Complexes between the nonsense-mediated mRNA decay pathway factor human upf1 (up-frameshift protein 1) and essential nonsense-mediated mRNA decay factors in HeLa cells

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mRNAs harbouring premature translation-termination codons are usually degraded by the nonsense-mediated mRNA decay (NMD) pathway. Human up-frameshift protein 1 (Hupf1) is an NMD factor that is conserved between yeast and mammals. To isolate cellular complexes that are formed with Hupf1 and to explore the role of cellular proteins in NMD, we generated a HeLa cell line that stably expresses Hupf1 bearing a double-affinity tag (termed Hupf1-2tag). Hupf1-2tag is localized in the cytoplasm similar to the endogenous Hupf1 protein, and the Hupf1-2tag cell line is fully NMD-competent. Using affinity chromatography, Hupf1-2tag-associated proteins were isolated. MS and immunoblotting identified the NMD factors Hupf2 and Hupf3a/b as interaction partners of Hupf1. Size-exclusion chromatography indicates that the NMD factors Hupf1, Hupf2 and the large isoform of Hupf3a might exist in a stable,

high-molecular-mass complex of approx. 1.3 MDa. Interestingly, the poly(A)-binding protein was also identified by MS to be associated specifically with Hupf1-2tag. In contrast with the interaction with Hupf2 and Hupf3a/b, the association of poly(A)-binding protein with Hupf1 is highly sensitive to treatment of the isolated complexes with RNase. Components of the exon–exon junction complex or the translational eukaryotic release factor (eRF) 3 were not identified in complexes associated with Hupf1-2tag. We discuss these findings in the context of current models of NMD.

Key words: human up-frameshift protein 1 (upf1)/Rent1, nonsense-mediated mRNA decay (NMD), poly(A)-binding protein (Pabp), RNA surveillance, tandem-affinity purification.

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) represents a conserved surveillance pathway by which cells can specifically eliminate mRNAs that carry premature translation-termination codons (PTCs; reviewed in [1–4]). Such mRNAs are potentially deleterious for cells or organisms, because they encode C-terminal-truncated polypeptides that might, when translated, exert dominant-negative effects. In B- and T-lymphocytes, somatic mutation and recombination events frequently generate PTC-containing mRNAs. The NMD pathway is assumed to prevent the expression of truncated immunoglobulin and T-cell-receptor polypeptides arising from such aberrant mRNAs [5]. The beneficial role of the NMD system is also illustrated by genetic disorders that normally follow a recessive mode of inheritance, but can manifest as dominant diseases when the mutant transcripts escape NMD. Examples of this include mutations that introduce PTCs into the human *ROR2* mRNA and cause brachydactyly type B [6] or mutations of the β -globin gene, which cause β -thalassaemias [7,8].

Mammalian cells distinguish normal and premature translation-termination codons from each other by referencing their position to that of the final intron: stop codons followed by an intron at 50 or more nucleotides downstream are considered ‘premature’, and normally trigger NMD [9–11]. It is thus not surprising

that most normal translation-termination codons are located in the 3′-terminal exon [12]. Since introns are removed from the nucleus and translation occurs in the cytoplasm, the position of removed introns is assumed to be marked by the so-called exon–exon junction complex (EJC). The EJC is deposited on mRNAs as a consequence of splicing, and some components of the EJC accompany spliced mRNAs into the cytoplasm [13–15]. According to current models of NMD [14,16,17], a stop codon is interpreted as ‘premature’ if the components of the EJC or factors associated with it are recognized by a translation termination-dependent process to be bound downstream of a stop codon.

Genetic studies in yeast initially identified three *trans*-acting NMD factors, upf1 (up-frameshift protein 1)–upf3 [18–20]. Mutations in either of these can impair NMD without affecting other mRNA degradation pathways (reviewed in [1,21]). Recently, the mammalian orthologues, termed Hupf1–3 (where Hupf1 stands for human upf1), have been identified in mammals and shown to be involved functionally in NMD (reviewed in [16,17]). When tethered to the 3′-untranslated region of a wild-type β -globin reporter mRNA, all three proteins can provoke NMD of this mRNA [22] and thus may mimic the presence of a downstream EJC. Hupf3 occurs in two forms, Hupf3a and Hupf3b, which are encoded by two different genes [22,23]. In addition, Hupf3a occurs in two isoforms with different molecular masses [22,23]. Hupf3 has been suggested to provide a biochemical link

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EJC, exon–exon junction complex; eRF, eukaryotic release factor; Hupf1, human up-frameshift protein 1; NMD, nonsense-mediated mRNA decay; ORF, open reading frame; Pabp, poly(A)-binding protein; PTC, premature translation-termination codon; SMG, suppressor with morphogenetic effect on genitalia; TEV, tobacco etch virus.

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between the NMD pathway and the EJC [13–15,24]: RNPS1 and Y14, both components of the EJC, have been reported to interact with Hupf3 [25,26] and to trigger NMD in a tethering assay [25,27].

Hupf2 is a protein that shares conserved domains with the eukaryotic translation-initiation factor eIF4G. It interacts with both Hupf1 and Hupf3a/b, and is involved functionally in NMD [22,23,28]. Furthermore, in a yeast two-hybrid assay, Hupf2 was shown to interact with the eukaryotic translation-initiation factor eIF4A and Sui1, a subunit of the translation-initiation factor eIF3 [28].

