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REPORT

The yeast antiviral proteins Ski2p, Ski3p, and Ski8p exist as a complex in vivo

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ABSTRACT

The yeast superkiller (*SKI*) genes were originally identified from mutations allowing increased production of killer toxin encoded by M "killer" virus, a satellite of the dsRNA virus L-A. *XRN1* (*SKI1*) encodes a cytoplasmic 5′-exoribonuclease responsible for the majority of cytoplasmic RNA turnover, whereas *SKI2*, *SKI3*, and *SKI8* are required for normal 3′-degradation of mRNA and for repression of translation of poly(A) minus RNA. Ski2p is a putative RNA helicase, Ski3p is a tetratricopeptide repeat (TPR) protein, and Ski8p contains five WD-40 (beta-transducin) repeats. An *xrn1* mutation in combination with a *ski2*, *ski3*, or *ski8* mutation is lethal, suggesting redundancy of function. Using functional epitope-tagged Ski2, Ski3, and Ski8 proteins, we show that Ski2p, Ski3p, and Ski8p can be coimmunoprecipitated as an apparent heterotrimeric complex. With epitope-tagged Ski2p, there was a 1:1:1 stoichiometry of the proteins in the complex. Ski2p did not associate with Ski3p in the absence of Ski8p, nor did Ski2p associate with Ski8p in the absence of Ski3p. However, the Ski3p/Ski8p interaction did not require Ski2p. In addition, *ski6-2* or *ski4-1* mutations or deletion of *SKI7* did not affect complex formation. The identification of a complex composed of Ski2p, Ski3p, and Ski8p explains previous results showing phenotypic similarity between mutations in *SKI2*, *SKI3*, and *SKI8*. Indirect immunofluorescence of Ski3p and subcellular fractionation of Ski2p and Ski3p suggest that Ski2p and Ski3p are cytoplasmic. These data support the idea that Ski2p, Ski3p, and Ski8p function in the cytoplasm in a 3′-mRNA degradation pathway.

Keywords: mRNA degradation; poly(A); RNA helicase; superkiller; tetratricopeptide (TPR) repeat; WD-40 beta-transducin repeat

INTRODUCTION

The superkiller (*SKI*) genes in yeast are encoded in the nuclear genome. They were initially identified from mutations that caused overexpression of a killer toxin encoded by the endogenous double-stranded RNA, denoted as M, a satellite of the double-stranded RNA virus L-A (Toh-e et al., 1978; Ridley et al., 1984; reviewed in Wickner, 1996a, 1996b). Subsequent work demonstrated that *SKI2*, *SKI3*, *SKI6*, *SKI7*, and *SKI8* are necessary to repress translation of poly(A) minus RNAs (Widner & Wickner, 1993; Masison et al., 1995; Benard et al., 1998, 1999), whereas *SKI1* is *XRN1* (Johnson & Kolodner, 1995) and encodes a 5′-exoribonuclease (Larimer et al., 1992). In strains lacking both M and L-A, double mutants of *xrn1Δ* and either *ski2* or *ski3* mutations are not viable (Johnson & Kolodner, 1995) or are temperature sensitive (Benard et al., 1999), depending on strain background. These observations indicate that the *SKI* genes are necessary for a general cellular function in addition to an antiviral activity (Johnson & Kolodner, 1995). *ski8* mutations also are synthetic lethal with *xrn1*Δ (Jacobs Anderson & Parker, 1998; J.T. Brown & A.W. Johnson, unpubl. observation). More recently, *SKI2*, *SKI3*, *SKI6*, and *SKI8* have been shown to be required for a 3′-mRNA degradation pathway (Jacobs Anderson & Parker, 1998). Ski6p/Rrp41p itself is a 3′-exoribonuclease and is an essential component of the exosome (Mitchell et al., 1997), a large complex of 3′-exoribonucleases that is involved in multiple RNA processing events in ribosome biogenesis (Mitchell et al., 1996, 1997; Zanchin & Goldfarb, 1999).

The relationship between 3′-degradation and repression of translation of poly(A) minus mRNA by the *SKI* genes is unclear. The *SKI* gene products may act directly in a 3′-mRNA degradation pathway with translation effects being secondary (Jacobs Anderson & Parker, 1998). Ski2p is a 146-kDa putative RNA helicase of the DEVH family (Widner & Wickner, 1993) and shares

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some similarity with Suv3p, a yeast mitochondrial RNA helicase (Stepien et al., 1992). Suv3p is a component of the mitochondrial degradosome that also contains the 3′-exoribonuclease Dss1p/Msu1p (Dziembowski et al., 1998) and is required for intron degradation in mitochondria (Margossian et al., 1996). Similarly, the *Escherichia coli* degradosome, a complex containing RNase E, polynucleotide phosphorylase, and the DEAD-box RNA helicase RhlB, is required for 3′ degradation of mRNA (Py et al., 1996; Coburn & Mackie, 1999; reviewed in Carpousis et al., 1999). In vitro analysis of the *E. coli* degradosome indicates that RhlB is required for degradation past secondary structures (Py et al., 1996). Because among the *SKI* genes only *SKI6* is known to encode an exonuclease activity, Ski2p, Ski3p and Ski8p may act to recruit the exosome to mRNA or act on the mRNP to allow access by the exosome (Jacobs Anderson & Parker, 1998).

