The Hrp65 self-interaction is mediated by an evolutionarily conserved domain and is required for nuclear import of Hrp65 isoforms that lack a nuclear localization signal

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Summary

Hrp65, an evolutionary conserved RNA-binding protein from the midge *Chironomus tentans*, has a conserved DBHS (Drosophila behavior, human splicing) domain that is also present in several mammalian proteins. In a yeast two-hybrid screening we found that Hrp65 can interact with itself. Here we confirm the Hrp65 self-interaction by in vitro pull-down experiments and map the sequences responsible for the interaction to a region that we refer to as the protein-binding domain located within the DBHS domain. We also show that the protein-binding domains of *Drosophila* NonA and human PSF, two other proteins with conserved DBHS domains, bind to Hrp65 in the yeast two-hybrid system. These observations indicate that the protein-binding domain can mediate homodimerization of Hrp65 as well as heterodimerization between different DBHS-containing proteins. Moreover, analyses of recombinant Hrp65 by gel-filtration chromatography show that Hrp65 can not only dimerize but also oligomerize into complexes of at least three to six molecules. Furthermore, we have analyzed the functional significance of the Hrp65 self-interaction in cotransfection assays, and our results suggest that the interaction between different Hrp65 isoforms is crucial for their intracellular localization.

Key words: RNA-binding protein, *Chironomus tentans*, Oligomerization, Yeast two-hybrid, Transfection, Nuclear import

Introduction

In eukaryotic cells, pre-messenger RNA precursors (pre-mRNAs) are cotranscriptionally assembled into ribonucleoprotein complexes (pre-mRNPs), processed into mature mRNA and exported to the cytoplasm to direct protein synthesis. The salivary gland cells of the dipteran *Chironomus tentans* have been used as a model system to study the intranuclear steps of gene expression, and several RNA-associated proteins of *C. tentans* have been characterized in recent years (reviewed by Daneholt, 2001). In previous studies, we identified Hrp65 as a basic protein of 65 kDa (Wurtz et al., 1996; Miralles et al., 2000). Hrp65 has a modular structure and contains two classical RNA recognition motifs (RRMs). Three Hrp65 isoforms have been characterized, which differ in their C-terminal sequences and which are generated by alternative splicing from a single pre-mRNA (Miralles and Visa, 2001). The C-terminal sequences of the Hrp65 isoforms are important for intracellular localization. The Hrp65-1 isoform contains a nuclear localization signal (NLS) in its C-terminus that mediates efficient import into the nucleus, whereas Hrp65-2 and Hrp65-3 lack the NLS (Miralles and Visa, 2001).

In the salivary glands of *C. tentans*, Hrp65 is located in non-chromatin nucleoplasmic fibers, referred to as connecting fibers (CFs), as shown by immuno-electron microscopy studies using an antibody that recognizes all the Hrp65 isoforms. The CFs interact with pre-mRNP particles in the cell nucleus, which suggests that Hrp65 is involved in mRNA biogenesis at the post-transcriptional level (Miralles et al., 2000). But recent studies have shown that the Hrp65-2 isoform plays a role in transcription by RNA polymerase II (Percipalle et al., 2003).

Hrp65 is evolutionary conserved in metazoans and has high sequence similarity to a group of RNA-binding proteins that includes the mammalian proteins paraspeckle protein 1 (PSP1), polypyrimidine tract-binding protein-associated splicing factor (PSF) and p54^{nrb}/NonO, and the *Drosophila* protein NonA/Bj6 (Fox et al., 2002; Patton et al., 1993; Dong et al., 1993; Yang et al., 1993; von Besser et al., 1990; Jones and Rubin, 1990; Shav-Tal and Zipori, 2002). These proteins, together with Hrp65, share a conserved central domain of 320 amino acids referred to as the DBHS domain, for *Drosophila* behaviour and human splicing (Dong et al., 1993). The DBHS domain consists of two RRMs followed by a downstream element of approximately 100 amino acids recently identified as a protein-protein interaction domain [(Peng et al., 2002) and this study].

The DBHS proteins appear to play several different roles in gene expression. The most studied DBHS protein, the human PSF, was first characterized as a splicing factor (Patton et al., 1993). PSF is often found in a complex with another DBHS protein, p54^{nrb}/NonO, and several studies have implied a function for the PSF/p54^{nrb} complex in transcriptional

regulation (Basu et al., 1997; Yang et al., 1997; Mathur et al., 2001; Sewer et al., 2002; Emili et al., 2002), splicing (Peng et al., 2002) and nuclear retention of viral RNA (Zhang and Carmichael, 2001). In *Drosophila*, mutations of the NonA gene cause defects in the central nervous system that affect vision and behavior in the fly (Jones and Rubin, 1990; Rendahl et al., 1992), but the function of NonA at the molecular level is unknown.

In mammalian cells, PSF and p54^{nrb}/NonO have been found to be stably associated with each other, which suggests that dimerization among proteins of the DBHS group is functionally important. In a yeast two-hybrid screening that we report here, we have found that Hrp65 can interact with itself. We have confirmed the Hrp65 self-interaction both in vivo and in vitro, shown that the different hrp65 isoforms can interact with each other, mapped the Hrp65 self-interaction domain to the downstream element of the DBHS domain, and found that Hrp65 can not only dimerize but also oligomerize into complexes of at least 400 kDa. Moreover, we have analyzed the functional significance of the Hrp65 self-interaction and found that the NLS of Hrp65-1 is required for the nuclear location of Hrp65-2 and Hrp65-3, which suggests that the interaction between different isoforms is necessary for the nuclear import of those isoforms that lack a NLS.

Materials and Methods

Plasmid constructions for expression in HeLa cells

To obtain the Flag-Hrp65-2 fusion construct, a PCR fragment encoding the complete open reading frame (ORF) of Hrp65-2, N-terminally fused to a Flag epitope, was amplified from pEGFP-C3-Hrp65-2 (Miralles and Visa, 2001) using a sense oligonucleotide *65Bgl-Flag* (5′-gaagatctgccaccatggactacaaagacgatgacgataaagcaaggg-atgtgaaagcggaagca-3′) and an antisense oligonucleotide *652-R-X* (5′-gtcagtcctcgagccattcctataacgtgctt-3′), and subsequently cloned in a mammalian expression vector to generate Flag-Hrp65-2.