Upf1 is conserved in yeast, nematode worms [29] and mammals [30,31]. Hupf1 may represent a biochemical link between upstream events in NMD and the process of translation termination at premature stop codons, because it can interact with the upstream NMD factor upf2 and with the translational eukaryotic release factors (eRFs) 1 and 3 [22,23,28,32]. Hupf1 has been demonstrated to be involved functionally in NMD, because a dominant-negative mutant of Hupf1 as well as knock-down of Hupf1 by RNA interference inhibits NMD in mammalian cells [30,31,33,34]. Hupf1 belongs to the superfamily of group I helicases, which display RNA-binding activity that can be modulated by ATP [35]. Hupf1 is a phosphoprotein [36] and a substrate for the human suppressor with morphogenetic effect on genitalia (hSMG)-1 kinase [37]. Inactivation of hSMG-1 leads to an accumulation of PTC-containing transcripts [37]. Taken together, these findings suggest that Hupf1 is a central component of the NMD pathway in mammals.

To examine the biochemical interactions of Hupf1 *in vivo*, we generated an affinity-tagged variant of Hupf1 and introduced it stably into the HeLa cells. Hupf1-associated protein complexes were isolated and characterized by MS, size-exclusion chromatography and immunoblotting. Using this approach, we found the poly(A)-binding protein (Pabp) to be a novel interaction partner of Hupf1 and show that Hupf1 forms biochemically distinct complexes *in vivo*.

EXPERIMENTAL

Plasmid construction

To generate the plasmid pcDNA (Hupf1-2tag), the transgenic Hupf1-2tag was first assembled in the vector pGEM (Hupf1-2tag) and then subcloned from pGEM (Promega, Mannheim, Germany) to pcDNA3.1(–) (Invitrogen, Karlsruhe, Germany) using the *XhoI*–*BamHI* sites. pGEM (Hupf1-2tag) was obtained as follows: 3'-end of the Hupf1 open reading frame (ORF) was modified by PCR using *Pfu* polymerase (Stratagene, Heidelberg, Germany) to delete the stop codon and insert an *NcoI* and *EcoRI* cleavage site for insertion of the affinity tag (see below). The primers for this PCR were 5'-GCAAGCCACGGAAGC-3' (sense: 2741–2755 nt of Hupf1 ORF) and 5'-CGAATTCCTC-ATGGCATACTGGGACAGCCCC-3' (antisense: *NcoI*, boldface; *EcoRI*, underlined). The PCR product was cleaved with *PmlI* and *EcoRI*, and then inserted into the *PmlI*–*EcoRI* sites of pGEM (Hupf1) [30]. The resulting vector was digested with *NcoI* and *HindIII*, and the *NcoI*–*HindIII* fragment of the plasmid pBS1479 containing the Protein A tag (tandem-affinity purification tag) [38] was inserted to yield pGEM (Hupf1-2tag). The sequence of these constructs was confirmed by DNA sequencing (GATC Biotech AG, Konstanz, Germany).

Cell culture and stable transfection

Adherent HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) under standard conditions. The DMEM

was supplemented with 10 % (v/v) foetal bovine serum (Cambrex, Apen, Germany), 2 mM L-glutamine and 1 × antibiotic–antimycotic solution (Invitrogen, Karlsruhe, Germany). Cells were transfected with 2 µg of linearized pcDNA (Hupf1-2tag) using Effectene reagent according to the manufacturer's instructions (Qiagen, Hilden, Germany). After transfection, for 2 days, the medium was supplemented with 600 µg/ml G418 (geneticin; Invitrogen) to select the transfected cells. Clonal descendents of the transfectants were grown in the presence of G418 and the expression of the transgenic Hupf1-2tag was assessed by Western blotting for each clone. Clone 32 was chosen for the purpose of the present study and maintained in DMEM, supplemented with 500 µg/ml G418 by the Computer Cell Culture Center (Seneffe, Belgium). Untransfected wild-type HeLa S3 cells grown in suspension culture were purchased from the same source and served as controls.

Preparation of cytoplasmic extracts

For the preparation of cytoplasmic extracts, 1×10^9 cells were re-suspended in an equal volume of hypo-osmotic lysis buffer [10 mM Tris/HCl (pH 8), 10 mM NaCl, 2 mM MgCl₂, 0.2 mM dithiothreitol, 0.1 mM PMSF and 1 × Complete (Roche, Mannheim, Germany)]. Cells were lysed with a loose-fit Potter–Elvehjem homogenizer and cleared by centrifugation at 15 000 g for 30 min at 6 °C. Finally, the cytoplasmic extracts were supplemented with NaCl to obtain a final concentration of 50 mM. Aliquots of the lysates were either used immediately or stored at –70 °C until used.

Treatment of extracts with RNase A and preparation of total RNA

RNA was digested by treating the cytoplasmic extracts with 20 µg/ml RNase A (Sigma) for 15 min on ice. Before and after the RNase treatment, total RNA was isolated from the extracts (1.5 mg of total protein) by phenol/chloroform extraction.

