interaction is not a non-specific association of two RNA-binding proteins to a single RNA molecule. If extracts of cells expressing each protein individually were treated with RNase prior to their mixing and immunoprecipitation, the NS1–thSTL interaction was slightly diminished (data not shown, but see below), suggesting that RNA is not required for, but may stabilise, the interaction.

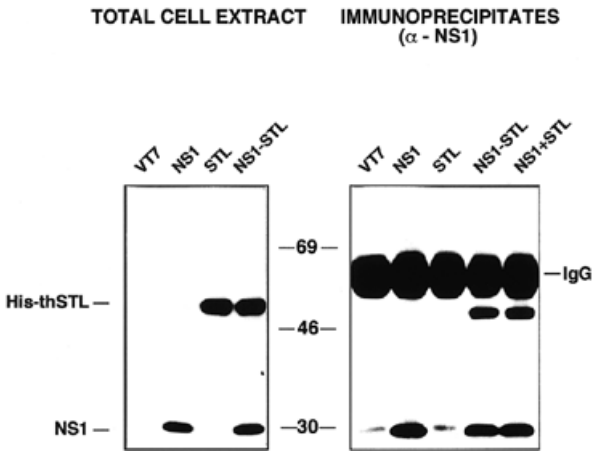

Figure 1. Interaction of STL and NS1 proteins *in vitro*. Cultures of COS-1 cells were infected with vaccinia vTF7-3 virus and transfected with plasmid pRSTL (STL) or plasmid pG4NS1(NS1), co-transfected with both plasmids (NS1–STL) or mock-transfected (VT7). Soluble cell extracts were prepared and immunoprecipitated with anti-NS1 serum. Samples of the extracts (left) or the immunoprecipitates (right) were analysed by western blot with a mixture of anti-NS1 and anti-STL sera. A mixture of NS1 and STL extracts was also immunoprecipitated (NS1+STL). The position of molecular weight markers (in kDa) is indicated between the two panels and the position of the heavy chain of the IgG used for immunoprecipitation is indicated to the right.

To test the binding of hStaufen and NS1 proteins in a more defined way, a recombinant His-tagged protein containing the 476 C-terminal amino acids of hStaufen (His-HST), which encodes the four dsRNA-binding domains, was purified to homogeneity and used to perform pull-down experiments. The NS1 protein was labelled *in vitro* in a transcription–translation coupled system and incubated with a His-HST-specific resin or an empty resin as a control. The protein present in the His-HST resin is shown in Figure 2C. The absence of RNA in the His-HST preparation was controlled and the identity of the protein as recombinant hStaufen was confirmed by western blot assays using either anti-STL or anti-T7 tag antibodies (data not shown). As an additional control, luciferase was also synthesised *in vitro* and used in binding experiments. After extensive washing, the material retained in the resins was analysed by polyacrylamide gel electrophoresis. The results are presented in Figure 2. Specific binding of NS1 protein to His-HST protein was observed, but not to the empty resin (Fig. 2A). About 10% of the labelled NS1 protein added to the specific resin was retained. No retention of luciferase was observed with either His-HST resin or empty resin (Fig. 2B). The involvement of RNA in the binding of NS1 protein to hStaufen was analysed with pull-down experiments analogous to those reported above. The labelled NS1 preparation was treated with RNase under conditions sufficient to completely degrade any RNA present in the mixture. The binding of such RNA-free NS1 protein was tested as above and the results are presented in Figure 2A. No significant reduction was observed in the amount of NS1 protein retained by His-HST resin. As expected, no binding was detected when an empty resin was used.

