The Roles of Intersubunit Interactions in Exosome Stability*

Received for publication, May 21, 2003
Published, JBC Papers in Press, June 23, 2003, DOI 10.1074/jbc.M305333200

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In eukaryotes, at least 10 proteins associate in a 3′-5′ exonuclease complex, the exosome, which is involved in the processing of many RNA species. A recent model for the exosome placed six RNase PH-related components in a hexameric ring core structure, with three S1 domain proteins associated with the ring surface. So far, however, this model lacks experimental support. Using a combination of RNA interference, complex affinity purification, and yeast two-hybrid approaches, we show here that the RNase PH homologues are important for maintenance of complex integrity. In contrast, the S1 domain proteins are not required for complex stability, although they are required for exosome function. Our results are partially consistent with the proposed model of the exosome, but indicate a different arrangement of the RNase PH proteins.

The exosome is a multiprotein complex involved in the 3′-5′ processing of many RNA species (1–3). It is localized in both the nucleus and the cytosol of the cell, and has been described in eukaryotes as diverse as Saccharomyces cerevisiae (4), Homo sapiens (5), Arabidopsis thaliana (6), Drosophila melanogaster (7), and the parasitic protozoa Trypanosoma brucei (8). The yeast exosome is composed of 10 different subunits present in apparent equal stoichiometry (9). Six subunits, Rrp41p, Rrp42p, Rrp43p, Rrp44p, and Mtr3p, are related to the bacterial 3′-5′ exonuclease RNase PH. Three others, Rrp44p, Rrp45p, and Csl4p, contain the RNA binding domain S1. The last component of the complex is Rrp6p, which is related to bacterial RNase H II; this protein does not seem to be associated with human or T. brucei exosomes (5, 8). In addition, the nuclear exosome of yeast and human cells contains another component, Rrp6p, which is related to bacterial RNase D; in trypanosomes TbrRrp6 is also located in the cytosol (8). Each exosome component has, or is predicted to have, 3′-5′ ribonuclease activity (2), with the possible exception of Csl4p (10). Prokaryotes do not have an exosome, but they do have another complex, the degradosome, that degrades RNA molecules from their 3′ end (11) and includes a 3′-5′ ribonucleasome component, polynucleotide phosphorylase (PNPase).1 Why so many exonucleases associate in a complex still remains an open question, but it is probably a way to regulate the degradation of RNA substrates in a very precise manner (1, 2). No detailed structural information is yet available for the exosome complex. Recently, a model for the structure of the yeast exosome has been proposed based on electron microscopy data and on the similarity between bacterial PNPase and exosome components (12). According to this model, the RNase PH-like proteins would be arranged in a hexameric ring, whereas the three S1 domain proteins would be laid on top of the ring structure. A systematic two-hybrid analysis performed with human exosome components in human cells partially supported the above “PNPase model,” but disagreed in the relative position of RNase PH-like proteins in the ring (10). Thus, in the PNPase model, the proposed interactions among RNase PH-like proteins would be Rrp41p-Rrp42p-Rrp43p-Rrp44p-Rrp45p-Rrp46p, whereas mammalian two-hybrid assay data suggest Rrp41p-Rrp42p-Mtr3p-Rrp43p-Rrp46p-Rrp45p. The only three interactions described for yeast exosome components using also a two-hybrid approach, Rrp42p-Mtr3p (13, 14), Rrp45p-Rrp41p (14), and Rrp43p-Rrp46p (15), support the organization proposed for the human exosome.

All yeast exosome components are essential for viability, with the exception of Rrp6p, and depletion of each of them yields broadly similar defects in RNA processing (16). Likewise, depletion of all exosome component homologues in T. brucei, with the possible exception of the Csl4p homologue, results in cell death and in very similar defects in 5.8 S rRNA processing (8). One possible explanation for these observations is that the absence of any subunit results in a failure to assemble the complex (1). We have now studied systematically the effect that the depletion of each exosome component has on the stability of the T. brucei complex and present an exhaustive yeast two-hybrid analysis of all the T. brucei exosome components.

EXPERIMENTAL PROCEDURES

Cell Culture—Procyclic form T. brucei 449 cells stably expressing the tetracycline repressor were grown in the presence of 0.5 μg/ml pleomycin (17). They were used to generate all the cell lines described in this work.

Preparation of Protein Extracts—Cells were harvested at a cell density of 8–12 × 10^6 cells/ml and washed with phosphate-buffered saline. To prepare total cell extracts, the cells were lysed in Laemmli buffer, boiled, and loaded directly in SDS-PAGE gels (2 × 10^6 cells/lane). To prepare protein extracts to be used in glycerol density gradient analysis and for the tandem affinity purification (TAP) method (see below), cells

1 The abbreviations used are: PNPase, polynucleotide phosphorylase; TAP, tandem affinity purification; RNAi, RNA interference; ORF, open reading frame; dsRNA, double-stranded RNA.
were harvested and washed as above, frozen in liquid N₂, and stored at −80 °C until use. Cell pellets were allowed to thaw in a water bath at room temperature. Cells were then resuspended in ice-cold buffer containing 10 mM Tris-HCl, pH 7.6, 10 mM NaCl, and protease inhibitors (Protease Inhibitor Mixture (EDTA-free); Roche Applied Science) and lysed by 10 strokes in a Dounce homogenizer equipped with a Teflon pestle (B. Braun, Germany) on ice. Igepal CA-630 (Sigma) was added at a final concentration of 0.1% and an additional 10 homogenization strokes were performed. The lysate was centrifuged at 4 °C for 20 min at 100,000 × g. The supernatant was ultracentrifuged for 1 h at 400,000 × g. NaCl was added to the final supernatant at final concentration of 150 mM. The protein extracts obtained using this method contain both the cytosolic and the nuclear fractions, as judged by immunoblot analysis using known cytosolic and nuclear protein markers (data not shown). We did not test for the presence of proteins from other cell compartments.

