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Rpg1p/Tif32p, a Subunit of Translation Initiation Factor 3, Interacts with Actin-Associated Protein Sla2p

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The yeast two-hybrid system was used to screen for proteins that interact *in vivo* with *Saccharomyces cerevisiae* Rpg1p/Tif32p, the large subunit of the translation initiation factor 3 core complex (eIF3). Eight positive clones encoding portions of the *SLA2/END4/MOP2* gene were isolated. They overlapped in the region of amino acids 318–550. Subsequent deletion analysis of Sla2p showed that amino acids 318–373 were essential for the two-hybrid protein–protein interaction. The N-terminal part of Rpg1p (aa 1–615) was essential and sufficient for the Rpg1p–Sla2p interaction. A coimmunoprecipitation assay provided additional evidence for the physical interaction of Rpg1p/Tif32p with Sla2p *in vivo*. Using immunofluorescence microscopy, Rpg1p and Sla2p proteins were colocalized at the patch associated with the tip of emerging bud. Considering the essential role of Rpg1p as the large subunit of the eIF3 core complex and the association of Sla2p with the actin cytoskeleton, a putative role of the Rpg1p–Sla2p interaction in localized translation is discussed.

Key Words: **yeast *Saccharomyces cerevisiae;* two-hybrid system; coimmunoprecipitation; protein–protein interaction; translation initiation factor 3; Rpg1p/Tif32p; actin associated cytoskeleton; Sla2p/End4p/Mop2p; localized translation.**

Association of specific mRNP particles and polysomes with the cytoskeleton has been suggested as the basis of a mechanism generating an asymmetric protein distribution by compartmentalization of the protein synthesis in the cell (1, 2). There is considerable evidence that at least a subfraction of mRNA, translation factors, and polysomes is co-localized or associated with cytoskeletal structures (3). It has been shown that, for example, the levels of initiation factors eIF-2, eIF-3, eIF-4A, and eIF-4B are enriched in the cytoskeletal fraction of HeLa cells compared to the soluble fraction (4). In addition, using fluorescence microscopy, eIF-2 and the elongation factor EF-2 were localized along microfilament bundles of cultured mouse embryo fibroblasts (5, 6). Another elongation factor (EF-1α) colocalized with the actin cytoskeleton in mammalian (7) and amoebae (8) cells.

Recent data imply that in budding yeast, *Saccharomyces cerevisiae,* the actin cytoskeleton plays an active role in generation of the asymmetric distribution of specific proteins (9, 10). Yeast may employ similar mRNA localization mechanisms as higher eukaryotes (11). With respect to association of polysomes with the cytoskeleton, the yeast ribosomal protein, Rpp2bp/Rpl45p, was found in a two-hybrid search for actin-interacting proteins (12). Another two-hybrid search (13) has revealed complex formation between the cytoskeletal assembly protein Sla1 and the prion-forming domain of the release factor Sup35 (eRF3). It was shown that yeast elongation factor 1 alpha, Tef1p/Tef2p, binds to Bni1p protein, a downstream target of Rho1p-Bni1p-mediated pathway functioning in reorganization of the actin cytoskeleton (14). All these interactions may be involved in the intracellular localization of translational complexes in yeast.

Here we characterize a direct physical interaction of Rpg1p with actin-associated protein Sla2p using the yeast two-hybrid system. Our immunofluorescence data suggest that portions of both proteins colocalize at specific cellular domains, especially at the patch associated with the tip of emerging bud.

Abbreviations used: ECL, enhanced chemiluminescence; eIF, eukaryotic translation initiation factor; HRP, horseradish peroxidase; MCS, multiple cloning sites; ORF, open reading frame; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; WT, wild type.

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MATERIALS AND METHODS

Cell strains and growth media. The following strains were used in this work: FY1679 (*MATa/MATα ura3/ura3 trp1/+ leu2/+ his3/+*), L40 (*his3 trp1 leu2 ade2 lexA-HIS3:LYS2 lexA-lacZ:URA3 gal4* (15)), RH2887 (*MATa lys2 leu2 ura3 his3 trp1 bar1* (16)), RH3395 (*MATa lys2 leu2 ura3 his3 bar1 end4Δ::HIS3 end4Δ376-501:TRP1* (16)), WAY205 (*MATa lys2 leu2 ura3 his3 trp1 bar1 end4Δ::HIS3* (A. Wesp)), WAY223 (*MATa lys2 leu2 ura3 his3 bar1 end4Δ::HIS3 end4Δ114-367:TRP1* (A. Wesp)).