Plasmid constructions for expression in *Escherichia coli*

The three Hrp65 isoforms were expressed in bacteria with an N-terminal T7 tag and a C-terminal 6×His tag by cloning their cDNAs into pET21b (Novagen). A DNA fragment containing the complete ORF of Hrp65-2 was subcloned from pEGFP-C3-Hrp65-2 (Miralles and Visa, 2001) into the *Bam*HI-*Sal*I site of pET21b to generate T7-Hrp65-2-His. This construct was subsequently digested with *Eco*RI and *Sal*I, releasing the C-terminus of Hrp65-2. The backbone was ligated with *Eco*RI-*Sal*I and *Eco*RI-*Xho*I fragments obtained from pCR2.1-Hrp65-1 and pCR2.1-Hrp65-3 (Miralles and Visa, 2001), to obtain T7-Hrp65-1-His and T7-Hrp65-3-His, respectively.

Plasmid constructions for expression in yeast

The bait construct DBD-Hrp65 was generated by inserting a PCR fragment encoding the complete ORF of Hrp65-1 into the *Sal*I site of the pBDGAL4 vector (Stratagene), generating pBDGAL4-Hrp65. PCR fragments encoding Hrp65-1 sequences corresponding to amino acids 1-95, 1-267 and 416-535 were inserted into the *Eco*RI-*Sal*I sites of pBDGAL4 to obtain pBDGAL4-Hrp65(1-95), pBDGAL4-Hrp65(1-267) and pBDGAL4-Hrp65(416-535).

PCR fragments encoding either the complete ORF of Hrp65-3 or amino acids 89-273 and 259-415 were each cloned into the *Bam*HI-*Xho*I sites of the pADGAL4 phagemid vector (Stratagene), generating pADGAL4-Hrp65, pADGAL4-Hrp65(89-273) and pADGAL4-Hrp65(259-415). For the construction of pADGAL4-Hrp65(Δ259-415), cloning of the deletion construct was first made in a mammalian expression vector. A PCR fragment encoding Hrp65 amino acids 1-258, N-terminally fused to a Flag-epitope, was amplified from pEGFP-C3-Hrp65-2 using the *65Bgl-Flag* oligonucleotide and a 3′ oligonucleotide that gives amplification from Hrp65 amino acid 258. Another PCR fragment encoding the Hrp65 amino acids 416-517 was amplified from the same template using specific oligonucleotides. The Hrp65(1-258) and Hrp65-2(416-517) fragments were joined in-frame by a two-step cloning of the fragments into the mammalian vector, generating pCS2-Hrp65-2(Δ259-415). From this plasmid, a new PCR fragment encoding the full sequence of Hrp65-2 with the 259-415 deletion (amino acids 1-258/416-517) was amplified with oligonucleotides *65Bgl-Flag* and *652-R-X*. This fragment was inserted into the *Bam*HI-*Xho*I site of the isolated pADGAL4 vector to obtain pAD-Hrp65-2(Δ259-415).

To generate DBD-NonA(448-603) and DBD-PSF(443-601) fusion constructs, total RNA was isolated from *Drosophila* S2 Schneider cells or human HeLa cells, respectively, and used as templates for reverse transcriptase-polymerase chain reaction (RT-PCR). PCR fragments corresponding to amino acids 448-603 of NonA and 443-601 of PSF were amplified using specific oligonucleotides and inserted into the *Eco*RI-*Pst*I sites of pBDGAL4 to generate pBDGAL4-NonA(448-603) and pBDGAL4-PSF(443-601).

Yeast two-hybrid system

The interaction screen was performed essentially according to the supplier's instructions (Stratagene). A HYBRI-ZAP cDNA library from *C. tentans* tissue culture cells was constructed and cloned into the *Eco*RI and *Xho*I sites of the pAD-GAL4-2.1 phagemid vector. The cDNA library was transformed into the yeast strain AH109 (Clontech) that had been pre-transformed with DBD-Hrp65. The expression of the fusion proteins encoded in the GAL4 DBD- and AD- fusion constructs was assayed by western blotting before assessment of protein-protein interactions. The fusion proteins were also expressed one by one in yeast to rule out reporter gene activation.

Multipex PCR

DNA from isolated library plasmids was used as template in a multiplex PCR reaction with a sense *65F5* oligonucleotide that is common to all known Hrp65 isoforms plus two antisense oligonucleotides *65S*, specific for Hrp65-2 cDNA, and *65L*, which anneals both Hrp65-1 and Hrp65-3 cDNAs (Miralles and Visa, 2001).

In vitro binding assay

Plasmids encoding T7-Hrp65-1-His, T7-Hrp65-2-His or T7-Hrp65-3-His were introduced into *E. coli* Bal21(DE3). After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), bacterial extracts were prepared in 50 mM MOPS at pH 7.0, 300 mM NaCl, 15 mM imidazole, and the recombinant proteins were purified on Ni-NTA-agarose (Qiagen). The rabbit reticulocyte coupled transcription/translation system (TnT; Promega) was used for the expression of ^{35}S-methionine-labeled proteins in vitro. Ni-NTA-agarose containing purified Hrp65-1, -2 or -3 was washed in radioimmunoprecipitation (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.1 mg/ml PMSF in PBS) and incubated with translation mixtures (^{35}S-methionine-labeled Hrp65-1, -2 or -3) in RIPA buffer containing 50 mM imidazole. After extensive washing in RIPA buffer containing 50 mM imidazole, proteins were eluted, separated by SDS-PAGE and analyzed by autoradiography.