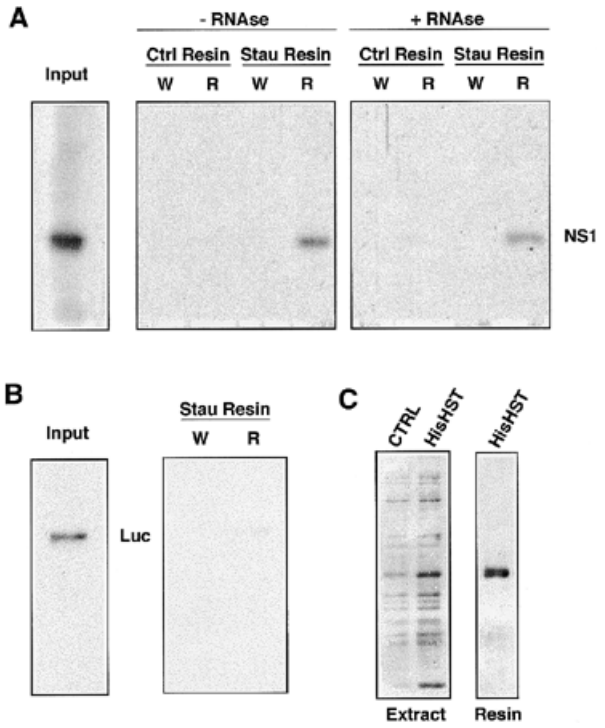

Figure 2. Interaction of NS1 protein with purified hStaufen protein. (**A**) The NS1 protein was translated *in vitro* using the TNT system and plasmid pG4NS1. The translation product was treated with RNase A or not treated and centrifuged in a table top ultracentrifuge to obtain the post-ribosomal supernatant (Input). Aliquots of this post-ribosomal supernatant were incubated with a Ni^{2+} resin containing purified His-HST protein or with an empty resin. After washing, the protein retained in the resin (R) was analysed by polyacrylamide gel electrophoresis, in parallel with the last wash (W). The position of NS1 protein is indicated to the right. (**B**) Similar experiment as indicated in (A), except that a plasmid expressing luciferase (Luc) was used instead of plasmid pG4NS1. (**C**) Characterisation of His-HST-containing resin. The left panel shows the analysis by polyacrylamide gel electrophoresis of extracts from *E.coli* cells transformed with plasmid pRSET (CTRL) or pRHST (HisHST). The right panel shows the protein content of an aliquot of His-HST-containing resin.

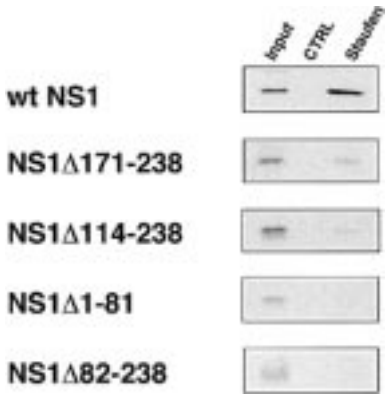

Figure 3. Mapping the NS1 domain interacting with hStaufen protein. Cultures of COS-1 cells were infected with vaccinia vTF7-3 virus and transfected with plasmid pG4NS1 or its mutant derivative pG4NS1Δ1-81, pG4NS1Δ82-238, pG4NS1Δ114-238 or pG4NS1Δ171-238. After incubation for 24 h in the presence of AraC, the cultures were labelled with [^{35}S]Met-Cys (200 μCi/ml) in Met-Cys free DMEM medium. Soluble cell extracts were prepared (Input) and used for incubation with either His-HST-containing resin (Staufen) or a control resin (CTRL). After extensive washing, the material retained was eluted with Laemmli sample buffer and analysed by gel electrophoresis and autoradiography.

To identify the region of NS1 protein involved in the interaction with hStaufen protein, *in vitro* binding experiments were carried out similar to those described above except that NS1 protein or mutants thereof were labelled with [^{35}S]Met-Cys *in vivo* as indicated in Materials and Methods. Extracts from COS cells infected/transfected with plasmid pG4NS1 or its mutant derivative pG4NS1Δ1-81, pG4NS1Δ82-238, pG4NS1Δ114-238 or pG4NS1Δ171-238 were incubated with either hStaufen-containing resin or a control resin. After extensive washing, the labelled protein retained was analysed by gel electrophoresis and autoradiography. As shown in Figure 3, retention of wild-type NS1 was clearly detected. In contrast, NS1Δ82-238 protein, which contains the RNA-binding domain and is fully able to bind RNA (27), was not retained. Similarly, NS1Δ1-81 mutant protein was unable to interact with hStaufen. Only partial binding activity was observed for mutants NS1Δ114-238 and NS1Δ171-238 (Fig. 3).