Yeast was grown in YPD and/or YPUADT rich medium (1% yeast extract, 2% peptone, 20 mg/L uracil, adenine and tryptophan, 2% glucose), and/or synthetic complete SC− medium (0.67% yeast nitrogen base without amino acids, 2% glucose); SC-T^-,L^-, +X-Gal was SC− medium without tryptophan, leucine and with 40 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Yeast transformations were performed by the lithium acetate method (17) and/or by the method described by Chen *et al.* (18).

Construction of plasmids. The plasmid containing the lexA DNA-binding domain fused with the entire coding region of Rpg1p was constructed as follows (constructs are shown diagrammatically in Fig. 1C). The *Bam*HI–*Pst*I fragment from plasmid pYCpAW20 was inserted into the multicloning site of pBTM116 (constructed by P. Bartel and S. Fields) in frame (blunting the overhangs of *Bam*HI cleavage sites) to generate plasmid pLBP. pLCP plasmid was created by removing the *Sma*I–*Cla*I (in the N-terminal sequence) fragment of pLBP. pLBN was created by removing the *Nde*I–*Pst*I fragment of pLBP. The *Eco*RI–*Pst*I fragment from plasmid pYCpAW20 was inserted into the multicloning site of pBTM116 in frame to generate pLEP. Every new ORF fusion was controlled by DNA sequencing and the expression of the protein was checked by Western blots (19).

Construction of plasmids containing the Gal4 DNA-activating domain fused to the entire coding region (pGCE) and/or deletion versions of the *SLA2* gene (pGLE and pGSE) was described elsewhere (16). Primers introducing *Bam*HI-sites 2 bp upstream of the initiator methionine and downstream of the stop codon, respectively, were used to amplify the *SLA2* coding region of the *sla2Δ318-373* allele (16). This PCR product was digested with *Bam*HI, gel purified and ligated into *Bam*HI-digested vector pGAD424 (15) to give pGME plasmid. Each plasmid complemented the growth defect of *sla2*Δ mutant (WAY205) at 35°C (16).

Standard techniques were used for plasmid isolation, plasmid analysis, and restriction fragment cloning (20). PCR was performed in 100-μl reactions using Vent polymerase (New England Biolabs, Beverly, MA) under conditions recommended by the manufacturer (21). DNA sequencing was accomplished by means of the dideoxy nucleotide chain termination method (22) using a T7 Sequencing Kit (Pharmacia). Autoradiography was performed with FUJI RX X-ray film (Tokyo, Japan), and the films were developed with an AGFA automatic film processor.

Two-hybrid analysis. Yeast strain L40 with two reporter genes (*lexA-lacZ* and *lexA-HIS3*) was transformed with pLBP and consequently with a yeast genomic library (23). The transformants were streaked on SC-T^-,L^-,H^- plates. After incubation at 30°C for 1 week colonies were restreaked on selective SC-T^-,L^-,H^- and SC-T^-,L^-,+X-Gal plates. Plasmids were recovered from colonies that both grew on plates without histidine and turned the chromogenic substrate X-Gal blue after incubation at 30°C for 3 days. The recovered plasmids were reintroduced into the L40 strain harboring pLBP. LacZ expression was quantitatively examined by measuring the activity of β-galactosidase by the method described by Miller (24).

Protein extracts, Western blotting and in vivo binding assay. Yeast protein extracts were prepared from exponentially growing cells as described elsewhere (16). Precleared extracts used for coprecipitation experiments were diluted with lysis buffer (0.1 M NaCl, 20 mM Mes pH 6.5, 5 mM $MgCl_2$, 1% Nonidet NP40) to give a final protein concentration of 1–2 mg per 0.5 ml. 5 μl of rabbit antiserum directed against the extreme C-terminal peptide of the Sla2p protein ((16); kindly provided by Andreas Wesp, Basel, Switzerland) was added and extracts were shaken for 1 h at 4°C. Immune complexes were collected after adding 50 μl of 50% protein G-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) per sample and incubating for 1 h at 4°C. Protein G-Sepharose beads were washed three times with lysis buffer and proteins were released into 50 μl SDS-sample buffer by heating the beads at 95°C for 5 min. 10 μl supernatant was separated by SDS–PAGE (25) and blotted to nitrocellulose membrane (Schleicher and Schuell) using standard procedures (19). The Rpg1p protein was detected using rabbit anti-Rpg1p antiserum (1:30,000 dilution). The level of Sla2p protein was checked with the Sla2p specific antibody. HRP-coupled donkey anti-rabbit Ig (Amersham Corp.) at 1:10,000 dilution was used as secondary antibody. Blots were developed using the ECL system (Pierce, Rockford, IL).