Gel filtration

Recombinant His-Hrp65-1 protein was expressed in *E. coli* as described for the in vitro binding assay and purified on Ni-NTA-

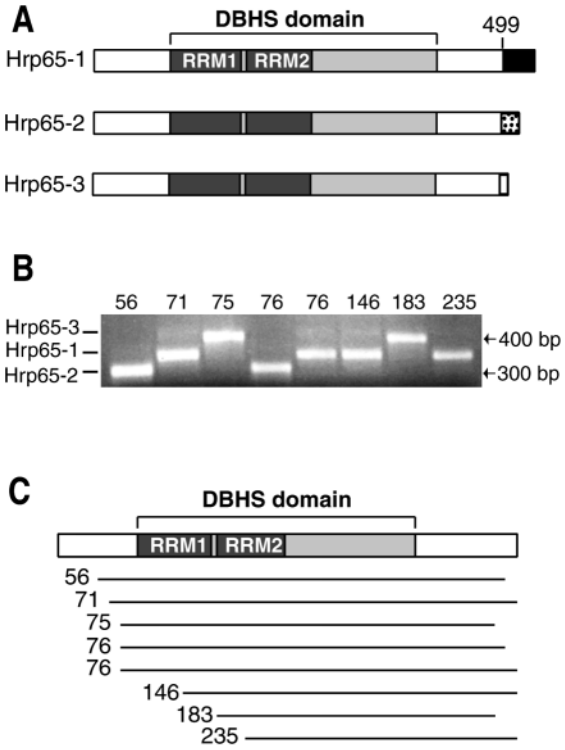

Fig. 1. Hrp65-encoding clones isolated in yeast two-hybrid screening. (A) Graphical depictions of the three Hrp65 isoforms. The sequence between amino acids 1-499 is common to all isoforms, but the C-terminal sequences are variable. (B) Identification of the Hrp65 isoforms isolated in the yeast 2-hybrid screening. Plasmids isolated from each Hrp65-encoding clone were used as templates for multiplex PCR reactions with one sense oligonucleotide that is common to all known Hrp65 isoforms and two antisense oligonucleotides that discriminate between the three Hrp65 isoforms. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The figures above the lanes indicate the first amino acid encoded in each cDNA. (C) Schematic representation of individual Hrp65 cDNAs isolated in the two-hybrid screening. For each clone, the first encoded amino acid downstream of the GAL4 AD is indicated to the left. The differences in length at the C-terminus indicate the isoform as determined in (B).

agarose (Qiagen). The purified protein was concentrated using a Nanosep device (Pall) and stored in 50 mM MOPS, 300 mM NaCl, 250 mM imidazole. The protein was fractionated on a Superose HR6 column (Amersham Biosciences) at a flow rate of 0.2 ml/minute, and fractions of 0.5 ml were collected. The proteins in the fractions were precipitated with acetone, separated on 12% SDS-PAGE and detected by Coomassie staining.

C. tentans protein extracts

Cytoplasmic and nuclear protein extracts were prepared as previously described (Miralles et al., 2000).

Transfection of HeLa cells

HeLa cells plated onto coverslips in 35 mm dishes were transiently transfected with pEGFP-C3-Hrp65-1, pEGFP-C3-Hrp65(1-499) (Miralles and Visa, 2001) or pCS2-Flag-Hrp65-2 using the Lipofectamine reagent (Invitrogen). In the same experiment, cells were cotransfected with pEGFP-C3-Hrp65-1 and pCS2-Flag-Hrp65-2 or with pEGFP-C3-Hrp65(1-499) and pCS2-Flag-Hrp65-2. Transfection medium was removed after 5 hours and cells were cultured in serum-containing medium for 12 hours before immunoflourescence. Transfection experiments were repeated at least three times with reproducible results.

Immunofluorescence

Transfected HeLa cells were treated for fluorescence using standard procedures. Briefly, cells were fixed for 15 minutes in 3.7% formaldehyde/PBS and permeabilized for 5 minutes in 0.5% Triton X-100 in PBS. Flag-tagged proteins were visualized using an anti-Flag monoclonal antibody (Sigma) and a Texas Red-conjugated secondary antibody (ICN/Cappel). Fluorescence was observed in a Zeiss Axioplan-2 microscope. Cells transfected with a single construct were also analyzed using both filters to rule out possible overlap between the GFP and the Texas Red signals.

Salivary glands from fourth instar larvae of *C. tentans* were fixed in PBS containing 4% paraformaldehyde for 60 minutes at room temperature, cryoprotected with 2.3 M sucrose and frozen by immersion in liquid nitrogen. Semi-thin sections were obtained in a cryoultramicrotome (Ultracut S/FC S, Reichert) and mounted onto glass slides as previously described (Visa et al., 1996). Indirect immunofluorescence was performed according to standard procedures using a FITC-conjugated secondary antibody.

Results

Hrp65 interacts with itself in the yeast two-hybrid system and in vitro

The full-length Hrp65-1 fused to the GAL4 DNA-binding domain (DBD-Hrp65) was used as a bait to screen an expression library obtained by fusion of the GAL4 transactivation domain (GAL4 AD) to cDNA from *C. tentans*. The interactions between bait and GAL4AD-fusion proteins were analyzed in a yeast strain containing three reporter genes (HIS3, ADE2 and lacZ/MEL1). After screening 4×10^6 clones, we isolated 54 positive clones, approximately 40% of which encoded the Hrp65 protein itself.

We have previously described three isoforms of the Hrp65 protein that are generated by alternative splicing of one pre-mRNA (Miralles and Visa, 2001). The three isoforms share amino acids 1-499 but vary in their C-termini (Fig. 1A). To determine the isoform-specificity of the Hrp65-encoding clones obtained from the two-hybrid screening, plasmid DNA isolated from individual cDNA clones was subjected to a multiplex PCR reaction with oligonucleotides designed to amplify the distinct Hrp65 cDNAs. All three Hrp65 isoforms were found in approximately equal proportions (Fig. 1B), which showed that Hrp65-1 could interact with all three Hrp65 isoforms.