**TAP of the T. brucei Exosome—**To create a C-terminal, TAP-tagged version of TbEAP1, the ORF was amplified by PCR and cloned in-frame with the TAP tag in the plasmid pDH918 (8), to yield plasmid pHDI335. The same approach was used to make a TAP-tagged version of TbRPP45, but the resulting plasmid was modified to exchange the hygromycin resistance marker for a blasticidin one, yielding pHDI337. Plasmids were linearized with NcoI and transfected by electroporation into 449 cells (pHDI335) or 449 cells already transfected with the expression vector pHDI146 (8) (pHDI337). Transfectedants were selected in the presence of 50 μg/ml hygromycin (pHDI335) or 50 μg/ml blasticidin plus 50 μg/ml hygromycin (pHDI337) and cloned by limiting dilution. The cell line expressing a TAP-tagged version of TbRPP45 has been described previously (8). The TAP procedure was performed as described (18, 19) from fresh extracts containing 30–60 mg of protein. The fractions containing the purified exosome were pooled and subjected to precipitation with trichloroacetic acid and sodium deoxycholate in siliconized tubes (20).

**Mass Spectrometry and Identification of New Exosome Components—**Proteins were identified using SDS-PAGE and visualized by Coomassie Blue staining (20). Individual bands were excised and subjected to tryptic digestion as described (21, 22). Tandem mass spectrometry was performed on a Q-TRAP electrospray ion source (PE SCIEX, Weiterstadt, Germany). The data were processed using BioAnalyst software (PE SCIEX, Weiterstadt, Germany). Proteins were identified using Mascot (Matrix Science), the SONAR protein identification program (Proteometrics), or TBLASTN (23) processed using Bioanalyst software (PE SCIEX, Weiterstadt, Germany) equipped with a nano-electrospray ionization ion source (MDS Protana, Odense, Denmark). The data were analyzed using the BioAnalyst software (PE SCIEX, Weiterstadt, Germany). Proteins were identified using Mascot (Matrix Science), the SONAR protein identification program (Proteometrics), or TBLASTN (23) processed using Bioanalyst software (PE SCIEX, Weiterstadt, Germany) equipped with a nano-electrospray ionization ion source (MDS Protana, Odense, Denmark). The data were analyzed using the BioAnalyst software (PE SCIEX, Weiterstadt, Germany). The reference database for Mascot was the Wellcome Trust Sanger Institute (Hinxton, United Kingdom; www.sanger.ac.uk/). The reference database for Mascot was the Wellcome Trust Sanger Institute (Hinxton, United Kingdom; www.sanger.ac.uk/).

**RESULTS**

Identification of New Components in the T. brucei Exosome—In a previous work, we described the purification and identification of some of the components of the T. brucei exosome using the TAP method with TbRPP4 as a bait (8). We showed that three proteins co-purified with TbRPP4-TAP: TbRPP6, TbRPP45, and TbCSL4. In addition, three other components with electrophoretic mobilities corresponding to 52, 32, and 30 kDa could not be identified by matrix-assisted laser desorption ionization/time of flight mass spectrometry. By increasing the starting amount of material used for the purification 5-fold and by using tandem mass spectrometry to identify the polypeptides, we were able to identify the remaining components. Fig. 1A shows a purification of the T. brucei exosome using the TAP method. The proteins detected seem to be genuine components of the exosome, as all of them could be visualized using either TbRPP4 or TbEAP1 (a novel component of the trypanosome exosome, see below) as baits for the purification; moreover, no proteins could be detected when the TAP tag peptide was expressed alone. The 52-kDa band corresponded to an RNase PH-like protein that we have designated EAP1 (protease with components of the yeast and human exosome subunits were transfected with plasmid pHDI337 (bearing a TAP-tagged version of TbRPP45; see above) and selected in the presence of 50 μg/ml hygromycin plus 10 μg/ml blasticidin. Clones that were chosen that were able to make RNAs and to express TbRPP45-TAP, both in a tetracycline-inducible manner. To purify exosome complexes from these cell lines, cultures were inoculated at 1 × 10⁸ millions/ml in the presence of 100 ng/ml tetracycline. Cells were grown for 48 h, and then harvested and processed as indicated above.

**Glycerol Density Gradient Analysis—**4 mg of protein extracts obtained as described above were made in 5 mM MgCl₂ and 10% glycerol and separated through 10–30% glycerol gradients essentially as described (13). The open reading frame of each T. brucei exosome subunit was amplified using PfX proofreading polymerase (Invitro) from genomic DNA using primers tagged with sequences homologous to both two-hybrid vectors (forward primer tag, gaagctacctgtgtaaaagcagggct; reverse primer tag, tctgacacctgtgtaagggggtga). Yeast strains pJ9–4A (baits) and pJ9–1alpha (preys) (25) were transformed with each PCR fragment (26) and linearized two-hybrid vector pGBAD-B (baits) or pACTD-B (preys) (vectors provided by D. Markie, University of Otago, New Zealand). Recombinant vectors were verified for the correct sized inserts. Pools of 11 transformed clones were streaked onto selective media and mated in all possible combinations by replica plating onto media containing 0.1% and 0.2% 3-aminotriazole (3-AT); the mating plates were incubated at 30 °C for 6h. Diploids were selected on media lacking tryptophan and leucine and reporter activation tested by replica plating onto media lacking tryptophan, leucine, and either histidine or adenine. Growth was recorded at 5 and 10 days. All matings were performed in triplicate, and only interactions isolated in two or more replicates were considered positives.
components of the exosome, \( T\). \( B\). \( E\). \( A\). \( P\) \( 4\) and \( T\). \( B\). \( R\) \( P\) \( 4\)\( 1\)B, co-migrated as a single band in SDS-PAGE gels prompted us to re-examine all the other protein bands by tandem mass spectrometry. All the tryptic peptides examined from the proteins \( T\). \( B\). \( R\) \( P\) \( 6\), \( T\). \( B\). \( R\) \( P\) \( 4\)\( 5\), and \( T\). \( B\). \( R\) \( P\) \( 4\) could be matched to the corresponding proteins. However, the \( T\). \( B\). \( C\) \( S\) \( L\) \( A\) band also contained peptides matching \( T\). \( B\). \( R\) \( P\) \( 4\)\( 0\), a protein previously thought not to be associated with the \( T\). \( B\). \( E\) exosome (8). A weaker band below \( T\). \( B\). \( C\) \( S\) \( L\) \( A\) \( T\). \( B\). \( R\) \( P\) \( 4\)\( 0\) was again identified as \( T\). \( B\). \( R\) \( P\) \( 4\)\( 0\) (Fig. 1A).

