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Dynamic association and localization of human H/ACA RNP proteins

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ABSTRACT
Mammalian H/ACA RNPs are essential for ribosome biogenesis, pre-mRNA splicing, and telomere maintenance. To form mature RNA–protein complexes, one H/ACA RNA associates with four core proteins. In the cell, this process is assisted by at least one nuclear assembly factor, NAF1. Here we report several unanticipated dynamic aspects of H/ACA RNP proteins. First, when overexpressed, NAF1 delocalizes to the cytoplasm. However, its nucleocytoplasmic shuttling properties remain unaffected. These observations demonstrate a subtle equilibrium between NAF1 expression levels and the availability of NAF1 nuclear binding sites. Second, although NAF1 is excluded from mature RNPs in nucleoli and Cajal bodies, NAF1 associates with mature H/ACA RNA in cell lysates. This association occurs post-lysis because it is observed even when NAF1 and the H/ACA RNA are expressed in separate cells. This documents a protein–RNP association in cell lysates that is absent from intact cells. Third, in similar experiments, all H/ACA core proteins, except NAP57, exchange with their exogenous counterparts, portraying an unexpected dynamic picture of H/ACA RNPs. Finally, the irreversible association of only NAP57 with H/ACA RNA and the conundrum that only NAP57 is mutated in X-linked dyskeratosis congenita (even though most core proteins are required for maintaining H/ACA RNAs) may be more than a coincidence.
Keywords: H/ACA RNP; snoRNP; dyskeratosis congenita; nucleocytoplasmic shuttling; RNP dynamics; telomerase; nuclear localization signal

INTRODUCTION
Mammalian H/ACA RNPs function mainly in the pseudouridylation of ribosomal and spliceosomal small nuclear RNAs but also in the processing of preribosomal RNA and in the stabilization of telomerase RNA. They all consist of one of the ~200 different H/ACA RNAs and the same four evolutionary conserved core proteins, NAP57 (dyskerin or Ch5p in yeast), NOP10, NHP2 (forming a core trimer), and GAR1 (Smith and Steitz 1997; Kiss 2001; Filipowicz and Pogacic 2002; Decatur and Fournier 2003; Meier 2005). In human cells, formation of these particles and consequent stabilization of the H/ACA RNA depends on at least one nuclear assembly factor, NAF1 (Darzacq et al. 2006; Hoareau-Aveilla et al. 2006).

It is commonly regarded that the four core proteins and the H/ACA RNAs form stable RNPs with no exchange of components. This is based on the following three observations. First, all core proteins, except GAR1, are essential for the stabilization of all H/ACA RNAs (Bousquet-Antonelli et al. 1997; Henras et al. 1998; Lafontaine et al. 1998; Watkins et al. 1998). Second, all H/ACA RNPs can be precipitated with antibodies directed against any of the core proteins, including GAR1 (Smith and Steitz 1997; Lafontaine et al. 1998; Watkins et al. 1998; Dez et al. 2001). Second, all H/ACA RNPs can be precipitated with antibodies directed against any of the core proteins, including GAR1 (Balakin et al. 1996; Ganot et al. 1997). Third, once an H/ACA RNA has assembled into an RNP, it cannot be competed for with an excess of the same RNA (Wang and Meier 2004). Surprisingly, we now demonstrate that the only protein irreversibly associated with H/ACA RNAs is NAP57.

H/ACA RNPs are also of general interest because some of their components are mutated in the often-fatal bone marrow failure syndrome dyskeratosis congenita (Dokal and Vulliamy 2003; Marrone and Mason 2003). The X-linked form of dyskeratosis congenita (X-DC) is caused by mostly missense mutations in NAP57, whereas the milder and more rare autosomal dominant form is caused by mutations in telomerase reverse transcriptase or RNA (Heiss et al. 1998; Mitchell et al. 1999; Vulliamy et al. 2003).
RESULTS AND DISCUSSION

Differential localization and abundance of endogenous and exogenous NAF1

Human NAF1 is concentrated in the nucleus but excluded from sites of mature H/ACA RNP, the nucleoli and Cajal bodies (Darzacq et al. 2006; Hoareau-Aveilla et al. 2006). Surprisingly, when NAF1 was fused to the green fluorescent protein (GFP) and transiently expressed, it accumulated mostly in the cytoplasm (Fig. 1A). To test if this discrepancy reflected a more difference in steady-state distribution and if GFP-NAF1 shuttled between the nucleus and cytoplasm like endogenous NAF1 (Darzacq et al. 2006), we blocked CRM1-mediated nuclear export by incubation with leptomycin B (LMB). As a consequence, GFP-NAF1 accumulated in the nucleoplasm, suggesting that it was rapidly imported but prevented from returning to the cytoplasm (Fig. 1B).