Production of antibodies

An anti-(Hupf1 peptide^{106–123}) serum was generated in rabbits as described previously [30]. The serum was purified using Protein A–Sepharose as described previously [39]. For Western blotting, the purified serum was used at a dilution of 1:600. An anti-Hupf2 serum was raised in rabbits as described previously [22]. The serum was used for Western blotting at a dilution of 1:600.

Immunopurifications and tobacco etch virus (TEV) elution

For immunopurifications, cytoplasmic extracts of the parental or Hupf1-2tag-expressing cell lines (12 mg of total protein/reaction) were used. For the identification of Hupf1-interacting proteins by MS, 120 mg of total protein was used for the immunopurifications. To avoid elution of IgG chains, IgG–Sepharose (Amersham Biosciences, Freiburg, Germany) was cross-linked using dimethyl pimelimidate as described previously [39]. Typically, three reactions were performed in batch; the corresponding amount of the extracts was incubated for 1 h at 6 °C with 60 µl of cross-linked IgG–Sepharose on a rotating wheel. After a brief wash with buffer T8N150 [10 mM Tris/HCl (pH 8)/150 mM NaCl/0.2 mM dithiothreitol], the bound material was washed three times for 5 min at 6 °C on a rotating wheel. Elution from the IgG–Sepharose was performed in a volume of 60 µl of T8N150 by incubation with 50 units of recombinant TEV (tobacco-etch-virus) protease at 15 °C for 90 min. The bead suspension was

transferred to disposable microcentrifuge spin columns (Mobicol; MoBiTec, Goettingen, Germany) and released complexes were eluted by centrifugation. The eluate was concentrated under vacuum and divided into equal volumes according to the number of reactions that were performed in batches. Proteins were separated by SDS/PAGE and transferred on to PVDF membranes. For MS analysis, one-tenth of the eluate was loaded on to an SDS/polyacrylamide gel column for immunoblotting and the rest was loaded on to an SDS/polyacrylamide gel column for silver staining.

MS

Silver-stained protein bands were excised from one-dimensional polyacrylamide gels, reduced, alkylated and in-gel-digested with trypsin (Roche) as described previously [40]. After extraction from the gel pieces, the peptides were desalted on POROS R2 reversed-phase material (PerSeptive Biosystems, Cambridge, MA, U.S.A.) in self-assembled mini-columns and eluted directly into the spraying capillary. Tryptic peptides were sequenced by nanoelectrospray tandem MS on an API III triple quadrupole mass spectrometer (PE-Sciex; MDX Sciex, Toronto, Ont., Canada; see [41]). Proteins were identified by searching manually assembled peptide sequence tags against the comprehensive non-redundant protein database (nrdb) from the European Bioinformatics Institute (Hinxton, Cambridge, U.K.), containing 840 000 proteins using PeptideSearch version 3.0 [42].

Immunofluorescence

Cells were grown on coverslips under standard conditions, fixed with 2 % (w/v) paraformaldehyde and permeabilized with 0.1 % Triton X-100 (Merck, Darmstadt, Germany) as described previously [39]. DNA was stained with Hoechst 33342 (Molecular Probes, Eugene, OR, U.S.A.). The fixed cells were then incubated for 1 h with an Alexa 488-coupled anti-rabbit IgG (Molecular Probes) and washed three times with PBS. The coverslips were mounted on glass slides with Vectashield (Vector Laboratories, Peterborough, U.K.). Images were acquired with a Leica fluorescence microscope and processed using Adobe Photoshop 6.0.

SDS/PAGE, silver staining and immunoblotting

For detection of proteins by silver staining or immunoblotting, samples were resolved by SDS/PAGE. For the identification of Hupf1-interacting proteins by MS, the gel was subsequently fixed and proteins were stained with silver as described previously [40]. For immunoblotting, the proteins were transferred from the gel on to a PVDF membrane (Immobilon P; Millipore, Eschborn, Germany) by wet blotting. Western-blot analyses were performed using the appropriate antibodies as described previously [39] using 3 % (w/v) skimmed milk in PBS/Tween for blocking. Proteins were visualized using Renaissance Western-blot detection reagents (PerkinElmer LifeSciences, Rodgau, Germany). In some cases (indicated in the Figure legends), the membranes were re-probed with additional antibodies without prior stripping of the membrane.

Ammonium sulphate precipitation and gel filtration

Saturated ammonium sulphate solution (pH 8.5) was added to extracts of the Hupf1-2tag-expressing cell line (25 mg of total protein) to reach a final saturation of 30 % saturated ammonium

sulphate. After incubation on ice for 30 min, the precipitate was pelleted by centrifugation at 12 000 *g* (6 °C). The pellet was re-dissolved in buffer T8N150 and loaded on to a 24 ml Superose 6 FPLC column (Amersham Biosciences) equilibrated with the same buffer. Fractions (300 μ l) were collected from 7 to 15.3 ml elution volume. Two subsequent fractions were pooled and concentrated. One-third of these pooled fractions was treated with 30 units of recombinant TEV protease at 6 °C for 90 min and then resolved by SDS/PAGE. Finally, proteins were detected by immunoblotting. A calibration curve was obtained by running the proteins thyroglobulin, ferritin and aldolase (Amersham Biosciences) on the same column under the same conditions, and plotting the respective elution volume versus the molecular mass on a semi-logarithmic scale.