In addition, three novel protein bands, named \( T\). \( B\). \( E\) \( A\) \( P\) \( 2\), \( T\). \( B\). \( E\) \( A\) \( P\) \( 3\), and \( T\). \( B\). \( E\) \( A\) \( P\) \( 4\), were seen that were not detected in our previous work; their electrophoretic mobilities correspond to 33, 23, and 26 kDa, respectively (Fig. 1A). \( T\). \( B\). \( E\) \( A\) \( P\) \( 2\) migrated as a doublet and corresponded to a RNase-like protein similar to \( R\) \( p\) \( 4\) \( 5\)p and \( R\) \( p\) \( 4\) \( 3\)p of yeast and human cells (Table I). \( T\). \( B\). \( E\) \( A\) \( P\) \( 3\) also migrated as a doublet; it is similar to yeast \( L\) \( r\) \( p\) \( 1\)p (YHR081w) (Table I), a protein that has been described as a substoichiometric component of the yeast exosome (12, 27). Finally, \( T\). \( B\). \( E\) \( A\) \( P\) \( 4\) was identified as another RNase PH-like protein similar to the exosome components Mtr3p and \( R\) \( p\) \( 4\) \( 1\)p of yeast and human cells (Table I).

In summary, the \( T\). \( B\). \( E\) exosome seems to be composed of 11 proteins. Six are related to \( E\). \( c\) \( o\)l RNase PH: \( T\). \( B\). \( R\) \( P\) \( 4\)\( 1\)A, \( T\). \( B\). \( R\) \( P\) \( 4\)\( 1\)B, \( T\). \( B\). \( R\) \( P\) \( 4\)\( 5\), \( T\). \( B\). \( E\) \( A\) \( P\) \( 1\), \( T\). \( B\). \( E\) \( A\) \( P\) \( 2\), and \( T\). \( B\). \( E\) \( A\) \( P\) \( 4\), and could be the counterparts of \( R\) \( p\) \( 4\) \( 1\)p, \( R\) \( p\) \( 4\) \( 6\)p, \( R\) \( p\) \( 4\) \( 5\)p, \( R\) \( p\) \( 4\) \( 2\)p, \( R\) \( p\) \( 4\) \( 3\)p, and Mtr3p, respectively, of yeast and human cells (see below). Three components are related to the S1 domain proteins present in yeast and human exosome complexes (\( T\). \( B\). \( R\) \( P\) \( 4\), \( T\). \( B\). \( R\) \( P\) \( 4\)\( 0\), and \( T\). \( B\). \( C\) \( S\) \( L\) \( A\) ), one is related to yeast \( R\) \( p\) \( 6\)p and human \( P\) \( M\) \( S\)c\( 1\)\( 0\) (\( T\). \( B\). \( R\) \( P\) \( 6\)), and the last one, \( T\). \( B\). \( E\) \( A\) \( P\) \( 3\), is similar to the substoichiometric component of the yeast exosome Lrp1p (YHR081w) and shows no significant similarity to any human protein. The yeast exosome also contains the RNase II-like protein \( R\) \( p\) \( 4\) \( 4\)p (4), which seems to be absent in the human exosome (5). \( T\). \( B\). \( E\) cells also express an
Rrp44-like protein, TbrRRP44 (8), but we were unable to detect it in the purified exosome fractions (Fig. 1 and Ref. 8). Interestingly, five proteins migrated consistently as doublets in SDS-PAGE gels: TbrRRP6, TbrRRP40, TbrRRP41A, TbrEAP2, and TbrEAP3. Whether this is the result of protein degradation or partial post-translational modification remains to be determined.

**Similarities among Yeast, Human, and T. brucei RNase PH-like Proteins**—The sequence conservation of the RNase PH-like subunits is restricted mainly to the PH domains, which makes assignment of direct homologues difficult (9, 12). Although there are clear homologues of Rrp41p, Rrp45p, and Rrp46p in the human exosome (hRrp41p, hRrp45p, and hRrp46p, respectively), finding counterparts of yeast Rrp42p, Rrp43p, and Mtr3p in the human exosome proved to be problematic (5, 9). In particular, two human RNase PH-like proteins, encoded by the genes KIAA0116 and OIP-2, are also more similar to yeast Rrp45p than to any other exosome component, although hRrp45p is by far the more similar to Rrp45p (9). The protein encoded by KIAA0116 is also similar to yeast Rrp42p, and human Oip2p also shows similarity to yeast Rrp43p. Therefore, it was suggested that the proteins encoded by the genes KIAA0116 and OIP-2 are the counterparts of the yeast proteins Rrp42p and Rrp43p, respectively (5).