Alternatively, it has been suggested that the *SKI* gene products act in some way on the ribosome to confer specificity for translating poly(A) mRNA (Wickner, 1996a, 1996b; Benard et al., 1998, 1999). This interpretation is supported by the finding that a *ski6-2* mutant displays a defect in 60S ribosomal subunit biogenesis (Benard et al., 1998) and poly(A) minus mRNA is translated at levels 10- to 34-fold higher when electroporated into *ski2*, *ski3*, *ski6*, or *ski8* mutants as compared to wild-type (Masison et al., 1995; Benard et al., 1998). Furthermore, the human homolog Ski2w is localized primarily to the nucleolus with a smaller amount in the cytoplasm in apparent association with 40S ribosomal subunits (Qu et al., 1998), suggesting a direct role in ribosome assembly or function.

The current models for Ski protein function make certain predictions about their localization. A role in 3′ mRNA degradation would be supported by a cytoplasmic localization and a role in ribosome biogenesis would be supported by a nuclear or nucleolar localization. In an effort to dissect the general cellular role of the antiviral Ski proteins, we examined the protein interactions of Ski2p, Ski3, and Ski8p and the intracellular localization of Ski2p and Ski3p. Ski3p is a 164-kDa protein that contains ten copies of a tetratricopeptide repeat (TPR) (Rhee et al., 1989). TPR proteins are typically found in protein complexes and often in association with WD-repeat proteins (Goebl & Yanagida, 1991; van der Voorn & Ploegh, 1992; Neer et al., 1994; Smith et al., 1999). Ski3p also contains a canonical leucine zipper motif ($LX_6LX_6LX_6L$) from amino acid residues 1232 to 1253 (J.T. Brown & A.W. Johnson, unpubl. observation), suggesting protein oligomerization. Ski3p in yeast is reported to be nuclear (Rhee et al., 1989). Ski8p is a 44-kDa protein containing five WD repeats (Matsumoto et al., 1993; Smith et al., 1999; "The WD-repeat Family of Proteins" at http://bmerc-www.bu.edu/wdrepeat/). Based on the similarity of phenotypes of *ski3* and *ski8* mutants and the protein families to which these proteins belong, it has been suggested that these proteins physically interact (Matsumoto et al., 1993; Masison et al., 1995). We now show that Ski2p, Ski3p, and Ski8p form a stable complex and provide evidence that this complex is cytoplasmic.

RESULTS

To determine if Ski2p is associated in vivo with other proteins, we carried out immunoprecipitation experiments with Ski2p. We made an internally epitope-tagged *SKI2* construct (Ski2p3xc-myc) in which amino acids 559–567 of Ski2p were replaced with three tandem copies of the c-myc epitope (see Materials and Methods). This construct was expressed under control of the *SKI2* promoter from a centromeric vector and fully complemented an *xrn1 ski2* double mutant, indicating it was functional (data not shown). Using quantitative Western blotting comparing the amount of Ski2p3xc-myc in extracts with immunopurified Ski2p3xc-myc, we estimate that there are approximately 5,000 molecules of Ski2p in haploid cells (data not shown). Immunoprecipitation of Ski2p3xc-myc from extracts prepared from [^{35}S]-methionine-labeled cells yielded a labeled band of the size expected for Ski2p (146 kDa) that was not observed when Ski2p was not epitope tagged (Fig. 1A). In addition, two other proteins were coimmunoprecipitated specifically with Ski2p3xc-myc (Fig. 1A, WT). These additional proteins were of the sizes expected for Ski3p (164 kDa) and Ski8p (44 kDa). Other protein bands observed in Figure 1A were not specific to tagged Ski2p. Quantitation of the Ski2p and putative Ski3p and Ski8p bands indicated that they were present in a 1:1:1 stoichiometry when accounting for the number of methionines in each species. We repeated this immunoprecipitation experiment in *ski3* and *ski8* deletion mutants. Coimmunoprecipitation of Ski2p in a *SKI3* deletion mutant abolished the coprecipitating band migrating in the position expected for Ski3p (Fig. 1A, *ski3Δ*). Surprisingly, deletion of *SKI3* also abolished the putative Ski8p band. Similarly, in a *SKI8* deletion mutant, the putative Ski3p and Ski8p bands were both absent (Fig. 1A, *ski8Δ*). Similar immunoprecipitation experiments performed in *ski6-2*, *ski4-1*, and *ski7::HIS3* strains showed no effect of these mutations on the Ski2p complex (Fig. 1A, *ski6*, and data not shown). These results strongly suggest that Ski2p is in a complex with Ski3p and Ski8p and that the interaction of Ski2p with Ski3p and Ski8p requires Ski8p and Ski3p, respectively. Upon overexposure of the autoradiograph shown in Figure 1A or in additional immunoprecipitation experiments, we did not observe additional proteins coprecipitating specifically with Ski2p.

To further support the conclusion that Ski3p and Ski8p were indeed the species coimmunoprecipitating with Ski2p, we carried out similar coprecipitation experiments with tagged Ski3p and Ski8p. We epitope tagged