RESULTS

Generation of an NMD-competent HeLa cell line stably expressing tagged Hupf1

For biochemical purification of Hupf1-containing cellular protein complexes assembled *in vivo*, we generated a tagged version of Hupf1 (Figure 1A). A double-affinity tag [38], consisting of the calmodulin-binding domain, a TEV protease cleavage site and the Protein A tag was added to the 3'-end of the Hupf1 ORF, and the construct was transfected stably into HeLa cells. To avoid 'artificial' non-specific biochemical interactions of the tagged protein arising from the strong overexpression of the transgene, we isolated and characterized a HeLa cell line that expresses tagged Hupf1 at a level below the expression of the endogenous protein (Figure 1B). Owing to the size difference generated by the calmodulin-binding domain, the expression of transgenic Hupf1 could be compared with that of endogenous Hupf1 by Western blotting directly after the removal of the Protein A part of the tag by cleavage with TEV protease. This step was necessary to avoid the strong interaction of IgG molecules of the α Hupf1 serum with the Protein A tag in an epitope-independent way. The cleavage product, termed 'Hupf1-1tag' (Figure 1A), migrates slower than the wild-type Hupf1 on an SDS/polyacrylamide gel and can therefore be distinguished readily.

Next, we investigated the subcellular localization of Hupf1-2tag. Owing to the ability of the Protein A part of Hupf1-2tag to interact with the IgG molecules, a dye-coupled secondary antibody could be used directly for immunofluorescence experiments. When compared with the parental cell line, the recombinant Hupf1 protein is localized within the cytoplasm and displays a somewhat punctuate, preferentially perinuclear, distribution (Figure 1C; left panel, control; right panel, Hupf1-2tag). This expression pattern matches previously published localization data for untagged Hupf1 [22,34] and we therefore conclude that Hupf1-2tag shows the same subcellular localization as wild-type Hupf1 [30]. Finally, we investigated whether the expression of Hupf1-2tag in the stable cell line interferes with its ability to support NMD. Transfection experiments showed that the Hupf1-2tag cell line degrades specifically a human β -globin NS39 mutant mRNA as effectively as the parental cell line (results not shown), indicating that the NMD pathway is fully active. Taken together, these results qualify the Hupf1-2tag cell line for the studies discussed below.

Purification and MS analysis of Hupf1-associated cellular proteins

To identify and characterize cellular proteins that associate with Hupf1, we prepared cytoplasmic extracts from the transgenic cell line and subjected them to immunoaffinity purification with

Figure 1 The stable cell line expresses cytoplasmic Hupf1-2tag at wild-type levels

(A) Schematic representation of the transgenic Hupf1-2tag protein along with the double-affinity tag and the TEV protease cleavage site at the C-terminus. The respective molecular masses of the endogenous protein Hupf1, of Hupf1-2tag, and the cleavage product of TEV cleavage, Hupf1-1tag, are indicated. (B) Extracts (100 μ g of total protein) of the parental HeLa S3 cell line (control) or the Hupf1-2tag-expressing stable cell line were left untreated (left-hand side) or treated with 2 units of recombinant TEV to remove the Protein A part of the tag (right-hand side). The untreated extracts and the cleavage products were resolved by SDS/PAGE and subjected to immunoblotting with an anti- α Hupf1 antibody. The cleavage product of the transgenic protein, Hupf1-1tag, can be distinguished from endogenous Hupf1 or Hupf1-2tag due to its intermediate size. Note that the signal of uncleaved Hupf1-2tag in the untreated extracts is very pronounced due to the ability of the Protein A part of the tag to react with IgG molecules of the antiserum in an epitope-independent way. (C) Immunofluorescence of the parental cell line (control, left panel) and the Hupf1-2tag-expressing cells (Hupf1-2tag, right panel). Cells were grown on coverslips, fixed and permeabilized. DNA was visualized with Hoechst 33342 (green) and Hupf1-2tag was detected with Alexa 488-coupled α -rabbit IgG (red; scale bars, 15 μ m). The image is representative of the vast majority of cells observed.

IgG-Sepharose (Figure 2A). After extensive washing, the retained fraction of tagged Hupf1 and its interaction partners were eluted under native conditions by cleavage with TEV protease. The eluted material was resolved subsequently by SDS/PAGE along with an eluate prepared in parallel from the parental cell line as a control. After silver staining, several bands were detected in the eluate from the Hupf1 transgenic cell line, which were absent from the eluate of the parental cell line (Figure 2B; lane 1, control;

Figure 2 Purification, identification and characterization of Hupf1-interacting proteins