Interaction of NS1 and hStaufen proteins *in vivo*

In addition to the interaction of NS1 and thSTL proteins revealed in the two-hybrid assay, we wished to confirm their association in mammalian cells in culture. To that aim, cultures of HeLa cells were transfected with plasmid pCMV-HST, which expresses the 476 C-terminal amino acids of hStaufen protein, with plasmid pSVa232NS1, which expresses NS1 protein, or co-transfected with both plasmids. The cultures were fixed and analysed by double immunofluorescence using antisera specific for hStaufen and NS1 proteins. The results are shown in Figure 4. The HST protein showed an intracellular distribution analogous to that reported for hStaufen protein and consistent with its localisation in the rough endoplasmic reticulum (35; Fig. 4B), while NS1 protein accumulated at large foci in the nucleus (27,43; Fig. 4A). However, when both proteins were co-expressed, recombinant HST protein co-localised with NS1 in the nucleus of the cell (Fig. 4D–F, arrow) in contrast to the situation in a cell that was expressing only HST protein (Fig. 4E and F, star). Similar results were obtained if thSTL protein was used instead of HST protein (data not shown). It should be pointed out that most of the fluorescence signal specific for hStaufen protein detected (Fig. 4) derives from the recombinant HST protein, since the level of accumulation of endogenous hStaufen protein is much smaller. Experiments in which only pSVa232NS1 plasmid was transfected indicated that the endogenous hStaufen protein did not accumulate in the nucleus (data not shown), suggesting that HST recombinant protein was not as strongly retained in the cytoplasm as wild-type hStaufen protein.

To test directly the possible interaction of NS1 and hStaufen proteins in the infection, HeLa cells were infected with influenza virus or mock-infected and soluble extracts were prepared and immunoprecipitated with anti-NS1 serum. The presence of endogenous hStaufen protein in the NS1-specific precipitates was assayed by western blot using anti-STL serum and the results are presented in Figure 5A. The endogenous hStaufen protein isoforms (Fig. 5, stars) were associated with NS1-specific immunoprecipitates (Fig. 5A, F) but absent in the control immunoprecipitates obtained from mock-infected cells (Fig. 5A, M) or obtained by precipitation with a control serum

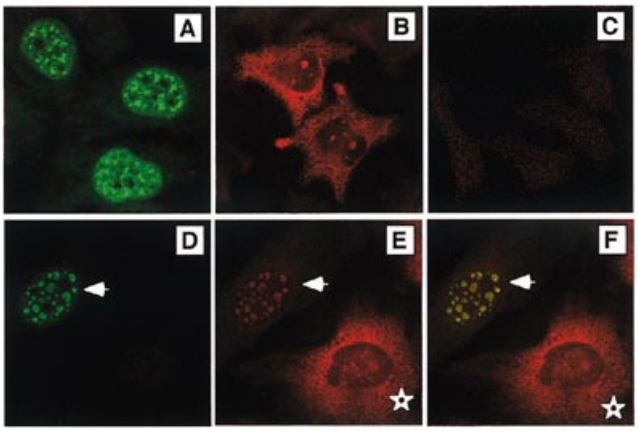

Figure 4. Intracellular relocalisation of hStaufen protein by co-expression with NS1 protein. Cultures of HeLa cells were transfected with either plasmid pCMV-HST or plasmid pSVa232NS1 or co-transfected with both plasmids. The cultures were fixed and analysed by double immunofluorescence using rat anti-NS1 serum and rabbit anti-STL serum. (**A**) Cells transfected with pSVa232NS1 plasmid and stained with anti-NS1 serum. (**B**) Cells transfected with pCMV-HST plasmid and stained with anti-STL serum. (**C**) Cells mock-transfected and stained with anti-STL serum. (**D**–**F**) The same field showing staining of NS1 protein (D), staining of HST protein (E) and overlay of both images (F). The arrow indicates a cell co-transfected with both plasmids. The star indicates a cell singly transfected with pCMV-HST plasmid.