We have performed phylogenetic analyses to ascribe homologues between *T. brucei* and yeast/human RNase PH-like proteins, but the bootstrap values were too low to be reliable (data not shown). We therefore have assigned homologues to the *T. brucei* proteins following the same approach used for the human RNase PH-like subunits (see above). There are two clear homologues of Rrp41p and Rrp45p, TbrRRP41A and TbrRRP45, respectively (8). However, there are two other proteins that show maximal similarities to yeast Rrp41p (although to a lesser extent than TbrRRP41A, TbrRRP41B and TbrEAP4 (Ref. 8 and Table I). TbrRRP41B is also similar to yeast and human Rrp46p (8), and TbrEAP4 is similar to the human Mrt3p homologue, even more than to human Rrp41p (Table I). Therefore, TbrRRP41B and TbrEAP4 could be the counterparts of Rrp46p and Mtr3p, respectively. Both TbrEAP1 and TbrEAP2 show maximal similarities to human Rrp43p, yet the latter is significantly more similar to hRrp43p than the former (Table I). Moreover, TbrEAP1 shows maximal similarities to yeast Rrp42p when compared with the yeast protein data base (Table I). Therefore, TbrEAP1 and TbrEAP2 could be the homologues of Rrp42p and Rrp43p, respectively.

**Requirement of Exosome Subunits for Cell Survival**—TbrRRP41A, TbrRRP41B, TbrRRP45, TbrRRP4, TbrRRP40, TbrRRP6, and TbrRRP44 are essential proteins involved in the maturation of 5.8 S rRNA, and depletion of TbrCSL4 results in a slower cell growth rate (8). To investigate the role of the novel proteins TbrEAP1, TbrEAP2, TbrEAP3, and TbrEAP4, they were depleted in *vivo* using RNA interference in a tetracycline-inducible fashion as described under “Experimental Procedures.” Depletion of any of these proteins resulted in cell death (Fig. 1B) and in an altered 5.8 S rRNA maturation phenotype that resembled that observed after depletion of other *T. brucei* exosome subunits (8) (data not shown).

**Effects of Depletion of Individual Exosome Components on Exosome Stability**—We have previously observed that overexpression of a TAP-tagged version of TbrRRP4 led to the depletion of the endogenous, untagged protein (8). Here we have investigated this phenomenon in more detail. Cell lines expressing TAP-tagged versions of TbrRRP4 and TbrRRP45 in a tetracycline-inducible fashion were generated, and the levels of both the tag and the untagged versions of the proteins were monitored using antisera specific for these two proteins (Fig. 2A). Expression of TAP-tagged TbrRRP4 or TbrRRP45 resulted in a depletion of the corresponding endogenous protein in a tetracycline-dependent manner. This phenomenon was not observed at the mRNA level (data not shown). Importantly, endogenous TbrRRP4 was not depleted by overexpression of TbrRRP4-TAP, nor was endogenous TbrRRP4 depleted by overexpression of TbrRRP45 (Fig. 2A). Addition of lactacystin, a known inhibitor of the proteasome of several species including *T. brucei* (28–30), restored the endogenous protein levels to those seen in the minus tetracycline samples. These results suggest that an excess of tagged protein competes out the endogenous protein from the complex, and that free TbrRRP4 and TbrRRP45 proteins are unstable and degraded, probably by the proteasome. In agreement with these observations, neither TbrRRP4 nor TbrRRP45 were detected as free entities in glycerol density gradient analysis (Ref. 8; see also Fig. 3).

We took advantage of this phenomenon to study the stability of the complex upon depletion of the individual subunits. We reasoned that if a given exosome subunit were important for exosome assembly or stability, its depletion would cause the exosome to disorganize, which in turn would cause TbrRRP4 and TbrRRP45 to be released from the complex and degraded. The effects of RNA interference-mediated depletion of every component of the exosome on the levels of TbrRRP4 and TbrRRP5 are shown in Fig. 2B. Depletion of any of the six RNase PH-like proteins, as well as depletion of TbrRRP6 and TbrEAP3, resulted in the concomitant depletion of TbrRRP4 (a phenomenon henceforth referred as “co-depletion”). In contrast, depletion of the S1 domain proteins TbrRRP4 and TbrCSL4 did not result in any apparent change in the levels of TbrRRP4. This was not the result of inefficient RNAi, because the corresponding mRNAs were depleted in all cases (Fig. 2B, *fifth row*). Depletion of the RNase PH-like proteins TbrRRP41B, TbrEAP2, and TbrEAP4 also resulted in co-depletion of TbrRRP45. The same phenomenon was observed upon depletion of TbrRRP6 and TbrEAP3. However, neither the depletion of the RNase PH-like proteins TbrRRP41A and TbrEAP1 nor depletion of any of the S1 domain proteins resulted in co-depletion of TbrRRP45. These results indicate that the RNase PH-like proteins and TbrRRP6 and TbrEAP3 are more important than the S1 domain proteins in keeping protein-protein interactions in the *T. brucei* exosome. Depletion of TbrRRP44 had little effect on either TbrRRP4 or TbrRRP45 levels (Fig. 2B). Moreover, TbrRRP44 levels were unaffected by the depletion of any of the exosome components (Fig. 2B, *third row*), which is expected for a protein that is not present in the exosome complex.

We next wanted to find out in detail the effects of depletion of individual subunits on the remainder. Repeated attempts to generate specific antisera to the other subunits have so far failed to yield reagents of adequate quality. As an alternative, therefore we set up the RNA interference in a cell line expressing TAP-tagged TbrRRP45. The exosome could then be purified from cell lines after subunit depletion. Results are shown in Fig. 4. In particular, Fig. 4A shows a Coomassie-stained gel of the purified exosomes. Note that once again several bands are co-migrating; for unknown reasons a resolution such as that shown in Fig. 1A is only intermittently attainable. A few other protein bands, intensity of which varied between preparations, were also seen (see Fig. 4 legend). Depletion of TbrCSL4 (*far right*, band indicated by asterisk to the left) had, as expected, no effect on the abundances of TbrRRP4 and the yield of exosome was equivalent to that seen from undepleted cells. Evidently TbrCSL4 is not essential for exosome integrity. Depletion of TbrRRP40 mRNA was effective (data not shown), but a reduction in the protein was difficult to assess, partly because of the co-migration with TbrCSL4. No effects of TbrRRP40 depletion on
and 5

DNA probes using the ethidium bromide stain as loading control. To check that mRNAs were being depleted, RNA samples obtained from the cultures were subjected to Northern blotting and hybridized to specific

at the amounts (in ng/ml) indicated

and

Tb

exosome composition were observed (Fig. 4B). TbRRP4 depletion was confirmed by Western blot (Fig. 4, B and C); the remainder of the exosome was intact (Fig. 4A).