Sequence analysis revealed that all the cDNAs encoding Hrp65 isolated in the screening were N-terminally truncated, as illustrated in Fig. 1C. Isolation of clones lacking amino acids 1-182 or 1-234 indicated that the Hrp65 self-interaction is mediated by a sequence downstream of the first RRM.

To verify whether the full-length Hrp65 can also self-interact, we co-expressed DBD-Hrp65 with a construct encoding the full ORF of Hrp65 fused to the GAL4 activation domain (AD-Hrp65). Yeast cells expressing both DBD-Hrp65 and AD-Hrp65 were able to grow in -His -Ade selective medium, which confirmed the ability of full-length Hrp65 to interact with itself.

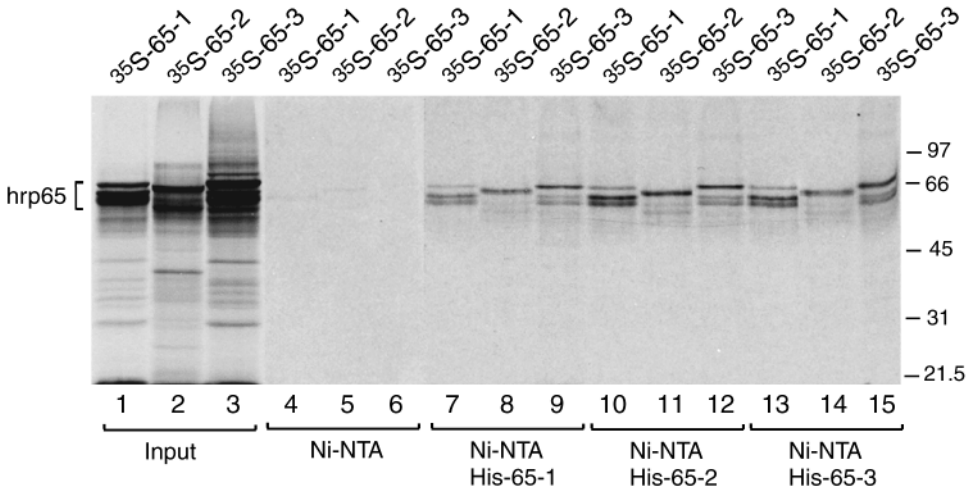

Fig. 2. In vitro binding of the three Hrp65 isoforms. Recombinant His-tagged Hrp65-1, -2 and -3 were purified on Ni-NTA-agarose and incubated in the presence of ^{35}S-methionine labeled Hrp65-1, -2 or -3 obtained by in vitro translation in rabbit reticulocyte lysate (lanes 7-15). As a negative control, Ni-NTA agarose beads without His-tagged proteins were incubated in the presence of each ^{35}S-labeled Hrp65 isoform (lanes 4-6). The bound proteins were eluted, resolved in a 10% SDS-PAGE gel and autoradiographed. ^{35}S-labeled Hrp65-1, -2 and -3 proteins were loaded as inputs (lanes 1-3). The multiple Hrp65 bands observed in each input lane (bracket) are due to partial degradation and to alternative translation initiation sites. The mobility of molecular mass standards is indicated on the right (kDa).

To confirm the results of the yeast two-hybrid screening, we assayed the interaction of Hrp65-1 with the three Hrp65 isoforms in vitro. Recombinant Hrp65-1 was expressed with a C-terminal 6×His-tag (His-Hrp65-1) and immobilized on Ni-NTA agarose. The immobilized His-Hrp65-1 was then incubated in a high stringency RIPA buffer with ^{35}S-labeled Hrp65-1, -2 or -3 obtained by in vitro translation. In parallel, ^{35}S-labeled proteins were incubated with Ni-NTA agarose without His-Hrp65 as a negative control. The ^{35}S-Hrp65-1, -2 and -3 bound with similar efficiencies to His-Hrp65-1 immobilized on Ni-NTA (Fig. 2, lanes 7-9), but showed very little or no binding to the Ni-NTA agarose alone (Fig. 2, lanes 4-6). A control ^{35}S-chloramphenicol acetyltransferase (CAT) protein did not bind to His-Hrp65 proteins immobilized on Ni-NTA agarose (not shown).

To investigate whether Hrp65-2 and Hrp65-3 can also interact with themselves and/or each other, we expressed recombinant His-tagged Hrp65-2 and -3 and assayed the interaction of these isoforms with the ^{35}S-Hrp65 isoforms. Similarly to His-Hrp65-1, His-Hrp65-2 and -3 bound all three Hrp65 isoforms to the same extent (Fig. 2, lanes 10-15). These observations confirm that the in vitro interaction of Hrp65 is isoform-independent, in agreement with the results from the yeast two-hybrid screening reported above (Fig. 1C).