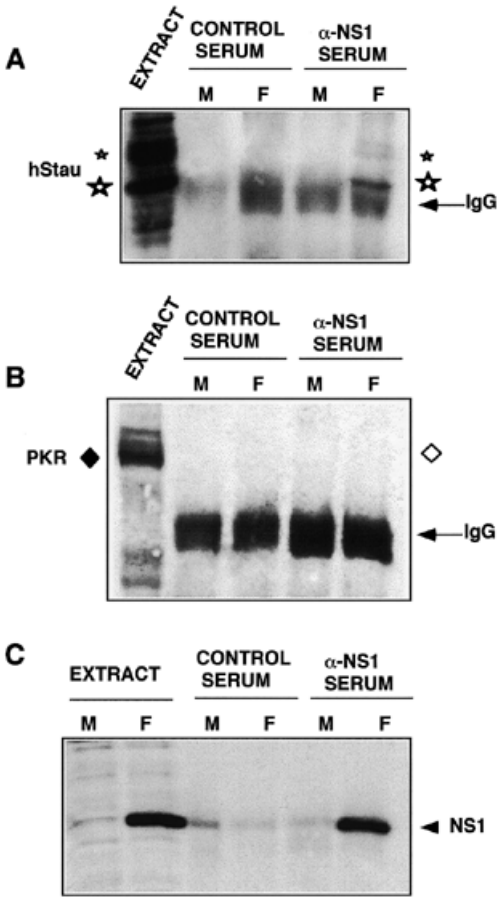

Figure 5. Interaction of NS1 with hStaufen protein in influenza virus-infected cells. Cultures of HeLa cells were infected with influenza virus (F) or mock-infected (M). Soluble extracts were prepared and used for immunoprecipitation with anti-NS1 serum or a pre-immune serum. Aliquots of the original extracts or the immunoprecipitates were analysed by western-blot using anti-STL serum (**A**), anti-PKR serum (**B**) or anti-NS1 serum (**C**). The stars indicate the presence of hStaufen protein in the extract and the NS1-specific immunoprecipitate. The arrowhead indicates the presence of NS1 protein in the infected cell extract and its NS1-specific immunoprecipitate. The filled rhomboid indicates the presence of PKR protein in the infected cell extract and the empty rhomboid shows its absence from the NS1-specific immunoprecipitate.

(Fig. 5A, control). Under the experimental conditions used, it could be estimated that 7% of the hStaufen protein present in the extract was associated with NS1 protein. Since both NS1 and hStaufen are RNA-binding proteins, the specificity of their interaction in the infection was tested by assaying the binding of NS1 to PKR (Fig. 5B, filled rhomboid), an RNA-binding protein related to hStaufen. The same immunoprecipitates were tested for the presence of PKR by western blot using a PKR-specific serum and the results are presented in Figure 5B. No co-immunoprecipitation was detectable (Fig. 5B, F), indicating that the interaction of hStaufen and NS1 in the infected cell is specific. The presence of NS1 protein in the NS1-specific immunoprecipitates was ascertained by western blot using anti-NS1 serum and is shown in Figure 5C.

NS1 and hStaufen proteins co-localise in the polysomes of infected cells

We have previously shown that hStaufen protein isoforms associate with polysomes in cultured cells (35). Likewise, it has been described that NS1 protein can be found in the polysome fractions in influenza virus-infected cells (11,13). We wished to test whether both proteins could be found in association with polysomes during the infection and hence polysomes were separated by centrifugation in sucrose gradients and the presence of both proteins was tested by western blot. The results are presented in Figure 6. Both NS1 and hStaufen proteins could be found in the 80S region of the gradient as well as in the fractions containing polysome complexes. The pattern of hStaufen in the polysomes was not affected by infection with influenza virus (compare Fig. 6 with the results previously published; 35). To verify that the proteins co-sedimenting with the largest complexes were indeed associated with polysomes, extracts from cells pretreated with puromycin were analysed in parallel. This treatment leads to premature termination of translation and is reflected by the disappearance of ribosomes from the fast sedimenting material in the gradient (Fig. 6, compare rRNA and rRNA+P). As shown in Figure 6 (NS1+P and hStau+P), incubation with puromycin led to the displacement of both NS1 and hStaufen proteins to the slow sedimenting fractions of the gradient. Therefore, we can conclude that NS1 and hStaufen proteins co-localise in the polysomes of influenza virus-infected cells.