CRM1 exports nuclear export signal containing proteins (Fornerod et al. 1997). Indeed, scanning the amino acid sequence of human NAF1 revealed several potential nuclear export signal consensus sequences. However, there was only one classical bipartite nuclear localization signal (NLS) present consisting of two monopartite NLSs, KQRK338. To test if this NLS was responsible for nuclear import of GFP-NAF1, four lysines were mutated to alanines KQR338. When this GFP-NAF1 with the mutated NLS was cotransfected with wild-type NAF1 fused to RFP (Fig. 1C) and the cells were incubated with LMB to monitor nuclear import, all of the wild-type RFP-NAF1 was imported, but GFP-NAF1 with the mutated NLS remained mostly excluded from the nuclei (Fig. 1D, cf. panels 1 and 2). This finding indicates that the above-identified NLS in NAF1 is at least partially responsible for the nuclear import of NAF1. Other factors that may contribute to its nuclear import could be cryptic or nonclassical NLSs or, more interestingly, its binding partner NAP57, which contains several classical and functional NLSs of its own (Meier and Blobel 1994; Heiss et al. 1999). Specifically, NAF1 and NAP57 may enter the nucleus in a complex aided by the NLSs of both proteins. Activity of NLSs from both proteins may be crucial for the import of the larger complex of the core trimer that includes the NLS-less NOP10 and NHP2 (Wang and Meier 2004; Darzacq et al. 2006).

The differential localization of the exogenous GFP-NAF1 and the endogenous NAF1 was surprising because GFP tagging of a protein in most cases does not interfere with its proper localization. To test if indeed the tag was responsible for the mislocalization of the exogenously expressed NAF1, we replaced the GFP tag with the lacI repressor or
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Exchange of H/ACA RNP proteins in cell lysates

We previously showed that NAF1, when tethered to a chromosomal locus in the nucleus, recruits NAP57 but not H/ACA RNAs (Darzacq et al. 2006). This lack of cellular association of NAF1 with mature H/ACA RNPs is further supported by the failure of NAF1 to concentrate in nucleoli and Cajal bodies, the sites of mature H/ACA RNPs (Darzacq et al. 2006; Hoareau-Aveilla et al. 2006). To test if overexpression of NAF1 in the nucleus could overcome this lack of association, GFP-NAF1 was transiently expressed in COS cells and concentrated in the nuclei by addition of LMB to prevent its export. Even under these excessively concentrated conditions, GFP-NAF1 remained excluded from nucleoli, marked by nucleolin, and failed to accumulate in Cajal bodies, identified by coilin (Fig. 1G,H). As previously noted in yeast and mammalian cells, therefore, NAF1 associates only to a very minor degree, if at all, with H/ACA RNA containing RNPs (Dez et al. 2002; Fatica et al. 2002; Yang et al. 2002; Darzacq et al. 2006). Unsurprisingly, a GFP-NAF1 interaction with H/ACA RNA was observed when analyzed biochemically.

