The protein Mago provides a link between splicing and mRNA localization

Hervé Le Hir, David Gatfield¹, Isabelle C. Braun¹, Daniel Forler¹ & Elisa Izaurralde⁺

Howard Hughes Medical Institute, Department of Biochemistry, Brandeis University, 415 South Street, Waltham, MA 02454, USA and ¹European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, D-69117 Heidelberg, Germany

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The proteins Mago and Y14 are evolutionarily conserved binding partners. Y14 is a component of the exon–exon junction complex (EJC), deposited by the spliceosome upstream of messenger RNA (mRNA) exon–exon junctions. The EJC is implicated in post-splicing events such as mRNA nuclear export and nonsense-mediated mRNA decay. *Drosophila* Mago is essential for the localization of *oskar* mRNA to the posterior pole of the oocyte, but the functional role of Mago in other species is unknown. We show that Mago is a bona fide component of the EJC. Like Y14, Mago escorts spliced mRNAs to the cytoplasm, providing a direct functional link between splicing and the downstream process of mRNA localization. Mago/Y14 heterodimers are essential in cultured *Drosophila* cells. Taken together, these results suggest that, in addition to its specialized function in mRNA localization, Mago plays an essential role in other steps of mRNA metabolism.

INTRODUCTION

Messenger RNAs (mRNAs) exist in the cell in dynamic association with multiple proteins, of which many bind cotranscriptionally and accompany the mRNA to the cytoplasm (reviewed by Shyu and Wilkinson, 2000). Components of the splicing machinery (including the spliceosomal U snRNPs) are also loaded onto nascent transcripts, but while U snRNPs and most splicing factors dissociate from the spliced mRNA after completion of the splicing reaction, specific proteins bind to mRNAs as a consequence of splicing (Luo and Reed, 1999). Indeed, it has been shown recently that the spliceosome imprints the mRNA product by depositing several proteins 20–24 nucleotides (nt) upstream of mRNA exon–exon junctions (Le Hir *et al.*, 2000a,b, 2001; Kim *et al.*, 2001a).

When assembled *in vitro*, the so-called exon–exon junction complex (EJC) contains at least five proteins: SRm160, DEK,

RNPS1, Y14 and REF/Aly (Le Hir *et al.*, 2000a,b). SRm160, DEK and RNPS1 are related to the splicing process (Blencowe *et al.*, 1998; Mayeda *et al.*, 1999; McGarvey *et al.*, 2000). REF/Aly have been implicated in mRNA nuclear export by interacting with members of the TAP/NXF family of mRNA export receptors (reviewed by Conti and Izaurralde, 2001). Consistently, *in vivo* the EJC facilitates the recruitment of the heterodimeric nuclear export receptor TAP/p15 (NXF1/p15) to spliced mRNAs (Le Hir *et al.*, 2001).

In contrast to the other EJC components, the RNA-binding protein Y14 (also known as RBM8) remains bound to the mRNP after translocation through nuclear pore complexes (Kataoka *et al.*, 2000; Kim *et al.*, 2001a; Le Hir *et al.*, 2001). In this context, it has been proposed that Y14 communicates the position of introns to the cytoplasm and may play a role in nonsense-mediated mRNA decay (NMD; Kim *et al.*, 2001a,b; Le Hir *et al.*, 2001; Lykke-Andersen *et al.*, 2001). NMD is the process by which, if an in-frame stop codon is located some distance upstream of at least one exon–exon junction, it is generally recognized as premature and the mRNA is targeted for degradation. The proteins UPF1, UPF2 and UPF3 are required for NMD (reviewed by Sun and Maquat, 2000). UPF3 and UPF2 are recruited to the mRNP via interactions with components of the EJC, such as Y14 and RNPS1 (Kim *et al.*, 2001a,b; Le Hir *et al.*, 2001; Lykke-Andersen *et al.*, 2001).