We had already seen that normal levels of TbRRP45 were retained after depletion of two RNase PH subunits, TbRRP41A and TbEAP1 (Fig. 2B), although TbRRP4 was depleted, as confirmed in Fig. 4B. The purified exosomes from these cells, however, looked fairly normal with the exception of the reduction in TbRRP4; in particular there was little apparent reduction in the tagged subunits. These results are very difficult to interpret in the absence of specific antisera to the depleted components. Because of variations in the purification efficiency, we cannot reliably judge whether the yield of exosome is reduced in parallel with the reduction in TbRRP41A and TbEAP1 mRNAs. It is therefore possible either that TbRRP41A and TbEAP1 are indeed required to build an exosome, or that other RNase PH subunits can substitute.

Protein-Protein Interactions in the T. brucei Exosome—To investigate how the different components of the T. brucei exosome interact with each other, we first addressed the question of whether there are one or more copies of each exosome component per complex. It has been suggested that the human exosome contains at least two copies of each subunit (10), but this does not seem to be the case in the yeast exosome (12). We made use again of the cell lines expressing TAP-tagged versions of TbRRP4 and TbRRP45 that were shown in Fig. 2. The recombinant proteins were expressed at low levels, so both the endogenous and the tagged proteins should be detected in the purified exosome fraction. However, we could detect only the tagged versions (Fig. 5, lane 3). This indicates that there is only a single copy of TbRRP4 and TbRRP45 in the T. brucei exosome. The tagged protein is smaller in size in lane 3 as compared with lane 1 because of the tobacco-etch virus protease treatment during the TAP procedure.

Finally, we performed an exhaustive two-hybrid analysis of the T. brucei exosome subunits. Fig. 6A shows the results of testing all possible pairwise interactions between different components of the complex, and a summary of these results is shown in Fig. 6B. Protein-protein interactions observed in two-hybrid studies carried out either using yeast (13–15) or human (31) exosome components were also included in Fig. 6B for comparison (dashed lines). We used two different reporter markers, ade (stringent) and his (less stringent) (25). Nineteen interactions were detected, 12 of which were seen using both markers. We could also observe self-interactions in the cases of TbRRP40 and TbEAP3. Pairwise interactions between the different RNase PH-like proteins were observed as both bait or prey, and the same was true for the interaction between TbRRP6 and TbEAP3 (represented as thick solid lines in Fig. 6B). The interactions among S1 domain proteins were usually unidirectional as bait or prey but not both (represented as solid thin lines in Fig. 6B). No interactions could be detected when using TbCSL4 as bait. The only interactions we could detect between an S1 domain protein and RNase PH-like proteins were those of TbCSL4 with TbRRP41A and TbCSL4 with TbEAP4. These interactions were found only when the RNase PH-like proteins were used as baits. As expected, interactions between TbRRP44 and other exosome components were not detected.

![Fig. 2. Effects of overexpression and depletion of exosome components. A. effect of overexpression of TAP-tagged versions of TbRRP4 and TbRRP45 on the levels of the corresponding endogenous proteins. TAP-tagged proteins were induced at increasing levels by adding tetracycline at the amounts (in ng/ml) indicated above each lane. LC, cells expressing TAP-tagged proteins grown in the presence of both 100 ng/ml tetracycline and 5 μM lactacystin. As controls, untransformed 449 cells grown in the presence of 100 ng/ml tetracycline (C) or 100 ng/ml tetracycline plus 5 μM lactacystin (C + LC) were included. Cells were grown for 24 h and total cell extracts obtained as described under “Experimental Procedures” were subjected to SDS-PAGE, transferred to nitrocellulose filters, and decorated with antisera against TbRRP4 and TbRRP45 (8) or p34/37 (36), which served as a loading control. B. effect of depletion of every individual exosome component on the levels of TbRRP4, TbRRP45, and TbRRP44. Total cell extracts were obtained from cells expressing dsRNA specific for the individual exosome subunit indicated on top of the panel after addition of 100 ng/ml tetracycline and incubation for 24 or 48 h. Samples obtained from each cell line grown in the absence of tetracycline for 48 h (−) were also included. Proteins were detected with antisera against TbRRP4, TbRRP45, TbRRP44 (8), and TbCSL4 (37), which served as loading control. To check that mRNAs were being depleted, RNA samples obtained from the cultures were subjected to Northern blotting and hybridized to specific DNA probes using the ethidium bromide stain as loading control.]
DISCUSSION