We established a stable human U2OS cell line that expresses the rat H/ACA RNA E3 from an inducible promoter (Darzacq et al. 2006). To test if H/ACA core proteins associated with this RNA, GFP-tagged proteins were transiently transfected into this cell line and H/ACA RNA transcription was induced, followed by cell lysis and immunoprecipitation of the fusion proteins with antibodies to GFP. The presence of the induced rat E3 (Fig. 2A, upper panel) and the endogenous human E3 snoRNA (lower panel) in the precipitates was analyzed by an RNase protection assay (Darzacq et al. 2006). Most GFP-H/ACA core proteins, but not the C/D RNP control protein GFP-fibrillarin, efficiently precipitated the full-length induced H/ACA RNA E3 (Fig. 2A, upper panel, lanes 3–7). The stabilization of the full-length rat E3 snoRNA suggested that these core proteins were correctly incorporated into an RNP. This conclusion was supported by our previous observation that knockdown of NAP57 causes a destabilization and consequent loss of the E3 snoRNA in these cells (Darzacq et al. 2006). The core protein GFP-NOP10 exhibited a reduced association likely caused by the size of the GFP tag, which was about four times that of NOP10 (Fig. 2A, lane 7). Specifically, the GFP tag may slow down NOP10 association with newly synthesized snoRNAs, as in the case of the induced E3 snoRNA (Fig. 2A, lane 7), but eventually incorporate into the snoRNP, as in the case of the snoRNP U19 (Fig. 2C, lanes 9,10). Nevertheless, GFP-NAF1, despite its lack of association with mature H/ACA RNPs in intact cells, precipitated the full-length E3 snoRNA as efficiently as did the H/ACA core proteins (Fig. 2A, lane 8).

Given the efficient association of GFP-NAF1 with mature E3 particles in cell lysates but not in intact cells, we tested if it bound to the induced H/ACA RNA only after cell lysis. For this purpose, we compared the outcome of two experimental approaches (Fig. 2B, I,II). In the first approach, we basically repeated the previous experiment (Fig. 2A) by transfecting the GFP-tagged constructs into U2OS cells expressing the rat E3 snoRNA followed by immunoprecipitation of the GFP-fusion proteins (Fig. 2B, I). In the second approach, the GFP-tagged constructs were transfected into parent U2OS cells (lacking the rat E3 gene), whereas the rat E3 snoRNA was expressed in separate cells (Fig. 2B, II). In both cases, 2 d post-transfection, an equal number of transfected and untransfected (but rat E3 snoRNA induced) cells were mixed, lysed, and processed for immunoprecipitation with anti-GFP antibodies. Endogenous and induced H/ACA snoRNAs were detected using RNase protection analysis (Fig. 2C). In both cases, all GFP-fusion proteins associated with the endogenous human H/ACA snoRNAs U19 and E3 (Fig. 2C, top and bottom panels). In addition, GFP-NAF1 precipitated the induced rat E3 snoRNA regardless of whether it was transfected into cells expressing E3 or not (Fig. 2C, center panel, cf. lanes 1 and 2). The latter indicated that GFP-NAF1 associated with the E3 snoRNA after cell lysis, presumably by binding to NAP57 and by replacing GAR1 in the H/ACA RNP as predicted from our competitive in vitro and in vivo binding studies (Darzacq et al. 2006).
In agreement with this interpretation, GFP-GAR1 also exchanged between mature rat E3 RNPs (Fig. 2C, center panel, cf. lanes 5 and 6). In contrast, GFP-NAP57 only associated with rat E3 snoRNA if it was transfected into the cells expressing rat E3 (Fig. 2C, center panel, cf. lanes 3 and 4). This demonstrated that the major core protein and pseudouridylase NAP57 bound only to newly synthesized H/ACA RNA, consistent with its presence at the H/ACA RNA transcription site (Darzacq et al. 2006), but not to that in preexisting RNPs. Consequently, NAP57 was unable to exchange post-RNP assembly. After transfection into non-E3 expressing U2OS cells, GFP-NHP2 and GFP-NOP10 also associated with the rat E3 snoRNA expressed in separate cells (Fig. 2C, center panel, cf. lanes 7 and 8, lanes 9 and 10, respectively). Specifically, when the amount of precipitated E3 snoRNA was adjusted relative to that of the endogenous E3 and U19 snoRNAs, exchange of GFP-NHP2 and GFP-NOP10 between mature snoRNPs was over twofold less than that of GFP-GAR1 and GFP-NAF1 (Fig. 2C,D). This finding was consistent with NOP10 and NHP2 forming part of a core trimer with NAP57. Nevertheless, NAP57 alone was primarily responsible for the stability of H/ACA RNPs and for our previously documented lack of RNA exchange between H/ACA RNPs (Wang and Meier 2004).