DISCUSSION

The interaction of NS1 and hStaufen proteins is genuine

The interaction of hStaufen and influenza NS1 proteins was first detected by the yeast two-hybrid test and has been confirmed both *in vivo* and *in vitro* by a variety of techniques. These included co-immunoprecipitation from extracts of cells in which both proteins were co-expressed by transfection (Fig. 1) and from

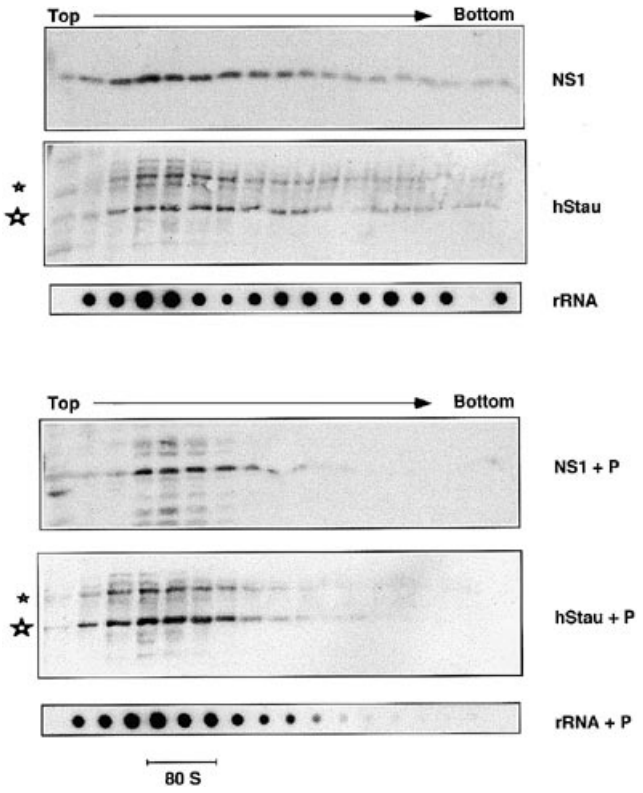

Figure 6. Co-localisation of NS1 and hStaufen proteins in polysomes of influenza virus-infected cells. Cultures of HeLa cells were infected with influenza virus and, at 5 h post-infection, nuclear and cytoplasmic fractions were prepared. The cytoplasmic fraction was separated by centrifugation on a sucrose gradient as indicated in Materials and Methods. Parallel infected cultures were treated with puromycin prior to fractionation (+P). Aliquots of each fraction were analysed by western blot using anti-NS1 serum or using anti-STL serum. The position of NS1 protein is indicated to the right. The position of hStaufen protein (stars) is indicated to the left. Other aliquots of each fraction were used to isolate total RNA that was analysed by dot-blot hybridisation with a rDNA probe (rRNA/rRNA+P). The approximate position of a monosome is indicated at the bottom. The first fraction of the gradient, corresponding to the sample, has not been included in the analyses.

extracts of influenza virus-infected cells (Fig. 5) as well as co-localisation in co-transfected cells (Fig. 4) and in influenza virus-infected cells (Fig. 6). The interaction *in vitro* has been detected by co-immunoprecipitation after mixing extracts containing each recombinant protein (Fig. 1) and by pull-down experiments in which purified hStaufen protein bound to an affinity resin-retained NS1 protein synthesised *in vitro* from cloned cDNA (Fig. 2). This accumulation of evidence makes it very unlikely that the interaction reported here is an experimental artifact and speaks for a functional role of this interaction in NS1 protein function during influenza virus infection. The co-localisation of NS1 and hStaufen proteins in the polysomes of infected cells, together with the absence of nuclear translocation of endogenous hStaufen to the nucleus in cells transfected with the NS1 gene, suggests that the association of both proteins during infection takes place in the cytoplasm.

Since both NS1 and hStaufen are RNA-binding proteins (23,26–28,30,31,35), it could be considered that their interaction merely reflects the non-specific binding to a single RNA molecule. This possibility was ruled out by three pieces of evidence. First, treatment of mixed extracts with RNase only slightly affected the capacity of the proteins to co-immunoprecipitate (data not shown). Second, an affinity resin containing purified, RNA-free hStaufen protein was able to retain NS1 protein synthesised *in vitro* and treated with RNase (Fig. 2). Finally, deletion mapping indicated that a NS1 mutant protein containing the N-terminal 81 amino acids, capable of binding RNA (27), does not interact with hStaufen (Fig. 3). In addition, the fact that PKR, an RNA-binding protein related to hStaufen, could not co-immunoprecipitate with NS1 protein in extracts obtained from influenza virus-infected cells (Fig. 5) speaks for a specific association of NS1 with hStaufen and not with other members of the same family of RNA-binding proteins. Nevertheless, these experiments do not preclude a role for RNA in the specific NS1–hStaufen interaction and more detailed studies will be required to ascertain such a possibility. In addition, the results presented here do not demonstrate a direct interaction between hStaufen and NS1 proteins, since the pull-down experiments were carried out with NS1 protein synthesised in reticulocyte lysates and we cannot exclude the possibility that a cellular protein present in the lysate could play a role in the interaction.