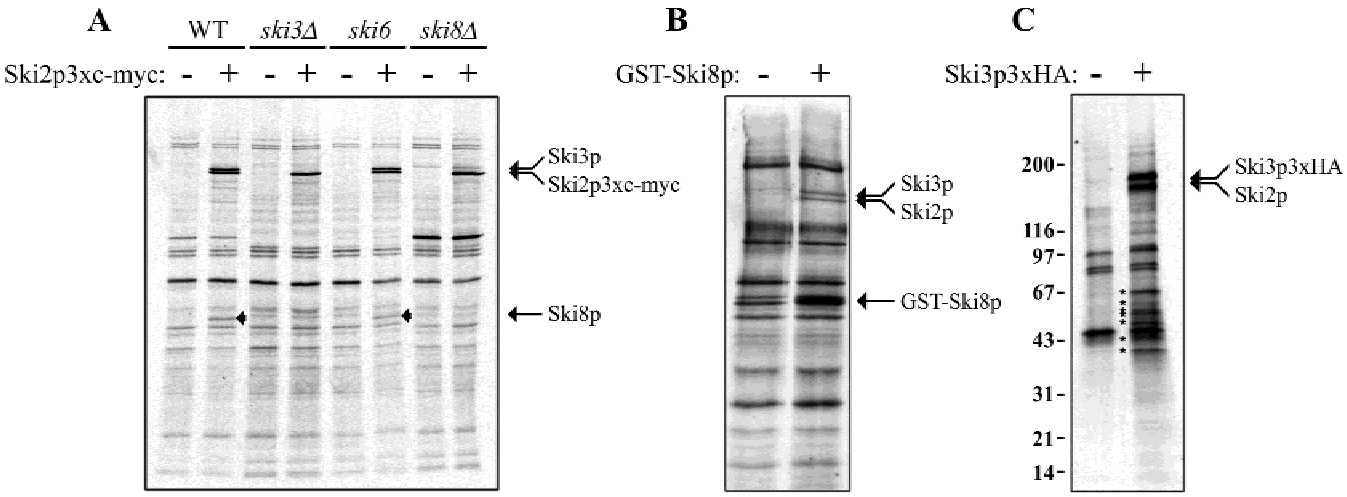

FIGURE 1. Coimmunoprecipitation of a protein complex containing Ski2p, Ski8p, or Ski3p. Yeast strains were labeled with [^{35}S]-methionine. Extracts were prepared and proteins were immunoprecipitated with tag-specific antibody and protein A beads. The immunoprecipitated proteins were fractionated by SDS-PAGE on 6–18% acrylamide gradient gels and autoradiographed. **A**: Extracts from strains BJ5464 (WT), RKY2033 (*ski3Δ*), RW2911 (*ski6-2*), and AJY685 (*ski8Δ*) carrying either pAJ39 (*SKI2*) or pAJ160 (*SKI2cmyc*) were immunoprecipitated with α-c-myc antibody. Short arrows identify putative Ski8p. **B**: Immunoprecipitations were performed on extracts from strain CH1305 carrying either pAJ267 (*SKI8*) or pAJ261 (*GST-SKI8*) using α-GST antibody. **C**: Immunoprecipitations were performed on extracts from strains CH1305 (*SKI3*) and AJY245 (*SKI3HA*) using α-HA antibody. The positions of protein bands consistent with being tagged or untagged Ski2p, Ski3p, and Ski8p are indicated. Additional protein bands in the Ski3p3xHA lane of **C** that were coprecipitated with Ski3p are noted with asterisks. + and − indicate the presence or absence of the respective tag.

the C-terminus of Ski3p with three tandem copies of HA (Ski3p3xHA) and we expressed Ski8p as a fusion to the C-terminus of glutathione S transferase (GST-Ski8p) (see Materials and Methods). Ski2p3xc-myc was immunoprecipitated from extracts prepared from strains coexpressing either Ski3p3xHA or GST-Ski8p. As seen in Figure 2A, Ski3p3xHA was specifically associated with Ski2p3xc-myc. No significant Ski3p3xHA signal was observed when the immunoprecipitation was done in the absence of tagged Ski2p. Similarly, GST-Ski8p was specifically immunoprecipitated with Ski2p3xc-myc (Fig. 2B). Again, no significant amount of GST-Ski8p was observed with untagged Ski2p. Thus Ski3p and Ski8p bind Ski2p in vivo.

We also examined whether the Ski2p-containing complex could be recovered by copurification with tagged Ski3p or tagged Ski8p. GST-Ski8p expression was induced by the addition of galactose and cells were labeled with [^{35}S]-methionine. Extracts were prepared and GST-Ski8p was purified by immunoprecipitation with α-GST antibodies. The proteins in the bound fraction were analyzed by SDS-PAGE and autoradiography. Under these conditions, a labeled protein of the size expected for GST-Ski8p (70 kDa) was specifically retained (Fig. 1B). In addition, labeled proteins of the sizes expected for Ski2p and Ski3p were also specifically retained when GST-Ski8p was used, but not when wild-type Ski8p without GST was used as a control. The higher level of radioactivity in the GST-Ski8p band compared to the putative Ski2p and Ski3p bands was due to galactose-induced overexpression of GST-Ski8p. A similar copurification from labeled cells was done using HA-tagged Ski3p (Ski3p3xHA) expressed from the genomic *SKI3* locus. As predicted, in addition to recovering Ski3p3xHA, a band in the expected position of Ski2p was also observed (Fig. 1C). However, several additional proteins ranging in size from about 40 kDa to 70 kDa were also specifically immunoprecipitated with Ski3p3xHA. These additional bands were not observed in control experiments using untagged Ski3p (Fig. 1C) nor were they seen in Western blots of Ski3p3xHA-containing crude extracts (data not shown), suggesting that they were not degradation products of Ski3p. Because Ski8p migrates in this size range, we could not identify an individual band as Ski8p. However, we have shown above that Ski8 and Ski3p associate. The stoichiometry of Ski3p to Ski2p in Figure 1C is approximately 2:1, suggesting that, unlike Ski2p, there is an excess of Ski3p over Ski2p. This excess Ski3p may then be free to associate with other proteins.