Recently, Y14 was found in yeast two-hybrid screens when the human protein Mago (Hs MGN) was used as a bait (Zhao *et al.*, 2000). The interaction between Mago and Y14 was independently confirmed by Mingot *et al.* (2001), who identified Mago/Y14 heterodimers as the import substrate of importin-13. *Drosophila* Mago (also known as *mago nashi*, Dm MGN) is required for the definition of the anteroposterior and dorso-ventral axis in *Drosophila* and for the localization of *oskar*

⁺Corresponding author. Tel: +49 6221 387 389; Fax: +49 6221 387 518; E-mail: izaurrealde@embl-heidelberg.de

Fig. 1. MGN/Y14 interaction is conserved. **(A)** Lysates from *E. coli* expressing the proteins indicated above the lanes were incubated with glutathione agarose beads. Bound proteins were eluted with SDS sample buffer. One-hundredth of the inputs and one-third of the bound fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining. Asterisks and dots indicate the position of GST-tagged or untagged proteins, respectively. **(B)** Lysates from SL2 cells expressing zz-tagged Dm MGN were incubated with IgG-Sepharose beads. Bound proteins were eluted with SDS sample buffer. One-hundredth of the inputs and one-half of the bound fractions were analyzed by SDS-PAGE followed by western blot using anti-Dm Y14 antibodies. The antibodies also recognize the zz tag fused to Dm MGN.

mRNA to the posterior pole of the oocyte (Micklem *et al.*, 1997; Newmark *et al.*, 1997).

In this study we show that the conserved MGN/Y14 heterodimer specifically associates with EJC containing spliced mRNAs both *in vitro* and *in vivo* and accompanies these mRNAs to the cytoplasm. Moreover, we show that both MGN and Y14 are essential in *Drosophila* cells and colocalize in the nucleus, both in *Drosophila* and HeLa cells. This raises the possibility that factors involved in mRNA localization in the cytoplasm are loaded onto the nuclear mRNA during splicing and escort it to its final cytoplasmic destination.

RESULTS

MGN/Y14 interaction is conserved and occurs *in vitro* and *in vivo*

Drosophila Y14 and MGN are 63 and 88% identical to their human counterparts (Micklem *et al.*, 1997; Zhao *et al.*, 1998, 2000; Kataoka *et al.*, 2000), suggesting that their interaction is conserved. Indeed, untagged Hs or Dm Y14 copurify with glutathione *S*-transferase (GST) fusions of Hs or Dm MGN when the proteins are coexpressed in *Escherichia coli* (Figure 1A, lanes 10 and 15). Conversely, GST-Hs Y14 pulls down untagged Hs MGN from total lysates of *E. coli* expressing both proteins (lane 5). The MGN/Y14 interaction occurs in the presence of RNase A, indicating that it is not RNA-mediated (data not shown). When the GST tag is removed by cleavage with TEV protease and MGN/Y14 complexes are further purified by gel filtration, the two subunits are recovered in a stoichiometric ratio and the apparent molecular weight of the complex is consistent with that of the heterodimer (data not shown).

The interaction between Dm MGN and Y14 was also investigated in *Drosophila* Schneider cells (line 2, SL2 cells). SL2 cells were transiently transfected with a plasmid expressing Dm MGN fused to two immunoglobulin-binding domains of protein A

from *Staphylococcus aureus* (zz tag). Total cell lysates were incubated with IgG-Sepharose beads; after extensive washes, bound proteins were eluted with SDS and analyzed by western blot. Endogenous Y14 specifically copurifies with zz-tagged Dm MGN (Figure 1B, lane 4), indicating that the interaction between these proteins also occurs *in vivo*.

MGN/Y14 localize within the nucleus and into nuclear speckles

In *Drosophila* oocytes MGN is predominantly nuclear, although a fraction of the protein accumulates within the posterior pole plasm (Micklem *et al.*, 1997; Newmark *et al.*, 1997). Consistently, in transiently transfected SL2 cells zz-tagged Dm MGN and Dm Y14 localize within the nucleus and are excluded from the nucleolus (Figure 2A and B, red). Similarly, when Hs MGN is transiently expressed in HeLa cells as a fusion with green fluorescent protein (GFP), it localizes in the nucleoplasm and is excluded from the nucleolus (Figure 2C and F). Although the GFP signal is widespread in the nucleoplasm, the staining is not homogenous and sites of higher concentration in speckled domains are observed (Figure 2C and F). These domains colocalized with the structures labeled by the antibody NM4 (Blencowe *et al.*, 1994) directed to SR proteins (Figure 2D). The localization of MGN in the nucleoplasm and in speckled domains is similar to that reported for Y14 (Figure 2G; Kataoka *et al.*, 2000).