In our previous analysis of the trypanosome exosome, we were unable to identify several subunits (8). The use of considerably more material, combined with increased resolution from tandem mass spectrometry, has now enabled us to describe all stoichiometric components. However, we cannot rule out that there are additional subunits that dissociate during purification or do not stain well. The results show that the trypanosome exosome is extremely similar to the yeast and human exosomes in composition. First, it contains six RNase PH-like subunits, TbRRP41A, TbRRP41B, TbRRP45, TbEAP1, TbEAP2, and TbEAP4. The sequence information suggests that these subunits are equivalent to Rrp41p, Rrp46p, Rrp45p, Rrp42p, Rrp43p, and Mtr3p, respectively, and it was supported by the yeast two-hybrid interaction data. The trypanosome exosome also has three S1 domain proteins, TbRRP4, TbRRP45, and TbCSL4. The TbRRP6 subunit, which is specific to the nuclear exosome in yeast and humans, is retained in the cytoplasmic exosome in trypanosomes. In addition, the trypanosome exosome contains TbEAP3, a protein that very distantly resembles Lrp1p (YHR081w), a substoichiometric yeast exosome component. The yeast exosome also contains a RNase II-like protein, Rrp44p (4), which is not detected in either T. brucei or human exosomes (Refs. 5 and 8; see also Fig. 1A). It is unlikely that the absence of the Rrp44p homologue from the latter two cell types is the result of the TAP technique, because the yeast Rrp44p protein could be co-purified when TAP-tagged versions of Rrp45p, Rrp46p, Skg6p (Rrp41p), or Cal4p were used as baits (12, 27). Moreover, TbRRP44 behaved as a free protein in glycerol density gradients, and an antiserum against TbRRP44 failed to detect this protein associated to the exosome (8). Interestingly, the exosome complexes from both T. brucei and human cells has a sedimentation coefficient of 11 S, whereas the yeast one sediments at 14 S. This difference may be explained by the absence of the Rrp44p homologue from the former two complexes. It is of course possible that Rrp44p is so loosely associated with the exosome that it is lost during cell lysis and purification. Indeed, yeast Rrp44p is the first protein to dissociate when a salt gradient is applied (9). Notably, depletion of TbRRP44 in T. brucei results in 5.8 S rRNA processing defects, which are very similar to those seen upon depletion of other exosome components (8).

Depletion of any of the components of yeast and T. brucei exosomes results in cell death and in very similar defects in RNA processing. The simplest explanation would be that the absence of any of the subunits causes a failure to assemble the whole complex or inactivates it (1). However, it was shown that a yeast strain lacking two exosome subunits showed stronger phenotypes than either single mutant (16), which suggests that the absence of one component does not inactivate the entire complex. In the present work we have studied systematically the effect that the depletion of any of the components of the T. brucei exosome has on the stability of the complex. Overexpression of tagged versions of two components of the complex, TbRRP4 and TbRRP45, resulted in a concomitant depletion of the endogenous protein (Fig. 2A), and the endogenous proteins could be raised back to their original levels by the addition of the proteasome inhibitor lactacystin. Thus, these two exosome components are indeed degraded when they are not associated in the exosome. Results of our experiments, however, showed conclusively that some subunits could be depleted without any apparent effects on the others. Thus, depletion of the S1 domain proteins did not alter the levels of TbRRP4 or TbRRP45 (Fig. 3B). When analyzed in glycerol density gradients, complexes depleted of any of the S1 domain proteins behaved like intact complexes (Fig. 3). Most importantly, when the exosome

**Fig. 3.** Glycerol density gradient analysis of protein extracts obtained from cell lines depleted on individual exosome components. Protein samples obtained from RNAi cell lines grown in the presence of tetracycline for 24 h were loaded in glycerol gradients. The same number of cells was used in each analysis. Aliquots taken from each fraction of the gradient were subjected to SDS-PAGE and immunoblotting analysis using antisera against TbRRP45 (left series of panels) or TbRRP4 (right series of panels). The protein being depleted is indicated at the left of each gradient. The control cell line was transfected with the empty expression vector pHDI146 (see “Experimental Procedures”). 10 µg of protein corresponding to the control cell line were also loaded at the rightmost lane in the SDS-PAGE gels (control extract).
was purified from cell lines depleted of TbRRP4, TbRRP40, or TbCSL4 using the TAP procedure and TbRRP45 as a bait, no apparent changes in the general appearance nor in the stoichiometry of the subunits was evident upon inspection of Coomassie-stained SDS-PAGE gels (Fig. 4). Depletion of TbRRP4 and TbRRP40 results in cell death and in an impaired maturation of 5.8 S rRNA, whereas the depletion of TbCSL4 confers a slow growth phenotype (8). Thus, the S1 domain proteins are essential even though they are not required for the association of the remaining exosome components. Either they have essential functions on their own unrelated to exosome stability, or the absence of these subunits has deleterious effects on higher order exosome structure that would not be detected in our analysis.

The six RNase PH-like proteins TbRRP41A, TbRRP41B, TbRRP45, TbEAP1, TbEAP2, and TbEAP4, as well as TbRRP6 and TbEAP3, seem to have different roles in exosome stability in T. brucei. The depletion of any of these proteins resulted in co-depletion of the S1 domain protein TbRRP4. However, the depletion of TbRRP41B, TbEAP2 and TbEAP4, TbRRP6 and TbEAP3, but not TbRRP41A or TbEAP1, also affected TbRRP45 levels (Fig. 2B). Interestingly, although TbRRP45 levels did not change after depletion of TbRRP41A or TbEAP1, its behavior in glycerol density gradients was affected in the sense that TbRRP45 was detected in both the normal position (11 S) and also in lighter fractions (Fig. 3). This suggests that some exosome components are assembled in the absence of either of these two RNase PH-like proteins. This was confirmed by purification of exosome complexes depleted of TbRRP41A and TbEAP1 using TbRRP45 as a bait (Fig. 4). These complexes were purified using the TAP method from cell lines expressing dsRNA and a TAP-tagged version of TbRRP45. The control cell line bore the empty dsRNA-expressing vector pHDI146 (see “Experimental Procedures”). A, Coomassie Blue-stained gel of exosome complexes purified from the depleted cells. The identity of the subunit targeted by RNAi is shown above the lanes. The positions of the proteins being depleted are indicated by asterisks to the left of the corresponding band. The amounts of total soluble protein (in mg) used in each purification are indicated below the gel. Bands in the region of 120 kDa were composed of aggregates of other exosome components, as judged by mass spectrometry analysis. The band migrating just above TbEAP1 is β-tubulin (accession no. AAA30261). Total cell extracts (B) or 1/50 of each purified exosome fraction (C) obtained from every cell line were subjected to immunoblot analysis and decorated with TbRRP4 antiserum (which also recognizes in panel B the TbRRP45-TAP protein because of the presence of the IgG-binding motifs in the TAP tag). The immunoblot shown in panel B was stripped and decorated with a TbCSM antiserum (loading control).