Based on the ideas that TPR and WD-repeat proteins are often found in association with each other (Goebl & Yanagida, 1991; van der Voorn & Ploegh, 1992; Neer et al., 1994; Smith et al., 1999) and speculation that Ski3p and Ski8p physically interact (Matsumoto et al., 1993; Masison et al., 1995), we examined whether Ski3p and Ski8p could interact in the absence of Ski2p. Ski3p3xHA was immunoprecipitated from extracts prepared from *SKI2* wild-type or *ski2Δ* strains and the immunoprecipitates were assayed for GST-

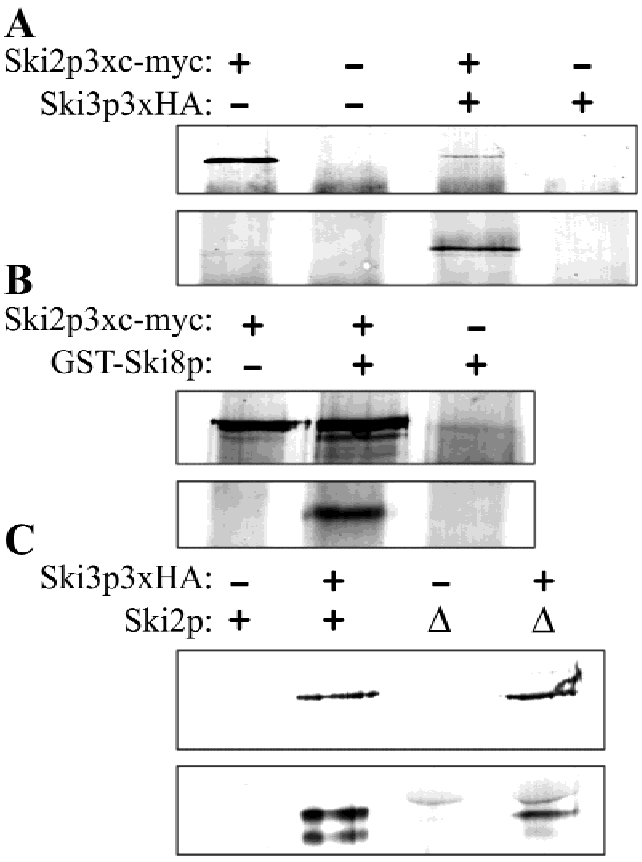

FIGURE 2. Ski3p and Ski8p are specifically coimmunoprecipitated with Ski2p3xc-myc and can associate in the absence of Ski2p. **A**: Extracts from yeast strains CH1305 (*SKI3*) and AJY245 (*SKI3HA*) carrying either pAJ39 (*SKI2*) or pAJ160 (*SKI2cmyc*) were immunoprecipitated using α-c-myc antibody. **B**: Extracts from yeast strain BJ5464 carrying either pAJ84 (*SKI2HA*) and pAJ581 (*GST-SKI8*), or pAJ160 (*SKI2cmyc*) and pAJ581 (*GST-SKI8*), or pAJ160 (*SKI2cmyc*) and pAJ267 (*SKI8*) were immunoprecipitated using α-c-myc antibody. **C**: Extracts from yeast strains CH1305 (*SKI3*), AJY245 (*SKI3HA*), RKY1973 (*ski2Δ*), or AJY1269 (*ski2Δ SKI3HA*) carrying pAJ581 (*GST-SKI8*) were immunoprecipitated using α-HA antibody. Immunoprecipitated proteins were fractionated by SDS-PAGE and analyzed by Western blotting using α-c-myc, α-GST, or α-HA primary antibodies. For Ski2p, + and Δ indicate the presence or absence of the *SKI2* gene and for all others + and − indicate the presence or absence of the respective tag.

Ski8p by Western blotting. As seen in Figure 2C, GST-Ski8p was readily immunoprecipitated from *SKI2* wild-type cells. GST-Ski8p was also immunoprecipitated from *ski2*Δ cells although the GST-Ski8p signal was reduced, suggesting weakened Ski3p/Ski8p interaction in the absence of Ski2p. Thus, although Ski2p cannot bind Ski3p or Ski8p in the absence of either of these proteins, the Ski3p/Ski8p interaction does not require Ski2p.

Ski2p, Ski3p, and Ski8p have been shown to have roles in 3′ mRNA degradation (Jacobs Anderson & Parker, 1998), suggesting a cytoplasmic function for this complex. An alternative explanation for their function is that they affect ribosome biogenesis or function, indirectly resulting in changes in 3′ degradation. Such a role in ribosome biogenesis would be supported by a nuclear localization for these proteins. Indeed, the human homolog of Ski2p is localized primarily to the nucleolus with a smaller amount in the cytoplasm in apparent association with 40S ribosomal subunits (Qu et al., 1998). In addition, yeast Ski3p fused to LacZ cofractionated with nuclei on Percoll gradients (Rhee et al., 1989). We examined the cellular localization of functional epitope-tagged Ski3p by indirect immunofluorescence and of Ski3p and Ski2p by cell fractionation. Indirect immunofluorescence showed that Ski3p3xHA was primarily cytoplasmic (Fig. 3A). Most cells revealed more intense signal around the nucleus but the nucleoplasm and nucleolus were depleted of signal. Control experiments using untagged Ski3p (Fig. 3B) indicated that the fluorescence signal we observed was highly specific for Ski3p3xHA. Attempts to visualize Ski2p3xc-myc by immunofluorescence failed when expressed from a centromeric plasmid. However, when expressed from a high-copy plasmid it showed a cyto-