MGN associates with spliced mRNAs carrying the EJC

Based on the heterodimerization of MGN with Y14 and its localization into nuclear speckles, we sought to determine whether MGN is a component of the EJC. To this end, β -globin pre-mRNAs containing either a 38- or a 17-nt exon 1 (named $\beta/38$ and $\beta/17$, respectively) were coincubated in HeLa cell nuclear

Fig. 2. MGN/Y14 complexes localize to the nucleoplasm and in nuclear speckles. (**A** and **B**) SL2 cells were transfected with plasmids expressing zz-tagged Dm MGN or Dm Y14 as indicated. Cells were fixed 4 days after transfection and the tagged proteins were detected by indirect immunofluorescence with a polyclonal anti-protein A antibody and a Cy3-coupled secondary antibody (red). The nuclear envelope (green) was stained with Alexa488-wheat germ agglutinin conjugates. (**C–H**) Subcellular localization of GFP–Hs MGN (**C** and **F**, green) and zz-tagged Hs Y14 (**G**, red) in HeLa cells. Nuclear speckles were labeled with antibodies directed to SR proteins (**D**, red).

extracts to generate spliced mRNAs. Because the EJC is deposited on spliced mRNAs more than 20 nt upstream of the exon–exon junction (Le Hir *et al.*, 2000b, 2001), the spliced $\beta/17$ mRNA with a 5' exon shorter than 20 nt does not carry the EJC (Le Hir *et al.*, 2001). Splicing reactions were supplemented with recombinant MGN or MGN/Y14 complexes having a GST tag fused N-terminally to MGN. Recombinant GST served as negative control. After splicing, these reactions were subjected to immunoprecipitation (IP) with anti-GST antibodies and the coimmunoprecipitated (coIP) RNA fragments monitored by denaturing PAGE (Figure 3A). In extracts supplemented with MGN/Y14 complexes, the spliced $\beta/38$ mRNA was precipitated above the background levels observed in extracts supplemented with GST (lane 8 versus 4). In contrast, RNAs without the EJC such as the pre-mRNAs or the spliced $\beta/17$ mRNA were not precipitated above background levels (lane 8 versus 4). GST–MGN alone did not significantly associate with spliced $\beta/38$ mRNA (lane 6 versus 4), suggesting that MGN is recruited to spliced mRNPs only as a heterodimer with Y14. These results also indicate that the association of recombinant MGN/Y14 complexes with $\beta/38$

Fig. 3. MGN/Y14 are deposited on spliced mRNAs carrying the EJC. (**A**) Labeled $\beta/38$ and $\beta/17$ pre-mRNAs were spliced in HeLa cell nuclear extracts supplemented or not with 5 ng/ μ l of recombinant GST, GST–Hs MGN (GST–M) or GST–Hs MGN/Y14 (GST–M/Y). Subsequently, splicing reactions were subjected to coIP with anti-GST antibodies. RNAs were separated by 10% denaturing PAGE. Splicing substrates, intermediates and products are indicated on the right. The coIP efficiencies for $\beta/38$ mRNA (boxes) are indicated. (**B**) AdML pre-mRNA was spliced in HeLa cell nuclear extracts supplemented with GST–Hs MGN/Y14. Subsequently, the reactions were incubated with cDNA oligos and the mRNA fragments resulted from RNase H cleavage (5', m and 3') were subjected to coIP with anti-GST antibodies. The coIP efficiencies for a given fragment (boxes) are indicated below the corresponding band. Symbols are as in (**A**). (**C**) *Xenopus* oocyte nuclei were injected with the indicated control RNAs and Ftz/36 and Ftz/18 pre-mRNAs. Where indicated, Hs MGN/Y14 complexes (GST–M/Y) were coinjected with the RNAs. Coinjection of GST served as a control. One hour after injection, RNA samples from nuclear (N) fractions were subjected to IP with anti-GST antibodies. RNAs were analyzed by 15% denaturing PAGE. Splicing substrates, products and control RNAs are identified on the left. The concentration of recombinant proteins in the injection mixtures was 1 mg/ml. In all panels, the coIP efficiencies (boxes) were calculated by dividing the intensity of a given precipitated fragment (lanes b: bound) by the intensity of the same fragment present in the input reaction (lanes i), taking into account that only one-tenth of each input reaction was loaded on the gel. i, Input; b, fraction bound to anti-GST antibodies; p, precursor mRNA; m, spliced mRNA; N, nuclear fraction; T, total oocytes collected immediately after injection.