**Fig. 4.** Purification of T. brucei exosome complexes lacking individual subunits. Exosome complexes were purified using the TAP method from cell lines expressing dsRNA and a TAP-tagged version of TbRRP45. The control cell line bore the empty dsRNA-expressing vector pHDI146 (see “Experimental Procedures”). A, Coomassie Blue-stained gel of exosome complexes purified from the depleted cells. The identity of the subunit targeted by RNAi is shown above the lanes. The positions of the proteins being depleted are indicated by asterisks to the left of the corresponding band. The amounts of total soluble protein (in mg) used in each purification are indicated below the gel. Bands in the region of 120 kDa were composed of aggregates of other exosome components, as judged by mass spectrometry analysis. The band migrating just above TbEAP1 is β-tubulin (accession no. AAA30261). Total cell extracts (B) or 1/50 of each purified exosome fraction (C) obtained from every cell line were subjected to immunoblot analysis and decorated with TbRRP4 antiserum (which also recognizes in panel B the TbRRP45-TAP protein because of the presence of the IgG-binding motifs in the TAP tag). The immunoblot shown in panel B was stripped and decorated with a TbCSM antiserum (loading control).
plexes presented reduced levels of TbRRP4, as expected from the co-depletion data. Regarding the other four RNase-like proteins, TbRRP41B, TbRRP45, TbEAP2, and TbEAP4, as well as TbRRP6 and TbEAP3, we did not attempt to purify what could be left from the complex using TbRRP45 as a bait, because this protein was co-depleted (Fig. 3B). Unfortunately, antibodies against components of the T. brucei exosome other than TbRRP4 and TbRRP45 are not available, and therefore we could not analyze the fate of the other subunits upon depletion of TbRRP41B, TbEAP2, TbEAP4, TbRRP6, and TbEAP3. However, it is tempting to suggest that the exosome complex is largely disorganized after depletion of these proteins. Interestingly, in yeast and in human cells, the cytosolic complex lacks Rrp6p (9), and therefore this protein is not essential for complex stability, at least in the cytosolic exosome. In T. brucei, however, the Rrp6p homologue TbRRP6 seems to reside in both the cytosolic and the nuclear complexes (Ref. 8 and Fig. 1A), and TbRRP6 depletion reduced TbRRP4 and TbRRP45 abundance. Therefore, TbRRP6 might have a more important role in keeping protein-protein interactions in the trypanosome exosome.

The results of the two-hybrid analysis shown in Fig. 6 support the hypothesis (10, 12, 32) that the six RNase PH-like proteins form a core within the exosome structure, and are consistent with our depletion experiments. The fact that depletion of TbRRP41A and TbEAP1 caused co-depletion of TbRRP4 but not TbRRP45 suggests that both proteins are located at the edge of the RNase PH core (Fig. 6B) and interact with TbRRP4. We could not detect those interactions in T. brucei. In fact, we could not observe interactions of TbRRP4 with any RNase PH-like protein. However, Rrp4p does interact with both Rrp41p and Rrp42p (TbEAP1-like) in the human exosome (Ref. 10; see also Fig. 6B). The three S1 domain proteins seem to interact with each other in T. brucei (Fig. 6), but these interactions were not detected in human cells (10). Human Rrp6p was not found to interact with any other component of the exosome (10). In T. brucei we could detect an interaction between TbRRP6 and TbEAP3, but neither of those two proteins interacted with other components, so it is difficult to determine their position within the complex. However, the fact that the depletion of either protein results in co-depletion of TbRRP4 and TbRRP45 suggests that they might be interacting with any of the RNase PH-like proteins TbRRP45, TbRRP41B, TbEAP2, or TbEAP4.

Based on structural similarities between bacterial nucleotidase phosphorylase and the exosome components, it has been suggested that the six RNase PH-like proteins are arranged in a ringlike structure (12). The two-hybrid study performed with human exosome subunits supports this model (10), although the predicted order of the subunits is different. The interactions between the RNase PH-like proteins detected in our two-hybrid experiments is in agreement with the arrangement of the RNase PH-like proteins proposed for the human exosome based on two-hybrid studies in human cells (10). Two of the predicted interactions could not be detected in the human study: Rrp41p-Rrp45p and Rrp45p-Rrp46p. The Rrp41p-Rrp45p interaction, however, has been detected in yeast (14).

We now confirm the Rrp45p-Rrp46p interaction in T. brucei (Fig. 6). On the other hand, we could not detect the predicted interaction TbRRP41A (Rrp41p-like)-TbEAP1 (Rrp42p-like) that would “close” the ring (Fig. 6), and we observed an interaction that is not predicted by the “ring model,” TbRRP41B-TbEAP4. Thus, our two-hybrid results do not imply the presence of an hexameric RNase PH ring in the T. brucei exosome.
The question of whether the six RNase PH-like proteins are in a ring structure can only be resolved by detailed structural analysis of the complex.

The proposed models for the exosome and the data presented here are consistent with a complex built around a core composed of the six RNase PH-like proteins (plus 75RRP6 and T6EAP5 in the case of T. brucei). An important conclusion from our work is that the S1 domain subunits are essential not because their absence results in complex breakdown, but because they have essential functions on their own. This is consistent with the proposed location of the S1 domain proteins either on top (12) or at the end (32) of the RNase PH core, and with a proposed role for the S1 domain proteins in RNA substrate recognition. The availability in T. brucei of exosome complexes devoid of given subunits, combined with the ease of genome sequencing project data available.