Immunofluorescence microscopy. Yeast cells (FY1679 strain) were fixed with 3.7% (w/v) formaldehyde for 45 min and processed for immunofluorescence microscopy as described elsewhere (26). Monoclonal Rpg1p-specific antibody PK1/1 (27) was applied as an ascitic fluid finally diluted 50-fold in 1% (w/v) BSA/PEMI at 25°C for 60 min. Cy3-labeled goat anti-mouse Ig antibodies (GAM-Cy3; Sigma) diluted 1:200 were used as secondary antibodies. Antibody against the C-terminal domain of Sla2p ((28); kindly provided by David Drubin, UC Berkeley, CA) and the secondary FITC-labeled goat anti-rabbit IgG antibody (Sigma, diluted 1:200) were used to visualize Sla2p. To diminish background fluorescence in yeast cells due to unbound antibodies to a minimum, all incubation and washing steps were done in suspension (26).

Samples were investigated with the Olympus BX-60 fluorescence microscope using standard filter sets. Images were recorded with 1280 × 1024 pixel resolution using Fluoview cooled CCD camera with the use of analySIS imaging software.

RESULTS

Rpg1p/Tif32p interacts with Sla2p in the two-hybrid assay. A screen using the two-hybrid system was undertaken for genes encoding Rpg1p/Tif32p-binding proteins. Originally, the entire coding region of Rpg1p was fused to the lexA DNA-binding domain (plasmid pLBP). Conditional mutant strain YLV041, expressing *RPG1* gene under the control of the *MET3* promoter (29), was transformed with control (pBTM116) and/or pLBP plasmid, respectively. Cells carrying lexA-Rpg1p hybrid protein were viable while the control cells exhibited a lethal phenotype under restrictive conditions (data not shown). These results and Western blot analysis of the protein extracts from transformants (data not shown) confirmed that the lexA-Rpg1p fusion protein is functional.

Plasmid pLBP and the yeast *S. cerevisiae* genomic library, expressed as fusion proteins with the Gal4 transcription-activating domain (23), were introduced into L40 cells that carried *lexA-lacZ* and *lexA-HIS3* reporters. The plasmids were then recovered from the colonies that grew on SC-T^-,L^-,H^- selective plates. After reintroduction of the plasmids, only 8 clones specifically increased the level of expression of both *HIS3* and *lacZ* reporter gene in L40 cells in a pLBP-dependent manner. Sequence analysis revealed that each of these eight clones was derived from the *SLA2/*

END4 gene originally identified during a screen for mutants that are defective in the internalization step of endocytosis (30) and during an independent screen for synthetic lethals with *abp1* null mutant (31). Among these clones (four of them—pHB21, pHB22, pHB29 and pHB201—were identical) longer fragments containing a second coiled-coil domain (aa 575–767) exhibited higher affinity to Rpg1p than shorter fragments (Fig. 1A). All *SLA2* clones overlapped in the region of amino acid residues 318–550 (Fig. 1A).

Amino acids 318–373 of Sla2p are essential for the Sla2p-Rpg1p interaction. The binding affinity of the full-length Sla2p hybrid protein (Fig. 1B, pGCE) was similar to the level observed for the shorter fragments (Fig. 1A; pHB21, pHB2 and pHB26). To identify residues essential for the two-hybrid interaction between Rpg1p and Sla2p, we constructed several truncated mutants (Fig. 1B). The results from the screen suggested amino acids 318–550 of Sla2p to be sufficient for the interaction with full-length Rpg1p protein (Fig. 1A). Deletion of amino acids 318–373 abolished the enhanced expression of the reporter genes (Fig. 1B, pGME). In contrast, deletion of any part of the central coiled-coil domain (aa 376–573) enhanced the interaction (Fig. 1B, pGLE and pGSE). These results suggested that the domain formed by amino acids 318–373 is essential for the Sla2p–Rpg1p interaction.