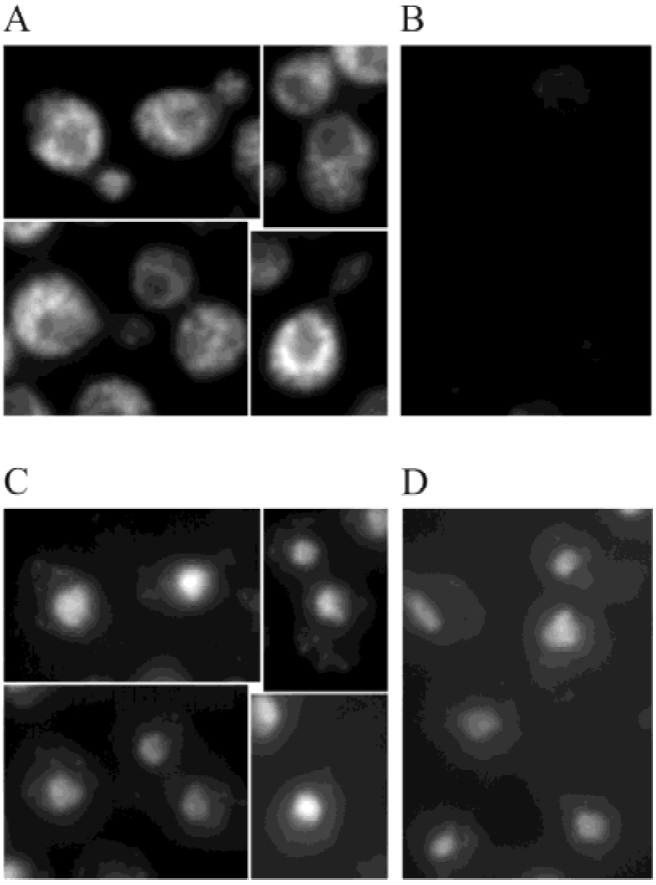

FIGURE 3. Indirect immunofluorescence of Ski3p3xHA. Log-phase cells were fixed and prepared for indirect immunofluorescence as described in the text. **A**,**C**: Strain AJY245 (*SKI3HA*); **B**,**D**: strain CH1305 (*SKI3*); **A**,**B**: indirect immunofluorescence using α-HA monoclonal antibody at a 1:5000 dilution; **C**,**D**: DAPI staining. Secondary antibody was Cy3-conjugated goat α-mouse at a dilution of 1:200.

plasmic localization (data not shown). A cytoplasmic localization of Ski2p and Ski3p was supported by results of cell fractionation experiments. Extracts were prepared from cells coexpressing Ski2p3xc-myc from a centromeric vector and Ski3p3xHA from the genomic locus. The extracts were fractionated into nuclear and cytoplasmic fractions. The resulting fractions were analyzed by Western blotting for c-myc and HA as well as for glucose 6-phosphate dehydrogenase (G6PDH) and topoisomerase II (Topo II) as cytoplasmic and nuclear markers, respectively. Samples were loaded to give similar signals for G6PDH in total and cytoplasmic fractions and for Topo II in total and nuclear fractions. As seen in Figure 4, Ski2p3xc-myc and Ski3p3xHA fractionated in a manner similar to G6PDH, the cytoplasmic marker, and were not observed at significant levels in the nuclear fraction. Ski2p3xc-myc regularly gave lower signals on Western blots than did Ski3p3xHA. In addition Ski2p appeared to be more susceptible to proteolysis than was Ski3p. In Figure 4 we have included fraction C2, the lower portion of the cushion, which contained higher amounts of cytoplasmic proteins but was also slightly contaminated with nuclei. Both Ski2p and Ski3p signals are slightly stronger in this fraction. However, their absence from the nuclear fraction clearly indicates that their enrichment in C2 is not due to the nuclear contamination of this fraction. Thus, two different lines of evidence, indirect immunofluorescence and cell fractionation, suggest that, in yeast, Ski2p and Ski3p and likely the Ski2p/Ski3p/Ski8p complex are cytoplasmic.

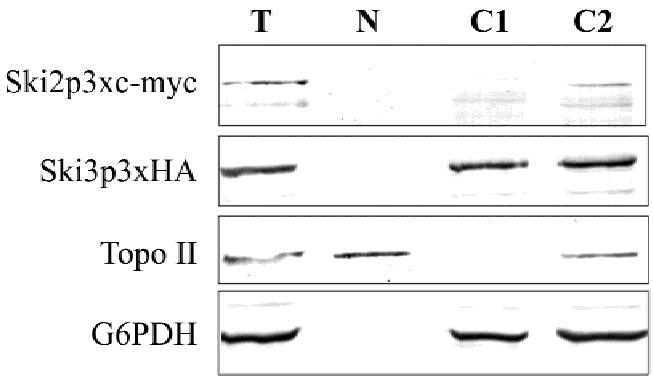

FIGURE 4. Subcellular fractionation of Ski2p3xc-myc and Ski3p3xHA. Strain AJY245 (*SKI3HA*) carrying pAJ160 (*SKI2cmyc*) was fractionated into nuclear and cytoplasmic fractions by centrifugation through a dense sucrose cushion as described in the text. T: total homogenate; N: nuclear fraction; C1: cytoplasmic fraction; C2: second cytoplasmic fraction corresponding to the lower portion of the cushion. Proteins were fractionated by SDS-PAGE and analyzed by Western blotting using antibodies specific for G6PDH and Topo II as cytoplasmic and nuclear marker proteins, respectively, and α-c-myc and α-HA antibodies for Ski2p3xc-myc and Ski3p3xHA, respectively. The protein amounts loaded were such that the signal for Topo II in the total and nuclear fractions and the signal for G6PDH in the total and cytoplasmic fractions were similar.