**Protein dynamics of H/ACA RNPs**

In summary, our observations show that H/ACA RNPs are dynamic RNA–protein complexes that allow exchange of all core proteins except for NAP57. NAP57 is the largest and catalytic subunit of the RNP protein moiety. It associates tightly with each newly synthesized H/ACA RNA, likely requiring de novo synthesis of both for every new particle. The tight NAP57–H/ACA RNA association marries substrate recognition by the RNA with catalysis by the pseudouridylase mirroring the unification of these two activities in pseudouridylases that only consist of a single protein. Therefore, this tight RNA–protein association in H/ACA RNPs may be rooted in the evolution of pseudouridylases. Conversely, the exchange of the other core proteins could reflect a possible rearrangement or breathing of the complex during catalysis. For example, recent crystal structures of partial archaeal H/ACA protein complexes demonstrate how NOP10 brackets the catalytic domain of NAP57 (Hamma et al. 2005; Manival et al. 2006; Rashid et al. 2006). During catalysis this NOP10–NAP57 interaction may need to be loosened to allow access to and/or release of substrate RNAs.

Unexpectedly, our studies reveal a differential localization of the exogenously and endogenously expressed assembly factor NAF1 and an H/ACA RNP association in cell lysates not observed in vivo. The differential localization was independent of protein tags and apparently caused by overexpression of the low-abundance NAF1. Meanwhile,
the NAF1 association with H/ACA RNPs in cell lysates may well be due to the loss of cellular compartmentalization in the lysates. Alternatively or as a result of compartmentalization, a post-translational modification of NAF1 or NAP57 that is usually sequestered (e.g., a phosphorylation or dephosphorylation event) could be responsible for the in vitro phenomenon. Regardless, our findings signify a caveat when working with RNPs in cell extracts, particularly in the light of similar observations with the RNA binding protein HuR and its interaction with c-fos mRNA (Mili and Steitz 2004).

Finally, our findings may shed light on a long-standing conundrum concerning the bone marrow failure syndrome X-linked dyskeratosis congenita. Two hallmarks of cells from patients with X-DC are shortened telomeres and loss of telomerase RNA (Mitchell et al. 1999). Similar to other H/ACA RNAs, human telomerase RNA is stabilized by the H/ACA core proteins (Mitchell et al. 1999; Dragon et al. 2000; Dez et al. 2001). Genetic depletion studies in yeast demonstrated that all H/ACA core proteins, except GAR1, are essential for the stability of H/ACA RNAs (Bousquet-Antonelli et al. 1997; Henras et al. 1998; Lafontaine et al. 1998; Watkins et al. 1998). Nevertheless, X-DC mutations have been described previously: GFP-NAF1 (pNK16, NAF1 fused to monomeric GFP), RFP-NAF1 (pNK31, NAF1 fused to monomeric RFP), and NAF1 (pNK40) (Darzacq et al. 2002). Briefly, after transfection of GFP-tagged constructs with FuGene 6 (Roche) into 1.2 × 10^6 cells and simultaneous induction of the transgene with 6 µg/mL doxycycline for 24 h, the cells were lysed and sonicated in 500 µL of 40 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.01% Triton X-100, and the RNPs precipitated with anti-GFP agarose beads (Medical & Biological Laboratories). After proteinase K treatment of the RNPs, the precipitated with anti-GFP agarose beads (Medical & Biological Laboratories).

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**MATERIALS AND METHODS**

**Tissue culture, transfection, and cell lines**

Hela and COS-1 cells were cultured in high-glucose DMEM (GIBCO) containing 10% fetal bovine serum (FBS, GIBCO). The rat E3 snoRNA inducible U2OS cells were generated as described previously (Darzacq et al. 2002). Briefly, after transfection of GFP-tagged constructs with FuGene 6 (Roche) into 1.2 × 10^6 cells and simultaneous induction of the transgene with 6 µg/mL doxycycline for 24 h, the cells were lysed and sonicated in 500 µL of 40 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.01% Triton X-100, and the RNPs precipitated with anti-GFP agarose beads (Medical & Biological Laboratories). After proteinase K treatment of the RNPs, the snoRNAs were extracted and analyzed by RNase protection.

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