(A) Outline of the experimental strategy. (B) Extracts (120 mg of total protein) of the parental (lane 1) or the Hupf1-2tag-expressing (lane 2) cell line were subjected to immunoaffinity purifications using cross-linked IgG-Sepharose. Bound material was washed and released by TEV protease cleavage, resolved by SDS/PAGE and proteins were visualized by silver staining. To the left, molecular-mass standards (in kDa) are indicated. The bands marked with arrows were identified by MS, as Hupf2 and Hupf1-1tag. Their identity was confirmed by immunoblotting (results not shown). The bands marked with I, II and III were identified by MS as keratin, IgG-binding protein and Pabp respectively. (C) Total RNA was prepared from extracts of the Hupf1-2tag or parental cell line (contr.) before (–) or after (+) treatment with 20 μ g/ml RNase A. Total RNA (7 μ g) was denatured in formamide containing buffer and loaded on to a gel stained with ethidium bromide. The arrows indicate the 28S and 18S rRNAs. Two prominent cleavage products of 28S rRNA are marked with arrowheads. (D) Extracts (12 mg of total protein) of the parental (control) or Hupf1-2tag-expressing cell lines were subjected to immunoaffinity purifications using cross-linked IgG-Sepharose without (–) or with (+) prior treatment of 20 μ g/ml RNase A. The bound material was washed and eluted from the beads by TEV protease cleavage. Proteins were resolved by SDS/PAGE and transferred on to a PVDF membrane. The membrane was first probed with a monoclonal α Pabp antibody (lower panel) and then re-probed with an α Hupf1 antiserum (upper panel). The input and supernatants (1% of total) of control (lanes 1 and 2) or Hupf1-2tag (lanes 3 and 4) reaction, 50 units of recombinant TEV protease (lane 5) and the TEV eluates of the untreated or treated control (lanes 6 and 7 respectively) or Hupf1-2tag (lanes 8 and 9 respectively) samples are shown.

lane 2, Hupf1-2tag). These bands were excised and subjected to identification by nanoelectrospray MS.

The most prominent band migrating at approx. 140 kDa was identified as Hupf1 (Figure 2B, lane 2), as expected. Using

immunoblotting of the same eluate, we could show that this band corresponds to Hupf1-1tag, i.e. the bait protein lacking the Protein A part (results not shown). The second most intensive band, a protein of higher molecular mass than that of Hupf1-1tag was identified as the NMD factor Hupf2 (approx. 170 kDa; Figure 2B, lane 2). This result shows that the known Hupf1-interacting protein Hupf2 was purified specifically under these experimental conditions. The MS identification of Hupf2 was confirmed independently by immunoblotting of the same eluate (results not shown). Careful inspection of the results might suggest that Hupf1 and Hupf2 occur with a near 1:1 stoichiometry (Figure 2B). However, it is important to note that silver staining does not allow quantitative comparisons between different proteins to be made.

In addition to Hupf1 and Hupf2, three further bands were analysed by MS (labelled I, II and III; Figure 2B, lane 2). Bands I and II were identified as keratin and IgG-interacting proteins respectively. These proteins represent common non-specific contaminants of the chosen experimental approach [40]. MS analysis of band III (Figure 2B, lane 2) identified the Pabp.

To characterize further the role of Pabp as a Hupf1-associated protein, we performed immunoprecipitations and TEV elution as described above, but with extracts that were pretreated with RNase A or remained untreated (Figure 3A). Total RNA was extracted before and after the RNase A treatment, and analysed on an agarose gel by ethidium bromide staining to assure that the RNA digest was effective and complete (Figure 2C). Pabp indeed co-purifies specifically with Hupf1 (Figure 2D cf. lane 8 with lanes 6 and 7), but this co-purification is abolished by RNase treatment (lane 9). By contrast, the immunoprecipitation of Hupf1 is unaffected by the RNase treatment. Thus the association of Pabp with Hupf1 requires the presence of RNA. This suggests that RNA may have an allosteric effect that is required for a more direct association of the two proteins, or that RNA bridges between Hupf1 and Pabp molecules that do not interact directly.

Interaction of Hupf1 with other Hupf factors

We next analysed the association of Hupf1 with other Hupf factors, namely Hupf2, Hupf3a and Hupf3b. Because these factors are thought to exert their functions in association with mRNAs [22], we also examined the role of RNA in this context. Immunoprecipitations with IgG–Sepharose from extracts of the Hupf1-2tag cell line with or without RNase A pretreatment were performed as described above (Figure 3A), and the efficiency of the RNase digest was confirmed (Figure 3G). Extracts from the parental cell line treated in a similar way served as specificity controls. The bound material was released with TEV protease, resolved by SDS/PAGE, and tested for the presence of Hupf1, Hupf2, Hupf3a and Hupf3b by immunoblotting (Figures 3B–3E).

As shown in Figure 3(B), both the endogenous and the recombinant Hupf1 can be discerned in the input material (lanes 1–4). As expected, the recombinant Hupf1 is effectively recovered in an RNase-independent way (Figure 3B, lanes 8 and 9). Interestingly, the endogenous Hupf1 remains fully in the supernatant (Figure 3B, lane 4) and is undetectable in the immunopurified eluate (Figure 3B, lanes 8 and 9). This suggests that only one Hupf1 molecule is present in each complex.

Consistent with the results in Figure 2(B), Hupf2 co-purifies with Hupf1 (Figure 3C). This association does not appear to require RNA (Figure 3C, lane 9), although RNase treatment diminishes Hupf2 recovery by approx. 2-fold.