DISCUSSION

The superkiller genes are required for normal 3′ mRNA degradation and for repression of translation of poly(A) minus mRNA. Whether mRNA 3′ degradation is the mechanism of translation repression or the superkiller genes act by directly affecting ribosome function or assembly has not been clearly established, although our results are generally more consistent with a cytoplasmic role in mRNA turnover. Our finding that Ski2p, Ski3p, and Ski8p form a stable complex helps to explain how these proteins all act in a concerted fashion in the same pathway.

The proteins in the Ski2p/Ski3p/Ski8p complex appear to be in a ratio of 1:1:1 with no significant free pool of Ski2p. However, when Ski3p was immunoprecipitated, the stoichiometry of Ski3p to Ski2p was approximately 2:1. Furthermore, additional proteins were observed in the Ski3p immunoprecipitation. Thus it is possible that there is an excess of Ski3p that interacts with additional proteins. Because mutations in any of the three genes have a similar phenotype, it is likely that complex formation is required for function of these proteins. We attempted to overexpress the complex in yeast by cooverexpressing *SKI2*, *SKI3*, and *SKI8*. However, we were unable to increase the amount of complex that could be immunopurified. Complex formation was normal in a *ski7* deletion mutant and in a *ski4-1* mutant, suggesting that these proteins are not required for complex formation. Ski7p is an EF1α-like protein and *SKI4* has not been cloned. It is possible that an additional component of the complex exists that we did not observe in our coimmunoprecipitation experiments, or that an additional limiting factor is needed transiently for complex formation.

Several lines of evidence support the notion that 3′ mRNA degradation is the primary defect in *ski* mutants. All mutants examined thus far display defects in 3′ RNA degradation and these defects are also observed on mRNA 3′ fragments that are not translated (Jacobs Anderson & Parker, 1998). No defects in 18S or 25S rRNA processing were observed in [^{3}H]-methylmethionine pulse-chase labeling experiments in *ski2* mutants (J.T. Brown & A.W. Johnson, unpubl. observation) and 5.8S rRNA processing appeared normal in *ski2*, *ski3*, and *ski8* mutants (Jacobs Anderson & Parker, 1998). Our finding that Ski2p and Ski3p are cytoplasmic supports the notion that the *SKI* gene products are directly involved in a cytoplasmic 3′ degradation pathway, although we cannot rule out the possibility that the Ski proteins act in the cytoplasm to provide poly(A) specificity to translation and not directly in a 3′ mRNA degradation pathway.

The eukaryotic exosome is a large complex of multiple 3′ exoribonucleases including Ski6p/Rrp41p (Mitchell et al., 1997; Decker, 1998) and is involved in numerous RNA processing and degradation reac-

tions including 3′ degradation of cytoplasmic mRNA (Mitchell et al., 1996; Jacobs Anderson & Parker, 1998; Zanchin & Goldfarb, 1999). The *E. coli* degradosome and the yeast mitochondrial degradosome are protein complexes that combine ribonucleases and RNA helicases in which the helicases apparently serve to disrupt RNA secondary structure and possibly RNA–protein interactions (Margossian et al., 1996; Carpousis et al., 1999). It has been suggested that the putative RNA helicase activity of Ski2p is required for exosomal degradation of mRNAs by the 3′ pathway (Jacobs Anderson & Parker, 1998). In this model, Ski2p would either act together with the exosome, recruiting it to substrates and removing secondary structure and/or proteins, or act in a sequential fashion allowing for subsequent mRNA degradation by the exosome. In preliminary experiments we were unable to detect association of a ProteinA-Rrp43p fusion with Ski2p or Ski3p under conditions in which we could detect Ski protein complex formation (J.T. Brown & A.W. Johnson, unpubl. observation). If the Ski protein complex physically interacts with the exosome, this interaction is weak or the ProteinA-Rrp43p fusion used disrupts interaction with the Ski protein complex.

We have shown by two independent means that Ski2p and Ski3p are cytoplasmic. This is contrary to expectations based on human Ski2w (Qu et al., 1998) and to previously published results for Ski3p (Rhee et al., 1989). In addition Ski2p contains two putative overlapping canonical bipartite nuclear localization signals (NLSs). Mutation of amino acids 622–624 (arginine-lysine-arginine) to three alanines deletes a cluster of basic residues that simultaneously disrupts both NLSs. This mutant complemented an *xrn1 ski2* double mutant (J.T. Brown & A.W. Johnson, unpubl. observation), indicating that the putative NLSs were not important with respect to Xrn1p activity. Previous work on Ski3p demonstrated cofractionation of Ski3p-LacZ with nuclei on Percoll gradients. We noted in our indirect immunofluorescence experiments that the signal for Ski3p was most intense around the nucleus. Thus the nuclear cofractionation of Ski3p-LacZ observed previously may have reflected a loose association of Ski3p with material surrounding the nucleus.

Our results suggest that there are significant differences between the human and yeast Ski2 proteins. The human protein, Ski2w, is primarily localized to the nucleolus, the site of ribosome biogenesis (Qu et al., 1998), with a smaller amount of the protein thought to be bound to ribosomal 40S subunits in the cytoplasm. We examined the sedimentation of yeast Ski2p on sucrose gradients and found that the protein sedimented throughout sucrose gradients (including polysomal fractions) but was not consistent with ribosomal association (X. Bai and A.W. Johnson, unpubl. observation). It is possible that an additional *SKI2* homolog exists in humans that is more similar in function to yeast *SKI2*. Alternatively the human protein may have gained additional functions.