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mRNA is specific and not due to an artificial tethering of recombinant GST–MGN to the EJC through endogenous Y14.

We next examined whether MGN binds to the mRNA at the position where the EJC is deposited. A pre-mRNA derived from the adenovirus major late transcription unit (AdML) was spliced in extracts supplemented with GST–MGN/Y14. The spliced mRNA was then subjected to RNase H digestion with two DNA oligos centered at positions –48 and +12, relative to the exon–exon junction (position 0). This divides the mRNA into three fragments: one containing exon 1 (band 5'), one containing the EJC (band m) and one comprising the remainder of exon 2 (band 3'). The anti-GST antibodies preferentially precipitated the full-length mRNA and fragment 'm' carrying the EJC (Figure 3B, lane 6).

The association of MGN with spliced mRNAs was also investigated *in vivo* in *Xenopus laevis* oocytes. Body-labeled Fushi tarazu (Ftz) pre-mRNAs containing either a 36- or a 18-nt exon 1 (named Ftz/36 and Ftz/18, respectively) were coinjected into oocyte nuclei along with recombinant GST–MGN/Y14 or GST. Following 1-h incubation, the ability of anti-GST antibodies to coIP Ftz/36 and Ftz/18 spliced mRNAs from oocyte nuclear fractions was tested (Figure 3C). U6Δss RNA, U5ΔSm RNA and human initiator methionyl-tRNA were coinjected with the pre-mRNAs to control for the specificity of the IP. In oocytes coinjected with MGN/Y14, the antibodies precipitated spliced Ftz/36 mRNA but not Ftz/18 mRNA, which does not carry the EJC (Figure 3C, lane 5).

Taken together, these results show that recombinant MGN/Y14 heterodimers specifically associate with spliced mRNAs and that deposition of MGN/Y14 complexes is spatially restricted to the mRNA fragment carrying the EJC.

MGN/Y14 heterodimers accompany the spliced mRNA to the cytoplasm

To investigate whether MGN remains associated with spliced mRNAs after export to the cytoplasm, full-length Ftz or β-globin pre-mRNAs were coinjected into oocyte nuclei along with recombinant GST–MGN/Y14 or GST and the mixture of control RNAs described above. Following 2-h incubation, oocytes were dissected and the ability of anti-GST antibodies to coIP spliced Ftz or β-globin mRNAs from nuclear and cytoplasmic fractions was tested (Figure 4A and B).

In oocytes injected with MGN/Y14, the antibodies specifically precipitated the spliced mRNAs (Figure 4A and B, lanes 6). No significant binding to RNAs that do not carry the EJC was detected, such as the pre-mRNAs, the excised introns or the control RNAs (Figure 4A and B, lanes 6). Furthermore, the antibodies precipitated spliced mRNAs in the cytoplasmic fraction as efficiently as in the nuclear fraction (Figure 4A and B, lanes 7 versus 6). Thus, like Y14 (Kataoka *et al.*, 2000; Kim *et al.*, 2001a; Le Hir *et al.*, 2001), MGN remains bound to the cytoplasmic mRNA after export. MGN alone did not significantly associate with mRNA (data not shown) suggesting that there is not a large pool of free endogenous Y14 in the oocyte.