Hupf3a exists in a small (S) and a large (L) isoform [22,23]. Both isoforms are expressed in the transgenic HeLa cell line (Figure 3D). Remarkably, Hupf3aS is almost undetectable in the

Figure 3 Interaction of Hupf1 with Hupf2 and Hupf3a/b is stabilized by the presence of RNA

(A) Outline of the experimental strategy. (B–E) Same as in Figure 2(C), but the membranes were probed with (B) an αHupf1 serum which detects Hupf1-2tag in input and supernatant of the Hupf1-2tag samples in an epitope-independent way. The shorter version Hupf1-1tag as well as wild-type Hupf1 is detected in an epitope-dependent way by the antiserum. (C) Immunodetection of Hupf2 with an αHupf2 antiserum. Note that Hupf1-2tag is detected due to the reaction of IgG with the Protein A part of the affinity tag. (D) Immunodetection of the small and large splice variant of Hupf3a with an αHupf3a serum. The proteins marked with (*) are cross-reacting proteins from the TEV protease preparation (lane 5). (E) Immunoblotting with an αHupf3b antiserum; and (F) re-probing with an αeRF3 antiserum. Loading of the SDS polyacrylamide gel was as described in Figure 2(C). (G) Total RNA (7 μg) was analysed before (–) and after (+) RNase A treatment as described in Figure 2(C). Results shown are representative of at least four independently performed experiments.

cytosolic extracts prepared from the parental cell line (Figure 3D, lane 1). This suggests that the expression of the Hupf1 transgene either increases the overall abundance of Hupf3aS or that it affects the subcellular distribution of Hupf3aS. Both Hupf3aS and Hupf3aL co-purify with Hupf1, even after RNase treatment (Figure 3D, lanes 8 and 9). Whereas the Hupf3aL signal is stronger

than the Hupf3aS signal in the input material, this ratio is reversed in the eluate (Figure 3D, cf. lanes 3 and 8). This result suggests that the Hupf3aS protein associates particularly strongly with Hupf1, and this interaction appears to be supported by RNA (Figure 3D, cf. lanes 8 and 9).

Like Hupf2, Hupf3b also co-purifies with Hupf1 in a RNA-supported, but not RNA-dependent, manner (Figure 3E). As for Hupf3aS, the amount of Hupf3b in the cytoplasmic extract of the parental cell line is lower than in the transgenic Hupf1-2tag-expressing cell line.

We conclude that Hupf1 forms stable complexes with the other Hupf factors *in vivo*. With the exception of the large isoform of Hupf3a, these associations are stabilized substantially with intact RNA, although none of these interactions depend strictly on the integrity of RNA. In contrast with the Hupf factors, we could not detect any stable interaction of eRF3 with these complexes (Figure 3F). This suggests that eRF3 associates with the Hupf factors in a more transitory fashion.

Interaction of Hupf1 with the components of the EJC

Current models of NMD envisage Hupf1 to interact with the components of the EJC indirectly, via Hupf3 and/or Hupf2 [14,25,26]. Thus we explored the interactions of Hupf1 with the EJC components in HeLa cells. Since Hupf2 and Hupf3a/b co-purified with tagged Hupf1 (Figure 3), we expected to detect at least some of the EJC components. However, we were not able to detect any specific interaction between the tagged Hupf1 and DEK, RNPS1, REF or Y14 (Figures 4C–4F). By contrast, Hupf3b co-purified as a positive control (Figure 4G). We could not detect any signal using an SRm160-specific antibody in the cytoplasmic extracts, although the antibody recognizes SRm160 in total cell extracts (results not shown), consistent with the nuclear localization of SRm160.

Biochemical evidence for distinct complexes associated with Hupf1

The experiments described above and previous studies [22,23,28] suggest that Hupf1 occurs in one or several stable complexes together with its interaction partners. To examine whether Hupf1 participates in the formation of one or several complexes *in vivo*, we analysed their native molecular mass by size-exclusion chromatography (Figure 5A). Cytoplasmic extracts were prepared from the Hupf1-2tag-expressing cell line as before, treated with RNase A (Figure 5C) and subjected to ammonium sulphate precipitation (results not shown). Hupf1-2tag-enriched fractions (30% saturated ammonium sulphate) were re-dissolved in a native buffer and subjected to FPLC with a Superose 6 size-exclusion column. Fractions were collected along the elution profile and one-third of each fraction was treated with TEV protease to remove the Protein A tag from the recombinant Hupf1-2tag protein and, therefore, allow a quantitative detection of transgenic Hupf1-1tag in comparison with the endogenous Hupf1.

Immunoblot analysis with Hupf1 antibodies (Figure 5D) shows that both the recombinant and the endogenous protein are distributed broadly across the entire elution profile. The co-fractionation of these two proteins that do not co-purify with each other (Figure 3B) further supports the notion that the tagged protein acts in a fashion similar to its unmodified endogenous counterpart. In contrast with Hupf1, Hupf2 displays a clear peak in fractions 12–16 (Figure 5E). Hupf3aL can be detected in the same fractions (Figure 5F), along with endogenous Hupf1 and recombinant Hupf1-1tag (Figure 5D), consistent with the formation of a high-molecular-mass complex (complex 1) among

Figure 4 Analysis of immunoaffinity-purified Hupf1 complexes for components of the EJC