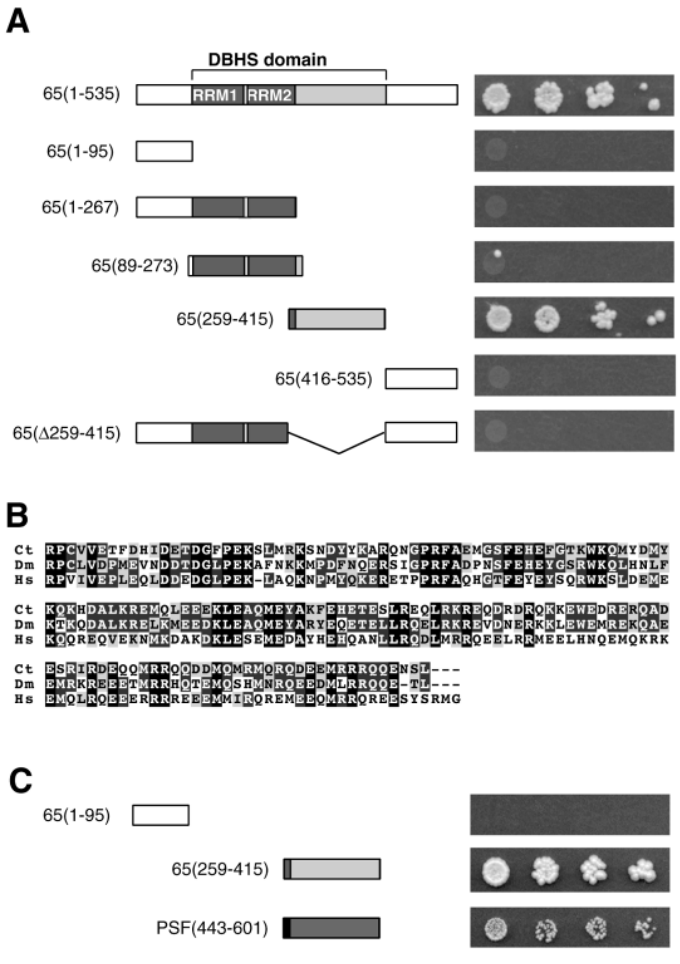

Fig. 3. Mapping of the self-interaction domain. Truncations and deletions of the Hrp65 protein (or PSF in part B) were expressed as GAL4-AD or -BD fusions and assayed for their ability to interact with full-length Hrp65 in the yeast two-hybrid system. Graphical representations of the assayed constructs are shown on the left. (A) Mapping of the Hrp65 self-interaction domain. Yeast cells were transformed with a full-length Hrp65 construct plus a truncation/deletion construct. The protein-protein interactions were detected by plating serial dilutions of the co-transformants onto double selective -His -Ade medium. (B) ClustalW alignment of the protein binding domains (PBDs) *of C. tentans* Hrp65 (residues 259-415), *Drosophila melanogaster* NonA (residues 448-603) and *Homo sapiens* PSF (residues 443-601). Residues identical in all three sequences are shown in black boxes, identical residues in two out of three compared sequences are shown in dark grey and similar residues in two out of three compared sequences are shown in light grey. (C) Interaction of the PBDs of Hrp65 and PSF with the full-length Hrp65. Protein-protein interactions were detected as in (A), except that the transformants were selected on -His plates.

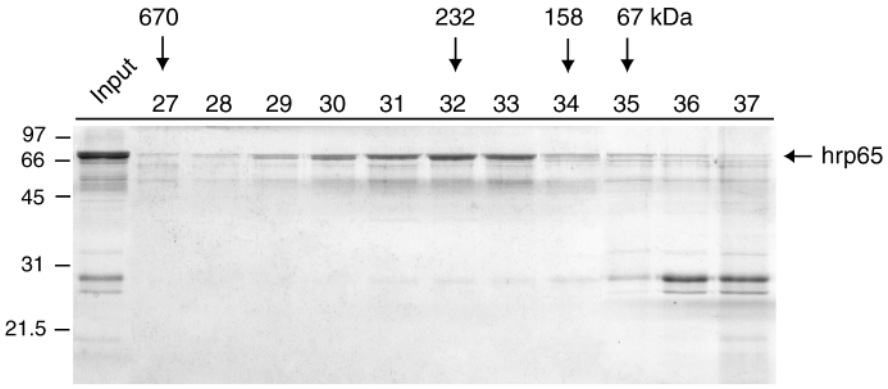

Fig. 4. Chromatographic analysis of Hrp65 oligomers. Recombinant Hrp65-1 purified on Ni-NTA agarose was fractionated on a Superose HR6^{TM} gel filtration column and the protein in each fraction was analyzed by 12% SDS-PAGE and Coomassie staining. Hrp65 was detected from fraction 27 to fraction 37. A major part of the protein fractionated as a broad peak between fractions 29-33. The fractionation of molecular mass standards in the gel filtration column is indicated at the top. The mobilities of molecular mass standards in the SDS-PAGE are shown on the left, in kDa.

Hrp65 self-interaction is mediated by the downstream element of the conserved DBHS domain

To identify the region of Hrp65 that mediates Hrp65-Hrp65 interaction, we expressed truncated Hrp65 mutants and assayed their ability to interact with full-length Hrp65 in the two-hybrid system. The results from these experiments are shown in Fig. 3A. Yeast transformed with two full-length Hrp65 constructs could grow readily under -His as well as -His -Ade selection (Fig. 3A, top). As expected from the isolation of N-terminally truncated clones in the two-hybrid screening, a truncation construct encoding the N-terminal domain of Hrp65, 65(1-95), showed no interaction with the full-length protein. A construct containing the C-terminal domain, 65(416-535), did not show any interaction either. We then tested two constructs corresponding to the central DBHS element, one containing the two RRMs, 65(89-273), and another containing the downstream region of the DBHS domain, 65(259-415). The transformants of the 65(89-273) construct showed poor growth on double-selective medium, but the transformants of the 65(259-415) construct were able to grow on -His -Ade medium, which showed that the downstream DBHS element interacted strongly with Hrp65. As no growth on double-selective medium could be detected with a construct comprising the entire N-terminal domain plus the two RRMs, 65(1-267), we concluded that the 259-415 region alone is responsible for mediating Hrp65-Hrp65 interaction. Moreover, removal of the same region (Δ259-415) totally abolished the interaction, showing that the 259-415 region is not only sufficient but necessary for mediating interaction between Hrp65 proteins.

In view of the present results, the DBHS domain can be described as a compound domain that contains two functional elements: an upstream element with two juxtaposed RRMs, and a downstream element that mediates protein self-interaction. We refer to this latter element as the protein binding domain, or PBD.