MATERIALS AND METHODS

Strain constructions, media, and plasmids

The yeast strains used are described in Table 1. Plasmids are listed in Table 2. Construction of plasmids and strains is described below. Standard media including synthetic complete medium (SC) were as described previously (Kaiser et al., 1994). Low Ade medium contained 6 mg/L adenine. Yeast transformations were performed as described elsewhere (Gietz & Woods, 1994).

Yeast strain AJY245, in which the C-terminus of Ski3p is tagged with a triple HA epitope, was constructed as follows. PCR was performed using pFA6a-3HA-kanMX6 as template and primers with 5′ moieties complementary to sequences immediately prior to and immediately following the *SKI3* stop codon and 3′ moieties complementary to sequence flanking the plasmid-borne 3xHA:kanMX6 cassette, as described (Longtine et al., 1998). The resulting DNA fragment was gel purified and cotransformed into strain CH1305 with pRS416 to facilitate selection of transformants. Resultant Ura$^+$ trans-

TABLE 1. Yeast strains used in this study.

Strain	Genotype	Source
AJY245	MATa *ade2 ade3 leu2 lys2-801 ura3-52 SKI3HA:kanMX6*	this work
AJY685	MATα *ade3 leu2 ura3 ski8::URA3*	this work
AJY836	MATα *ade2 ade3 leu2 ura3-52*	this work
AJY1269	MATα *ade2 ade3 leu2 ura3-52 ski2::LEU2 SKI3HA:kanMX6*	this work
BJ5464	MATα *ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*	Johnson & Kolodner (1991)
CH1305	MATa *ade2 ade3 leu2 lys2-801 ura3-52*	Kranz & Holm (1990)
RKY1973	MATα *ade2 ade3 leu2 ura3-52 ski2::LEU2*	Johnson & Kolodner (1995)
RKY1978	MATα *ade2 ade3 leu2 his3 ura3-52 xrn1::URA3*	Johnson & Kolodner (1995)
RKY2033	MATa *leu2 trp1 ura3 ski3::TRP1* L-A-o	Johnson & Kolodner (1995)
RW2911	MATa *ura3-52 leu2::hisG ade3 ski6-2* K1+	R. Wickner, pers. comm.
RW3082	MATa *leu2 ura3 his3 nuc1::LEU2 pep4::HIS3 ski8::URA3* K1+	R. Wickner, pers. comm.

TABLE 2. Plasmids used in this study.

Plasmid	Description	Source
pAJ39	pRS315-*SKI2*	this work
pAJ63	YEp213-*SKI2* library clone p2-18/8-4	Johnson & Kolodner (1995)
pAJ84	pRS315-*SKI2HA*	this work
pAJ159	YEp351-*SKI2cmyc*	this work
pAJ160	pRS315-*SKI2cmyc*	this work
pAJ261	pEG(KT)-*GAL-GST-SKI8*	this work
pAJ267	pRDK249-*GAL-SKI8*	this work
pAJ581	pRS416-*CYC-SKI8*	this work
pEG(KT)	*GAL1-10/CYC-GST* vector	Chevray & Nathans (1992)
pFA6a-3HA-kanMX6	3xHA-kanMX6 cassette	Longtine et al. (1998)
pKB241	3xc-myc cassette	S. Kron and G. Fink, pers. comm.
pRDK249	*GAL-XRN1*	Johnson & Kolodner (1991)
pRDK331	YEp351-*SKI2*	Johnson & Kolodner (1995)

formants were replica-plated to YPD supplemented to 200 μg/mL with G418 (Geneticin; Gibco BRL). G418-resistant colonies were passaged on 5-fluoroorotic acid plates to force loss of pRS416, then screened by Western blotting for production of Ski3p3xHA. Such an isolate (AJY245) was tested for functionality by mating with RKY1978 (*xrn1::URA3*). The recovery of G418-resistant, Ura$^+$ spore clones after sporulating the resulting diploid indicated that *SKI3HA* was functional (data not shown), as null mutations in *SKI3* are synthetic lethal with *xrn1Δ* mutations (Johnson & Kolodner, 1995). Yeast strain AJY685 (*ski8::URA3*) was created by mating AJY836 (*SKI8*) with RW3082 (*ski8::URA3*) followed by sporulation and tetrad dissection. Yeast strain AJY1269 (*ski2::LEU2 SKI3HA*) was created by mating AJY245 (*SKI3HA*) with RKY1973 (*ski2::LEU2*) followed by sporulation and tetrad dissection.

Plasmid constructions

A centromeric *SKI2* plasmid (pAJ39) was constructed by moving a *SKI2*-containing *Xba*I/*Sst*I fragment from pAJ63 (p2-18/8-4) (Johnson & Kolodner, 1995) into *Xba*I/*Sst*I-digested pRS315.

Because epitope tagging Ski2p at the amino or carboxyl terminal ends yielded a nonfunctional protein (Widner & Wickner, 1993), we created an internally triple-c-myc-tagged Ski2p by inserting the epitope-containing 123-bp *Bam*HI fragment from pKB241 (S. Kron and G. Fink) into *Bam*HI-digested pRDK331, thus replacing a 27-bp *Bam*HI fragment of the *SKI2* gene. *SKI2cmyc* was moved from the resultant plasmid (pAJ159) to pRS315 on an *Sst*I fragment, creating pAJ160. Both *SKI2cmyc*-bearing plasmids complemented the lethality of *xrn1Δ ski2-1-14* (data not shown).