(A) Outline of the experimental strategy. (B) Extracts (12 mg of total protein) of the parental (control) or Hupf1-2tag-expressing cell line were subjected to immunoprecipitations as described in Figure 2(B). TEV eluates were resolved by SDS/PAGE and transferred on to a PVDF membrane. Immunodetection of (B) Hupf1, the components of the EJC, (C) RNPS1, (D) REF, (E) DEK, (F) Y14 or (G) Hupf3b. Input and supernatant (1% of total) of the control (lanes 1 and 2 respectively) or the Hupf1-2tag (lanes 3 and 4 respectively) samples, 50 units of recombinant TEV protease (lane 5) and the TEV eluates from the control (lane 6) or the Hupf1-2tag sample (lane 7) are shown. The band marked with (*) is a cross-reacting protein from the TEV protease preparation (lane 5).

Hupf1, Hupf2 and Hupf3aL. Hupf3aS is detected in fractions corresponding to a lower molecular-mass complex (complex 2; Figure 5F, fractions 18–20), which lacks detectable Hupf3aL and Hupf2 (Figures 5F and 5E respectively). This suggests that Hupf3aS and Hupf1 may form a distinct complex.

Using molecular-mass markers that were separated on the same molecular-mass-exclusion column in a separate run under the same conditions (indicated in Figure 5B), we established a calibration curve to estimate the native molecular mass of protein complexes (results not shown). Based on this calibration curve, we estimated a native molecular mass of approx. 1–1.3 MDa for the putative complex 1 and 400–600 kDa for complex 2. Although these experiments cannot prove that the co-fractionating proteins are in the same complex, we suggest that Hupf1 probably forms multiple distinct complexes with cellular proteins, including the other Hupf factors.

DISCUSSION

We have established a stable HeLa cell line expressing an affinity-tagged form of the central NMD factor Hupf1 to generate a tool for the biochemical investigation of Hupf1 and its associated cellular

Figure 5 High-molecular-mass complexes containing Hupf1, Hupf2 and Hupf3 isoforms revealed by molecular-mass-exclusion chromatography

(A) Outline of the experimental strategy. (B) Elution profile of the molecular-mass-exclusion chromatography on a Superose 6 column. RNase A-treated extract (25 mg of total protein) of the Hupf1-2tag-expressing cell line was subjected to ammonium sulphate precipitation at 30 % saturation. The pellet was re-suspended in T8N150 buffer and loaded on to a Superose 6 column. The absorbance at 280 nm, the elution volume (in ml) and the fraction numbers are indicated. Arrows in the elution profile mark the start and end point of fractionation. The position of molecular-mass markers that were separated in parallel on the same column is indicated above the graph: dimer of thyroglobulin (1300 kDa); monomer of thyroglobulin (669 kDa); ferritin (440 kDa); aldolase (158 kDa); and void volume (vv). (C) Total RNA (10 µg) was analysed before (–) and after (+) RNase A treatment as described in Figure 2(C). (D, E) Fractions from gel filtration shown in (B) were concentrated and one-third of each fraction was subjected to TEV protease cleavage, loaded on to an SDS/polyacrylamide gel, and the proteins were transferred on to a PVDF membrane. (F) Another one-third was loaded directly on to an SDS/polyacrylamide gel for Western blotting without prior TEV cleavage. (D) Probing with an αHupf1 antiserum. Note that the TEV protease cleavage was not complete; therefore uncleaved Hupf1-2tag can be detected in most of the fractions. (E) Reprobing with αHupf2 antiserum. (F) Detection of the two splice variants of Hupf3a with an αHupf3a antiserum. Note that the prominent band marked with (*) is a cross-reacting protein. As a control, the last lane shows untreated (F) or TEV protease-treated (D, E) extract (0.15 mg of total protein). Along with the elution volume (in ml), the fraction number of the first of the two fractions that were pooled is indicated at the bottom of each lane.

proteins. We wanted to ascertain that the tagged Hupf1 protein shares important functional, cell biological and biochemical characteristics with the unmodified endogenous protein. We found that (1) the cytoplasmic localization of the transgenic Hupf1 fully corresponds to the previously published results on the localization of wild-type Hupf1 [22,34]; (2) the NMD pathway is fully active

in the stable cell line; (3) tagged Hupf1 interacts with Hupf2, Hupf3aS, Hupf3aL and Hupf3b; and (4) the elution profiles of recombinant and wild-type Hupf1 in gel-filtration experiments closely resemble each other. Although we cannot exclude the possibility that currently unknown properties of Hupf1 differ for the tagged protein, we conclude that the chosen cell line is a valid experimental system with which to study the biochemistry of Hupf1 in HeLa cells.