Other proteins of the DBHS group have been found to be associated with each other (see Discussion), and sequence comparisons show that the PBD is evolutionarily conserved among proteins of the DBHS group (Fig. 3B). The PBD of Hrp65 is 57% and 38% identical to the PBDs of *Drosophila* NonA and human PSF, respectively. To analyze the ability of the PBD to mediate interactions between DBHS proteins, we expressed the PBDs of the NonA (amino acids 448-603) and

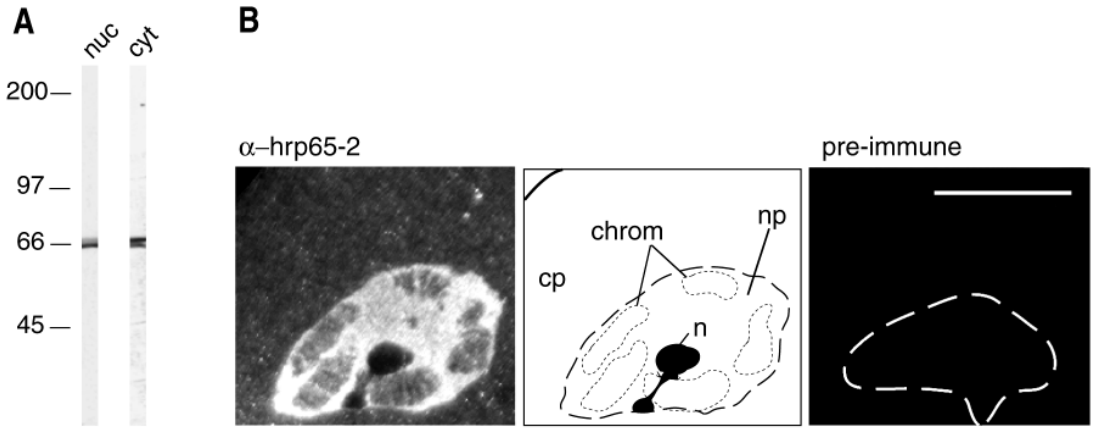

Fig. 5. Subcellular localization of the Hrp65-2 isoform in *C. tentans* cells. (A) Immunoreactivity of the anti-Hrp65-2 antibody analyzed by western blot analysis of nuclear and cytoplasmic protein extracts prepared from *C. tentans* tissue culture cells. The proteins in each extract were separated by SDS-PAGE and blotted to transfer membranes. The membranes were cut into strips and incubated with the anti-Hrp65-2 antibody. The mobilities of molecular mass standards, in kDa, are shown on the left. (B) Semi-thin cryosections of *C. tentans* salivary gland cells were stained with the anti-Hrp65-2 specific antibody followed by a FITC-conjugated secondary antibody. An illustration of the subcellular structures that are visible after immunofluorescent labeling is shown in the middle. Cp, cytoplasm; chrom, polytene chromosomes; n, nucleolus; np, nucleoplasm. The broken line represents the nuclear envelope and the solid line represents the cell surface. The pre-immune serum was used in parallel as a negative control, and the picture was overlaid with a broken line to demarcate the border between nucleus and cytoplasm.

the PSF (amino acids 443-601) in the yeast two-hybrid system and analyzed their interaction with the full-length Hrp65. We found that both PSF(443-601) and NonA(448-603) interact with Hrp65 (Fig. 3C and data not shown). This result shows that the PBD is a conserved self-interaction domain for DBHS-proteins, capable of mediating both homodimerization and heterodimerization.

Hrp65 oligomerization in vitro

The observed Hrp65 self-interaction raised the possibility that the Hrp65 could form oligomers, which could be important for the formation of CFs in vivo. To learn more about the Hrp65-Hrp65 association, we analyzed oligomerization of recombinant Hrp65 in vitro. Electrophoretic analysis of purified Hrp65 by SDS-PAGE in the absence of reducing agents revealed the formation of high-molecular-weight complexes that were not affected by RNAse A digestion (data not shown). To analyze the molecular mass of the detected Hrp65 complexes, we purified recombinant Hrp65 and fractionated it on a size-exclusion Superose HR6^{TM} chromatography column. The proteins in each fraction were collected and separated by SDS-PAGE. Most of the protein was detected in fractions 29-33, corresponding to molecular masses in the 200-400 kDa range (Fig. 4). This range corresponds to Hrp65 complexes between the size of a trimer and a hexamer. Thus, we conclude that in our experimental conditions most of the Hrp65 protein is in complexes that consist of three to six Hrp65 molecules.

Hrp isoforms that lack NLS can enter the nucleus by interacting with Hrp65-1

We have previously shown by transient transfection assays that the variant C-terminal sequences of the Hrp65 proteins are relevant for the subcellular location of each isoform (Miralles and Visa, 2001). When expressed in human and *Drosophila* cells, GFP-Hrp65-2 and GFP-Hrp65-3 locate mainly to the cytoplasm, while GFP-Hrp65-1 is efficiently imported to the nucleus due to the presence of a NLS in its C-terminus (Miralles and Visa, 2001). To analyze the cellular distribution of endogenous Hrp65-2 in *C. tentans* cells, we used a rabbit antibody against the C-terminal sequence of Hrp65-2 (Percipalle et al., in press). This antibody labeled a protein of approximately 65 kDa in both cytoplasmic and nuclear extracts of *C. tentans* tissue culture cells (Fig. 5A). We also used the anti-Hrp65-2 antibody for immunoflourescent labeling of *C. tentans* salivary gland cells. The Hrp65-2 antibody labeled chromosomes and nucleoplasm, and also gave a faint spotted staining in the cytoplasm (Fig. 5B). Thus, a significant fraction of the endogenous Hrp65-2 is present in the nuclei of *C. tentans* cells, despite the absence of an NLS in Hrp65-2. This suggested that in vivo nuclear import of Hrp65-2 is mediated by interaction with another protein. Given the strong interaction observed between Hrp65 isoforms, we decided to investigate the possibility that Hrp65-2 enters the nucleus by binding to Hrp65-1. For this purpose, we analyzed the simultaneous expression of different Hrp65 isoforms. Owing to the absence of methods for transfecting *C. tentans* cells, we performed transient transfections of Hrp65 constructs in HeLa cells. To be able to analyze co-expression of Hrp65-1 and Hrp65-2, we expressed Hrp65-1 as a fusion with GFP and Hrp65-2 with a Flag tag. Transfection of the Flag-Hrp65-2 construct showed that Flag-Hrp65-2 localizes mainly to the cytoplasm (Fig. 6A), which agrees with the previously reported localization of GFP-Hrp65-2 (Miralles and Visa, 2001). GFP-Hrp65-1 localizes to the nucleus, while a truncation of the C-terminal sequence of Hrp65-1 that contains the NLS [GFP-Hrp65-1(1-499)] renders the protein cytoplasmic. We then proceeded to cotransfection experiments to analyze the distribution of Hrp65-2 in the presence of Hrp65-1. Interestingly, when co-expressed with GFP-Hrp65-1, the distribution of Flag-Hrp65-2 became mainly nuclear (Fig. 6B). Moreover, when Flag-Hrp65-2 was co-expressed with GFP-Hrp65-1(1-499) instead of GFP-Hrp65-1, both GFP-Hrp65-1(1-499) and Flag-Hrp65-2 remained in the cytoplasm. Similar results were obtained for Hrp65-1 and Hrp65-3: Hrp65-3 was cytoplasmic when expressed alone, but nuclear when co-expressed with Hrp65-1 (not shown). Our experiments show that Hrp65-2 and -3 can enter the nucleus by association with Hrp65-1.