The *SKI8* gene was amplified by PCR from wild-type genomic DNA using primers containing *Sal*I and *Hin*dIII sites and then digested and ligated into *Sal*I/*Hin*dIII-digested pEG(KT), creating a galactose inducible *GST-SKI8* fusion (pAJ261). A galactose-inducible wild-type *SKI8* construct (pAJ267) was made by moving *SKI8* on a *Sal*I/*Hin*dIII fragment from pAJ261 into *Xho*I/*Hin*dIII-digested pRDK249 (Johnson & Kolodner, 1991). The *SKI8* gene was also moved on a *Xho*I/*Hin*dIII fragment from pAJ261 into *Xho*I/*Hin*dIII-digested pRS416, placing *SKI8* under the control of the *CYC1* promoter (pAJ581).

Immunoprecipitation experiments

[^{35}S]-methionine-labeled extracts were prepared by growing cells to mid-log phase in 7.5 mL of appropriate synthetic selective medium lacking methionine. The cells were concentrated to 1 mL, 80 μCi of [^{35}S]-methionine (Expre^{35}S^{35}S Protein Labeling Mix, DuPont NEN) was added, and the cells were incubated for an additional 30 min at 30 °C with shaking. Cells were collected, washed once with ice-cold extraction buffer (20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A), resuspended in 0.25 mL of ice-cold extraction buffer, and disrupted by vortexing with glass beads. The extract was removed, the beads washed once with 0.25 mL of extraction buffer, and the extract and wash were combined. The sample was clarified by centrifugation for 10 min at 15,000 $\times$ *g* at 4 °C. Clarified extract (0.2 mL) was mixed with an equal volume of extraction buffer supplemented to 500 mM $(NH_4)_2SO_4$ and 0.2% NP-40, 50 μL of BSA-blocked Protein A agarose (Gibco BRL) was added, and the samples were incubated at 4 °C with rocking. After 30 min, the beads were removed by centrifugation. The supernatant was recovered and the appropriate primary antibody was added, followed 1 h later by the addition of BSA-blocked Protein A agarose beads. After 2 h of rocking at 4 °C, the beads were collected by centrifugation and washed three times with extraction buffer containing 250 mM $(NH_4)_2SO_4$ and 0.1% NP-40. Proteins were eluted from the beads by heating at 100 °C in Laemmli buffer. Extracts for immunoprecipitations to be visualized by Western blotting were prepared similarly except cells were not labeled with [^{35}S]-methionine and the extract buffer was composed differently (20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A). For these reactions, immunoprecipitates were washed in extract buffer supplemented with 0.1% NP-40. Western blot analysis was carried out as previously described (Johnson, 1997). In Western blots the only immunoreactive

protein bands were those expected and no immunoreactive bands were observed in the absence of tagged protein (data not shown).

Immunofluorescence and subcellular fractionation

Indirect immunofluorescence was performed as previously described (Heyer et al., 1995) with α-HA (mouse 12CA5 ascites fluid from Berkeley Antibody Co.) diluted 1:5,000 in phosphate-buffered saline (PBS) + 0.1% bovine serum albumin (BSA) + 0.1% Triton X-100 as primary antibody and Cy3-conjugated goat α-mouse antibody (Amersham Life Science) diluted 1:200 in PBS + 0.1% BSA as secondary antibody.

For subcellular fractionation, yeast cells were spheroplasted using a protocol adapted from Aris and Blobel (1991) and then fractionated by a protocol adapted from Wise (1991). One liter of mid-log cells were concentrated to 15 mL and spheroplasted in synthetic dropout SPH media (1 M Sorbitol, 1% glucose, 0.2% yeast nitrogen base without amino acids and with ammonium sulfate, 0.1% synthetic complete dropout amino acid mix, 25 mM HEPES (free acid), 50 mM Tris base) supplemented with 2 mM dithiothreitol and 333 μg/mL Zymolyase T100 (ICN Biomedical Research Products) and then shaken gently at 30 °C for 50 min. The spheroplasting solution was then supplemented with 1 mM PMSF, 0.5 μg/mL leupeptin, and 0.7 μg/mL pepstatin A. The spheroplasts were centrifuged for 3 min at 4 °C at 6,000 $\times$ *g* over a 5-mL high-sucrose cushion (1 M sucrose, 25 mM HEPES (pH 7.6), 5 mM magnesium acetate, 1 mM PMSF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A) then washed in 15 mL HMC (0.5 M sucrose, 25 mM HEPES (pH 7.6), 5 mM magnesium acetate, 1 mM PMSF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A) and finally resuspended in 12 mL HMS (0.25 M sucrose, 25 mM HEPES (pH 7.6), 5 mM magnesium acetate, 1 mM PMSF, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin A). Nonidet P-40 was added to 0.1% prior to homogenization by repeated pipetting. The homogenate was then centrifuged for 2 min at 4 °C at 2,500 $\times$ *g* to remove cell debris. The supernatant was transferred to a fresh tube, a sample was taken to represent the total homogenate (T), and a 5-mL HMC cushion was layered under the remainder. The sample was centrifuged for 8 min at 4 °C at 8,000 $\times$ *g* and fractions were collected: C1 represented the supernatant and the top portion of the cushion; C2 represented the lower portion of the cushion; and N represented the nuclei-containing pellet.

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