Acknowledgments—We thank Cristina Guerra for technical help; George A. M. Cross, Brian Chait, and the NCRR-funded National Resource for the Mass Spectrometric Analysis of Biological Macromolecules at Rockefeller University (New York, NY) for making available the SONAR protein identification program; Frank Voncken for the characterization of the structure of the exosome. Because the two-hybrid results from three very diverged species are internally consistent, our work is that the S1 domain subunits are essential not because their absence results in complex breakdown, but because they have essential functions on their own. This is consistent with the proposed location of the S1 domain proteins either on top (12) or at the end (32) of the RNase PH core, and with a proposed role for the S1 domain proteins in RNA substrate recognition. The availability in T. brucei of exosome complexes devoid of given subunits, combined with the ease of genome sequencing project data available.

REFERENCES
1. van Hoof, A., and Parker, R. (1999) Cell 99, 347–350
2. Mitchell, P., and Tollervey, D. (2000) Nat. Struct. Biol. 7, 843–846
3. Butler, J. S. (2002) Trends Cell Biol. 12, 90–96
4. Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997) Cell 91, 457–466
5. Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Rajmakers, R., Prijn, G. J., Stockelin, G., Moroni, C., Mann, M., and Karin, M. (2001) Cell 107, 451–464
6. Chekanova, J. A., Shaw, R. J., Wills, M. A., and Belostotsky, D. A. (2000) J. Biol. Chem. 275, 33158–33166
7. Andrulis, E. D., Werner, J., Nazarian, A., Erdjument-Bromage, H., Tempst, P., and Lis, J. T. (2002) Nature 420, 837–841
8. Estevez, A. M., Kempf, T., and Clayton, C. (2001) EMBO J. 20, 3831–3839
9. Allmann, C., Petfalski, K., Podtelejnikov, A., Mann, M., Tollervey, D., and Mitchell, P. (1999) Genes Dev. 13, 2148–2158
10. Rajmakers, R., Eghbert, W. V., van Venrooij, W. J., and Prijn, G. J. (2002) J. Mol. Biol. 323, 653–663
11. Carposius, A. J., Van Houwe, G., Ehretmann, C., and Krisch, H. M. (1994) J. Mol. Biol. 239, 889–900
12. Aloy, P., Ciccarelli, F. D., Leutwein, C., Gavin, A. C., Superti-Furga, G., Bork, P., Bottcher, B., and Russell, R. B. (2002) EMBO Rep. 3, 628–635
13. Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conver, D., Kulafeisievich, T., Vijayadamodor, G., Yang, M., Johnston, M., Fields, S., and Rothberg, J. M. (2000) Nature 403, 628–635
14. Itò, T., Chiba, T., Ozawa, Y., Yoshida, M., Hattori, M., and Sakaki, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4569–4574
15. Oliveira, C. C., Gonzales, F. A., and Zanchin, S. I. (2002) Nucleic Acids Res. 30, 4186–4198
16. Allmann, C., Kufel, J., Chanfregu, F., Mitchell, P., Petfalski, E., and Tollervey, D. (1999) EMBO J. 18, 5399–5410
17. Bielinger, J., Wirtz, L. E., Lorenz, P., and C. (1997) Mol. Biochem. Parasitol. 85, 99–112
18. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) Nat. Biotechnol. 17, 1030–1035
19. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouweret, E., Bragado-Nilson, E., Wilm, M., and Seraphin, B. (2001) Methods 24, 218–229
20. Marshak, D. R., Kadenaga, J. T., Burgess, R. R., Kauth, M. W., Brennan, W. A., and Lin, S.-H. (1996) Strategies for Protein Purification and Characterization, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal. Chem. 68, 850–858
22. Rosenfeld, J., Capdevielle, J., Guillemot, J. C., and Ferrara, P. (1992) Anal. Biochem. 203, 173–179
23. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
24. Shi, H., Dijkeng, A., Mark, T., Wirtz, E., Tschudi, C., and Ullu, E. (2000) RNA 6, 1069–1076
25. James, P., Halliday, J., and Craig, E. A. (1996) Genetics 144, 1425–1436
26. Oldenburg, K. R., Vo, K. T., Michaelis, S., and Paddon, C. (1997) Nucleic Acids Res. 25, 451–452
27. Gaveau, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Ment, A., Klein, K., Hudaik, M., Dickson, D., Rudi, T., Gruau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Coll, E., Edelmann, A., Sperfuir, E., Rybin, V., Dovers, G., Raida, M., Boumweister, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) Nature 415, 41–47
28. Cox, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
29. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Oldenburg, K. R. (2002) J. Biol. Chem. 277, 7404–7409
30. Rajmakers, R., Noordman, Y. E., van Venrooij, W. J., and Prijn, G. J. (2002) J. Mol. Biol. 315, 809–818
31. Symmons, M. F., Williams, M. G., Luisi, B. F., Jones, G. H., and Carposius, A. J. (2002) Trends Biochem. Sci. 27, 11–18
32. Imer, H., and Clayton, C. (2001) Nucleic Acids Res. 29, 4707–4715
33. Quijada, L., Guerra-Giraldez, C., Drozdz, M., Hartmann, C., Imer, H., Ben-Dor, C., Cristodero, M., Dung, M., and Clayton, C. (2002) Nucleic Acids Res. 30, 4414–4424
34. Milone, J., Wilusz, J., and Bellafato, V. (2002) Nucleic Acids Res. 30, 4040–4050
35. Zhang, J., Rayechavan, W., and Williams, N. (1998) Mol. Biochem. Parasitol. 92, 79–88
36. Guerra-Giraldez, C., Quijada, L., and Clayton, C. (2002) Cell Sci. 115, 2651–2658