Gel-filtration analysis indicates that most of the Hupf1 exists as a monomeric protein (Figure 5). There is no indication that Hupf1 forms homodimers or that Hupf1-containing complexes contain more than one Hupf1 molecule per complex (Figure 3). The broad distribution of Hupf1 and the occurrence of the other Hupf proteins in distinct high-molecular-mass fractions suggest that Hupf1 polypeptides participate in the formation of cellular complexes. One of these Hupf1-containing complexes may have a native molecular mass of approx. 1–1.3 MDa ('complex 1') and, in addition to unknown proteins, contain Hupf2 and Hupf3aL, but not Hupf3aS. A smaller complex of approx. 400–600 kDa ('complex 2') appears to contain Hupf3aS, but not Hupf2 or Hupf3aL (Figure 5). It is important to appreciate that the co-fractionation of the respective Hupf proteins cannot be taken as direct evidence of complex formation with each other. Unfortunately, technical difficulties prevented our performing immunoprecipitation experiments on individual fractions and, therefore, more direct evidence could not be obtained. However, the co-immunoprecipitation of the other Hupf proteins with Hupf1 (Figures 1 and 2; [22,23,28]) supports the notion of complex formation. If complexes 1 and 2 exist as the co-fractionation results indicate, a comparison of their compositions suggests several interesting possibilities. First, the small (S) and large (L) isoforms of Hupf3a are associated with distinct complexes. The differences in complex association may indicate that the two versions of Hupf3a either fulfil different functions or that they contribute similar functions to different assemblies. They certainly indicate an unexpected level of complexity in the NMD pathway. Secondly, complex 2 harbours the Hupf1 and Hupf3 proteins, but does not contain detectable levels of Hupf2. According to the current models of NMD, Hupf3 proteins are assumed to recruit Hupf2, which in turn recruits Hupf1 [22,23]. The composition of complex 2 suggests that Hupf2 is not required for the association of Hupf1 and Hupf3aS. Complex 2 may arise from a Hupf2-independent assembly pathway or it may represent a product of the standard Hupf assembly pathway from which Hupf2 has dissociated. Future experiments will shed light on these interesting issues. Thirdly, the Hupf2 signal in fractions 12–16 (Figure 5E) is disproportionately strong compared with the Hupf1 (Figure 5D) and Hupf3aL (Figure 5F) signals, although the signal strengths for the three proteins in the unfractionated extracts (right lanes) do not show similar differences. This may suggest that complex 1 might contain a stoichiometric excess of Hupf2 in comparison with the other Hupf proteins.

Does Hupf1 provide a link between the EJC and translation termination?

Recent studies suggest a link between the EJC and the NMD machinery [14,25,26]. Using a tethering approach, components of the EJC have been involved functionally in NMD [25]. We therefore examined the interaction of Hupf1 with the components of the EJC (Y14, DEK, REF, RNPS1 and SRm160).

The lack of detectable SRm160 in the starting material for the immunoprecipitations is explained by its nuclear localization and by using cytoplasmic extracts for the present study. However, we

also could not detect interactions between Hupf1 and any of the other components of the EJC that has been tested, i.e. RNPS1, Y14, DEK and REF (Figure 4). In principle, these results could be attributed to an interference of the C-terminal tag of Hupf1 with the interacting proteins. However, we could detect clearly interactions between tagged Hupf1 and other proteins (Hupf2 and Hupf3a/b), arguing that, generally, the presence of the tag does not perturb the structure of the transgenic Hupf1. Furthermore, it has been found that Hupf1 does not specifically co-immunoprecipitate EJC-containing mRNAs [14], indicating that there might not be a direct link between Hupf1 and EJC. Conceivably, EJC proteins could have dissociated from 'NMD complexes' by the time that Hupf1 joins.

We also could not document an interaction between Hupf1 and eRF3 (Figure 3). It is possible that the interaction of eRF3 with the tagged Hupf1 *in vivo* is too weak or transient to withstand the co-purification procedures used in the present study; this interaction could be documented *in vitro* [32].

A role for the Pabp in NMD?

In yeast, NMD is a de-adenylation-independent mRNA degradation process that involves the removal of the protective 5'-cap structure and subsequent 5'- to 3'-exonucleolytic degradation [43]. The poly(A) tail and the Pabp may thus not be involved in the NMD pathway in yeast, or the protective function of the poly(A) tail and the Pabp is circumvented by the NMD pathway without requirement for de-adenylation.

Although the definitive information regarding the fate of the poly(A) tail in the NMD of mammalian mRNAs is currently lacking, we were intrigued to find the Pabp in association with Hupf1 (Figure 2). One may speculate that the binding of a Hupf1-containing NMD complex with Pabp may interfere with the cap-protective role of Pabp. Possibly, the interaction of eIF4G and Pabp could be competed for by Hupf1. The finding that the co-purification of Pabp and Hupf1 is RNA-dependent (Figure 2D) may reflect a structural role of RNA for this interaction. However, it is also possible that Hupf1 and Pabp are simply located on the same messenger ribonucleoprotein, and that their co-purification results from an mRNA-mediated tethering effect. Future studies will have to explore further the intriguing connection between Hupf1 and Pabp.

In summary, the present study describes a novel biochemical tool for the analysis of the NMD pathway in human cells. In addition to confirming previously known interactions, we did not expect to find an association between Hupf1 and Pabp. The possibility of multiple, distinct Hupf1-containing high-molecular-mass complexes also warrants further exploration. Finally, a recent understanding is that Hupf1 exists in different phosphorylation states and the identification of human SMG proteins that mediate the phosphorylation and dephosphorylation of Hupf1 raises important questions about the association of these proteins with different Hupf complexes [37]. The transgenic Hupf1-2tag cell line may prove to be a valuable tool for such studies.

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