Discussion

The ability of Hrp65 to interact with itself was first detected in a yeast two-hybrid screening. We have subsequently carried out in vitro binding experiments, electrophoretic analysis of recombinant Hrp65, and gel filtration assays to confirm and further characterize the Hrp65-Hrp65 interaction. Using these methods we have shown that all three Hrp65 isoforms can bind to themselves and to each other, that the Hrp65-Hrp65 interaction is direct and RNA-independent and that, at least in vitro, Hrp65 can build oligomers of up to 400 kDa. Furthermore, cotransfection studies have revealed that the Hrp65-Hrp65 interaction is necessary for intracellular targeting of different Hrp65 isoforms.

The DBHS domain contains a protein-binding domain

We have mapped the sequences responsible for Hrp65 self-interaction to a part of the protein that we refer to as the PBD located next to the RRMs in the DBHS domain. The PBD of Hrp65 is conserved in all the members of the DBHS group and the degree of similarity in this region is comparable to that observed in the RRMs.

In mammalian cells, PSF and p54^{nrb} have been found to be associated with each other (e.g. Mathur et al., 2001; Zhang and Carmichael, 2001), and deletions covering the PBD region of p54^{nrb} have been shown to disrupt the p54^{nrb}-PSF association (Peng et al., 2002). We have now shown that the PBDs of NonA and PSF are sufficient to mediate the interaction with full-length Hrp65 in vivo. Altogether, these observations indicate that the PBD is an evolutionary conserved domain able to mediate not only self-interaction but also heterodimerization between different proteins of the DBHS group.

The DBHS proteins appear to be able to interact with a large number of proteins in different physiological situations. For instance, PSF has been associated with several pre-mRNA splicing factors (Patton et al., 1993; Lutz et al., 1998; Peng et al., 2002). PSF and p54^{nrb}, in a complex with matrin 3, have been implicated in nuclear retention of inosine-rich RNA, as part of a cellular defense mechanism aimed at preventing

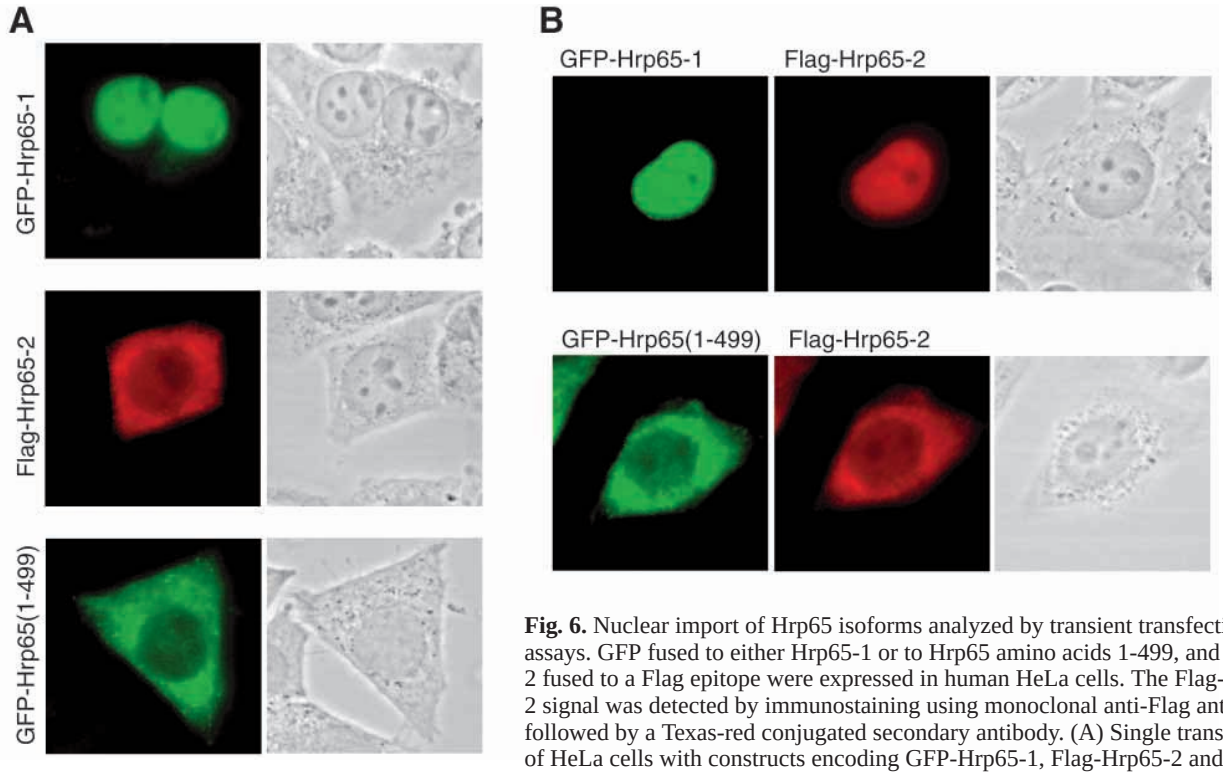

Fig. 6. Nuclear import of Hrp65 isoforms analyzed by transient transfection assays. GFP fused to either Hrp65-1 or to Hrp65 amino acids 1-499, and Hrp65-2 fused to a Flag epitope were expressed in human HeLa cells. The Flag-Hrp65-2 signal was detected by immunostaining using monoclonal anti-Flag antibody followed by a Texas-red conjugated secondary antibody. (A) Single transfection of HeLa cells with constructs encoding GFP-Hrp65-1, Flag-Hrp65-2 and GFP-Hrp65(1-499). (B) Flag-Hrp65-2 was expressed together with either GFP-Hrp65-1 or GFP-Hrp65(1-499) by cotransfection of two constructs into HeLa cells. Transfected cells are shown in phase contrast on the right.