Given the fact that MGN and Y14 accompany mRNAs to the cytoplasm, we also investigated a possible role of MGN/Y14 dimers in mRNA export. When Ftz/36 and Ftz/18 mRNAs are generated by splicing in *Xenopus* oocytes, only Ftz/36 is efficiently exported. Export of spliced Ftz/18 mRNA is inefficient but can be partially rescued by coinjection of TAP/p15

Fig. 4. MGN/Y14 are components of the EJC *in vivo*. *Xenopus* oocyte nuclei were injected with the indicated control RNAs and Ftz (A) or β-globin (B) derived pre-mRNAs. Where indicated, Hs MGN/Y14 complexes (GST–M/Y) or GST were coinjected with the RNAs. One hour after injection, RNA samples from nuclear (N) or cytoplasmic (C) fractions were subjected to IP with anti-GST antibodies. Splicing substrates, products and control RNAs are identified on the left of each panel. Abbreviations are as in Figure 3. The coIP efficiencies were calculated as described in Figure 3.

heterodimers or of REF proteins (Le Hir *et al.*, 2001). Coinjection of equivalent amounts of purified recombinant Y14 or of MGN/Y14 heterodimers, however, did not stimulate Ftz/18 mRNA export nor the export of other inefficiently exported mRNAs (Le Hir *et al.*, 2001; data not shown).

MGN and Y14 are required for cell viability

In order to gain additional insight into the role of MGN and Y14 *in vivo*, we depleted the endogenous proteins from SL2 cells by

means of double-stranded (ds) RNAi. The effect of these depletions was compared with the phenotype observed when the essential mRNA export receptor NXF1 or the NMD factor UPF1 are depleted. Depletion of MGN or Y14 inhibits cell growth (Figure 5A). This inhibition is detected 4 days after transfecting the corresponding dsRNAs and parallels the inhibitory effect observed when UPF1 is depleted. However, it is not as dramatic as the inhibition observed when cells are depleted of NXF1 (Figure 5A).

The efficiency and specificity of the depletion was investigated by western blot with antibodies raised against the recombinant proteins. The steady-state expression level of MGN was reduced to ~5–10% of the level detected in untreated cells, 4–6 days after transfecting MGN dsRNA (Figure 5B, days 3–8 versus 0). Surprisingly, a similar reduction of MGN protein level was observed when cells were transfected with Y14 dsRNA (Figure 5B, lanes 5–12). Conversely, Y14 protein levels were reduced in cells transfected with MGN dsRNA, although not as efficiently as when Y14 dsRNA was transfected (Figure 5B). The effects of MGN and Y14 dsRNAs are specific, as the expression level of tubulin or other unrelated proteins was not affected (Figure 5B and data not shown). These results are consistent with the observation that MGN and Y14 form heterodimers and suggest that depletion of one subunit affects the expression level of the second component of the heterodimer.

A possible role of MGN or Y14 in mRNA export was investigated by determining the intracellular distribution of bulk polyadenylated [poly(A)⁺] by *in situ* hybridization with a Cy3-labeled oligo(dT) probe. MGN or Y14 depletion resulted occasionally in a partial accumulation of poly(A)⁺ RNAs within the nucleus. This accumulation was not observed in all cells and occurred 14 days after transfecting dsRNAs, suggesting that these effects are indirect. The lack of mRNA export inhibition was further confirmed by the observation that depletion of MGN/Y14 heterodimers reduces, but does not abolish, incorporation of [³⁵S]Met into newly synthesized proteins (data not shown). These results also indicate that depletion of MGN/Y14 does not result in a general inhibition of splicing.

DISCUSSION

MGN and Y14 are highly conserved and ubiquitously expressed proteins in metazoans. The genome of *Schizosaccharomyces pombe* also encodes MGN and Y14 homologs, but no obvious homologs are encoded by the *Saccharomyces cerevisiae* genome (Micklem *et al.*, 1997; Newmark *et al.*, 1997; Zhao *et al.*, 1998, 2000; Conklin *et al.*, 2000; Kataoka *et al.*, 2000). The conservation of MGN/Y14 in metazoans and in *S. pombe* and their ubiquitous pattern of expression suggest an essential role for these proteins, in addition to the specialized role of MGN in the localization of *oskar* mRNA in *Drosophila* oocytes. Indeed, as components of the EJC, MGN/Y14 are likely to associate with most, if not all, spliced mRNAs and may have a more general function in post-splicing mRNA metabolism.