Little is known about the domain of hStaufen that interacts with NS1. The protein fragment identified in the two-hybrid screen includes amino acids 92–417. These sequences comprise the two internal, long dsRNA-binding domains. In humans, four Staufen mRNAs are generated by alternative splicing that encode hStaufen proteins that differ in their N-terminus (36). Interestingly these different protein isoforms contain the NS1-binding region (Fig. 5, stars).

Is NS1–hStaufen interaction relevant for infection?

The data presented in this report constitute circumstantial evidence in favour of a role for hStaufen–NS1 interaction during influenza virus infection, but we cannot provide a direct proof for it. The main reason is that the function of hStaufen in mammalian cells is not as yet clear. The role for dmStaufen during fly development and neural cell differentiation involves the intracellular localisation of specific mRNAs and the stimulation of their translation at the site. Thus, dmStaufen protein specifically binds *oskar* mRNA or *bicoid* mRNA and allows their localisation at opposite poles in the oocyte and early embryo and the expression of their gene products only at those sites (44). In addition, dmStaufen protein interacts with *prospero* mRNA by binding to its 3′-UTR and cooperates with *inscuteable* to allow its accumulation in the basal area of neuroblasts (45–47) and eventually its asymmetrical distribution in the progeny cells. Similarly to what has been observed with dmStaufen, hStaufen can bind RNA and microtubules *in vitro* (35,36) and it is localised in the rough endoplasmic reticulum in association with polysomes (35,36; this report). In hippocampal neurons, mammalian Staufen accumulates in the somatodendritic region, where localisation of several RNAs has been described (48). Again, Staufen staining in these cells co-localises with microtubules and with ribonucleoprotein particles (48). These results suggest that hStaufen could be implicated in the transport of RNAs, via its interaction with microtubules, to places of enhanced translation. On the other hand, NS1 protein stimulates the translation of viral mRNAs (13,24) by enhancing the initiation step (13) and can be cross-linked to the 5′-UTR of viral mRNAs (32). Therefore, it is tempting to speculate that the association of NS1 with hStaufen

may help in the localisation of viral mRNAs in the appropriate milieu for their efficient translation. In this context, it is worth mentioning that deleted NS1 mutant NS1Δ82-238 is unable to interact with hStaufen and is deficient in the enhancement of viral protein synthesis (27).

Possible interplay of hStaufen and NS1 proteins in the translation of viral mRNAs

It has been proposed that the expression of NS1 protein might avoid the activation of PKR in infected cells by sequestering any dsRNA generated (49) or by direct interaction with PKR (50). Although we could reproduce such an interaction in pull-down experiments similar to those reported in Figure 2 (data not shown), we were consistently unable to detect co-immunoprecipitation of both proteins from influenza virus-infected cells (Fig. 5), even with the use of three different anti-NS1 sera. This suggests that, although NS1 and PKR can interact, probably by simultaneous binding to dsRNA, they do not share the same compartment in the infected cell and hence do not interact *in vivo*. In fact, most of the PKR present in the infected cell appeared in the soluble fraction when analysed by sucrose gradient sedimentation (data not shown), whereas both NS1 and hStaufen were ribosome and polysome associated (Fig. 6). In view of the specific NS1–hStaufen interaction in infected cells, it is possible to speculate that NS1 expression might avoid the activation of PKR in a more subtle way, by blocking access of the kinase to viral mRNA-containing polysomes and hence avoiding its activation at the site. Alternatively, or in addition to the possibility discussed above, the interaction of NS1 with hStaufen could result in a more efficient association of viral mRNAs with polysomes, if we assume that hStaufen plays a role in directing cellular mRNAs for translation. Further experiments are needed to clarify whether these mechanisms, or others, are responsible for a role of hStaufen in influenza virus gene expression.

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