expression of viral genomes (Zhang and Carmichael, 2001). PSF has been found associated with nuclear structures such as the nuclear envelope (Otto et al., 2001) and the nucleolus (Fox et al., 2002). PSF and p54^{nrb} have also been found associated with the RNA polymerase II (Emili et al., 2002), and the p54^{nrb}-PSF complex has been implicated in transcriptional regulation through interactions with nuclear hormone receptors and Sin3A (Mathur et al., 2001; Sewer et al., 2002). In *C. tentans*, Hrp65 has been found to be associated, directly or indirectly, with a large number of proteins, including the hnRNP protein hrp36 (Wurtz et al., 1996), the putative transcription factor p2D10 (Sabri et al., 2002), and actin (Percipalle et al., 2003). These many different interactions and the multiple functions attributed to the DBHS proteins, suggest that a general role of this family of proteins is to serve as platforms for protein-protein interactions in different cellular processes. In this context, the dimerization or oligomerization of the DBHS proteins may constitute a mechanism to mediate long-range intermolecular interactions.

A role for Hrp65 in the formation of connecting fibers?

Hrp65 was identified in the salivary gland cells of *C. tentans* as a protein located in connecting fibers (CFs), thin fibers associated with pre-mRNPs in transit from the gene to the nuclear envelope (Miralles et al., 2000). Whether hrp65 is a structural component of the CFs or a protein bound to the CFs remains to be established. However, our present finding that Hrp65 can build oligomeric complexes suggests that this protein could be a structural component of the CFs. On the basis of the dimensions of the CFs, characterized by a diameter of approximately 5-7 nm and a variable length in the 50-100 nm range, it can be estimated that a typical CF is composed of 50-100 protein molecules of average size. Thus, the molecular mass of the oligomers detected in our in vitro assay is not sufficient to explain the formation of CFs as it occurs in vivo. Perhaps Hrp65 contributes only to the proximal part of the CF, in contact with the pre-mRNP, as suggested by previous immuno-electron microscopy experiments showing that Hrp65 is not located along the full length of the CFs but only in the proximal part (Miralles et al., 2000). It is also likely that other proteins contribute to the architecture of CFs.

We attempted to study the functional significance of Hrp65 self-interaction in CF formation by microinjecting recombinant PBD into live salivary gland cells. However, such experiments failed beause we could not prevent the recombinant PBD from polymerizing in vitro under the physiological conditions required for microinjection.

It is also important to notice that both *C. tentans* Hrp65 and recombinant Hrp65 produced in *E. coli* are prone to form large insoluble aggregates under native conditions. The buffers used in our protocol for Hrp65 purification were optimized to keep the recombinant Hrp65 in solution (see Materials and Methods), and by doing so we may have impaired the formation of larger Hrp65 multimers. In summary, our results indicate that the Hrp65 protein has the ability to form oligomeric complexes, but the molecular mass of Hrp65 oligomers in vivo and their contribution to the formation of CFs must be further investigated.

The Hrp65-Hrp65 interaction is required for intracellular localization of Hrp65 isoforms

Our present results reveal that the Hrp65-Hrp65 interaction is required for the localization of the Hrp65 isoforms inside the cell. The nuclear import of Hrp65-1 is mediated by an NLS located in the C-terminus of the protein, whereas Hrp65-2 and Hrp65-3 lack this NLS and are cytoplasmic when transiently expressed in heterologous systems (Miralles and Visa, 2001). We have now reported that the PBD of PSF can interact with Hrp65, which suggests that the endogenous PSF could potentially mediate nuclear import of Hrp65 isoforms in transfected mammalian cells. Indeed, in single transfection experiments, a certain increase in nuclear fluorescence was observed after long post-transfection times for both Hrp65-2 and Hrp65-3. However, in the conditions of our experiments, Hrp65-2 and Hrp65-3 appeared as mainly cytoplasmic due to the high overexpression levels.

Immunoblot and immunofluorescence analysis with an antibody specific for Hrp65-2 showed that the endogenous Hrp65-2 isoform is not restricted to the cytoplasm but is also present in the nucleus of *C. tentans* cells, in agreement with the reported location of Hrp65-2 at transcription sites on *C. tentans* polytene chromosomes (Percipalle et al., 2003). Our cotransfection experiments indicate that the import of Hrp65-2 and Hrp65-3 is dependent on the presence of Hrp65-1 and requires the C-terminal NLS of Hrp65-1. Thus, we conclude that Hrp65-2 and Hrp65-3 are imported into the nucleus associated with Hrp65-1. Given the role of Hrp65-2 in transcription (Percipalle et al., 2003), it is tempting to speculate that this heterodimerization-dependent import could be regulated and could influence other intranuclear events.

NonA, PSP1 and PSF also exist in several isoforms with variable C-terminal sequences, and in each case one of the isoforms is similar to Hrp65-1. Moreover, at least in the case of PSF, one isoform lacks the C-terminal NLS (Dye and Patton, 2001), as does Hrp65-2. The present results about Hrp65, together with the conservation of isoform structure among DBHS proteins, suggest that the intracellular localization of other DBHS proteins is also based on dimerization/oligomerization mechanisms.

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