Depletion of MGN or Y14 from SL2 cells inhibits growth. Our data suggest that this inhibition cannot be attributed to a general block of splicing or mRNA export. However, we cannot rule out that MGN/Y14 are required for splicing and/or export of a subset of essential mRNAs. Y14 has been implicated in NMD (see Introduction); remarkably, depletion of UPF1 from SL2 cells inhibits

Fig. 5. Depletion of MGN or Y14 inhibits cell growth. (A) SL2 cells were transfected with dsRNA specific for Dm MGN, Y14, UPF1 or NXF1. Cell numbers were determined up to 10 days post-transfection. Data are given as the *n*-fold increase in cell numbers relative to the amount of cells used for transfection on day 0 (where *n* represents a number on the y-axis). (B) SL2 cells were transfected with the indicated dsRNAs and analyzed by western blotting with antibodies raised against the recombinant proteins. The steady-state expression level of Dm MGN and Y14 were reduced relative to the expression level of tubulin (lanes 5–12). In lanes 1–4, dilutions of the sample isolated on day 0 were loaded to assess the efficiency of the depletion.

growth with similar kinetics as those observed when MGN or Y14 are depleted. The growth arrest upon MGN or Y14 depletion may then arise from a deficiency in NMD. Certainly, this possibility requires further investigation, in particular the analysis of mRNAs carrying premature stop codons.

Although Dm MGN is not an RNA-binding protein, mutations in the *mago nashi* gene affect the localization of *oskar* mRNA to the posterior pole of *Drosophila* oocytes (Micklem *et al.*, 1997; Newmark *et al.*, 1997). mRNA localization depends on the presence of different *cis*-acting RNA sequences, many of which fall within the 3' untranslated region (3' UTR) of the mRNA. These sequence elements recruit specific *trans*-acting factors (reviewed by Jansen, 2001). Thus, a possible role of MGN in *oskar* mRNA localization may be to facilitate the recruitment and/or to stabilize proteins bound to the localization signals present at the 3' UTR of this transcript. In this context, it is interesting to note that the protein Barentsz, which is also required for the posterior localization of *oskar* mRNA, fails to associate with *oskar* mRNA in *mago nashi* mutants (van Eeden *et al.*, 2001). Thus, despite their association with bulk mRNA, MGN/Y14 heterodimers may

play specific roles in multiple steps of RNA metabolism in both the nucleus and the cytoplasm.

METHODS

Hs and Dm cDNAs were amplified by PCR using Human testis Marathon-Ready cDNA (Clontech) or Dm Quick-clone cDNA (Clontech) as templates. All PCRs were performed with the Expand™ high-fidelity PCR system (Roche). The amplified cDNAs were cloned and sequenced. The cloned Dm cDNA sequences are identical to the predictions in the Flybase (<http://flybase.bio.indiana.edu>). A plasmid allowing the expression of untagged or GST- or GFP-tagged versions of Hs MGN or Y14 were generated by inserting the corresponding cDNAs into pET28c, pGEXCS or pEGFP-C1 vectors, respectively. A plasmid allowing the expression of a zz-tagged version of Hs Y14 was generated by inserting the corresponding cDNA at the *HindIII*–*NotI* sites of vector pRcCMVzz. For expression of Dm MGN and Y14 in SL2 cells, the corresponding cDNAs were cloned into a pBS-derived vector having the Dm actin promoter and the zz tag inserted upstream of a multicloning site. Additional plasmids used in this study have been described elsewhere (Le Hir et al., 2001). For expression of MGN/Y14 complexes, *E. coli* BL21 (DE3) LysS competent cells were cotransformed with plasmids pGEXCS-HsMGN and pET28c-HsY14.

Splicing reactions, oocyte injections and RNA analysis were performed as described previously (Le Hir et al., 2001). dsRNAi was performed essentially as described by Herold et al. (2001).

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