N-terminal part of Rpg1p mediates the interaction with Sla2p. To identify the Rpg1p domain that participates in the interaction with Sla2p, truncated versions of Rpg1p were introduced into L40 cells harboring pGCE plasmid encoding full-length Sla2p. Deletions in the evolutionary conserved N-terminal half (29) abolished expression of the reporter genes (Fig. 1C, pLCP and pLEP), while deletion of the C-terminal part (aa 615–968) did not disrupt the interaction (Fig. 1C, pLBN). These results suggested that the Rpg1p–Sla2p interaction is mediated by the N-terminal half of Rpg1p/Tif32p.

Rpg1p interacts with Sla2p in vivo. Rpg1p and Sla2p were also tested for interaction in cell extracts. Wild-type, *sla2Δ376-501,* and *sla2Δ114-367* strains were used for immunoprecipitation of Sla2p by the antiserum directed against the extreme Sla2p C-terminal peptide. Rpg1p was followed in these immune complexes by Western blotting analysis using anti-Rpg1p antiserum. Rpg1p was coimmunoprecipitated with Sla2p (Fig. 2, lane 1) but not with the *sla2Δ114-367* (Fig. 2, lane 3). A sixfold higher Rpg1p level was observed in an immune complex obtained from the *sla2Δ376-501* mutant (Fig. 2, lane 2). The Sla2p level observed in the *sla2Δ376-501* mutant was similar to the wild-type level (data not shown). These results from *in vivo* binding experiments are consistent with the two-hybrid data.

Rpg1p partially colocalizes with Sla2p. We have shown recently that Rpg1p is a microtubule-interacting protein (26). To compare Rpg1p localization with Sla2p distribution by immunofluorescence microscopy the double-labeling procedure was applied using the antibody against the C-terminal domain of Sla2p (28). The individual images of the double-labeled cells (FY1679 strain) are shown in Fig. 3. In the most of unbudded cells both proteins displayed a punctuate distribution. They colocalized at the nucleus-associated patch that probably corresponds to spindle pole body (Fig. 3A). In some unbudded cells Rpg1p co-localized with Sla2p in the cortical domain, probably at the presumptive bud site (Fig. 3B). In budded cells, Rpg1p is accumulated in a patch at the very end of cytoplasmic microtubules reaching the bud tip (26). In these cells, we found that a portion of Sla2p obviously colocalized with the patch of Rpg1p near the bud tip (Fig. 3C). In cells of the other cell cycle stages Sla2p accumulates in the neck region where the actin filaments are nucleated before cytokinesis (28) whereas Rpg1p is usually spread through the cytoplasm displaying colocalization with anaphase spindle microtubules (26). In these budded cells no obvious colocalization of both proteins was observed (data not shown). More information could be obtained by the confocal laser scan microscopy of double labeled cells.

DISCUSSION

Based on purification and *in vivo* binding assays it has been proposed that five yeast proteins (Rpg1p/Tif32p, Prt1p, Tif34p, Tif35p and Nip1p), homologous to human eIF3 subunits, are components of the conserved core of yeast eIF3 (32–34). In this respect, the Sla2p has not been classified as a stable component of the eIF3 complex. In agreement with this, Sla2p was observed to colocalize only partially with Rpg1p at specific cellular domains. Furthermore, the immunoprecipitated Sla2p complex contained only a small portion of the total cellular Rpg1p (J. Palecek, unpublished data) suggesting that the Rpg1p–Sla2p interaction is transient. It has been shown that the coiled-coil domains of Sla2p influence its distribution between the cytoplasm and the plasma membrane (28) due to modulation of Sla2p interactions with different protein complexes (16, 28). Interestingly, genetic manipulation of the Sla2p coiled-coil domains significantly increased the affinity of the protein to Rpg1p (Figs. 1A, 1B, and 2) suggesting that Rpg1p may specifically associate with some of these complexes to mediate its *SLA2*-related function.

Na *et al.* (35) have studied a specific function of the *SLA2/END4/MOP2* gene product with respect to expression of Pma1p (the plasma membrane proton-translocating ATPase). *mop2* mutations isolated in a search for modifier of *pma1* (*mop*) mutants, conferred

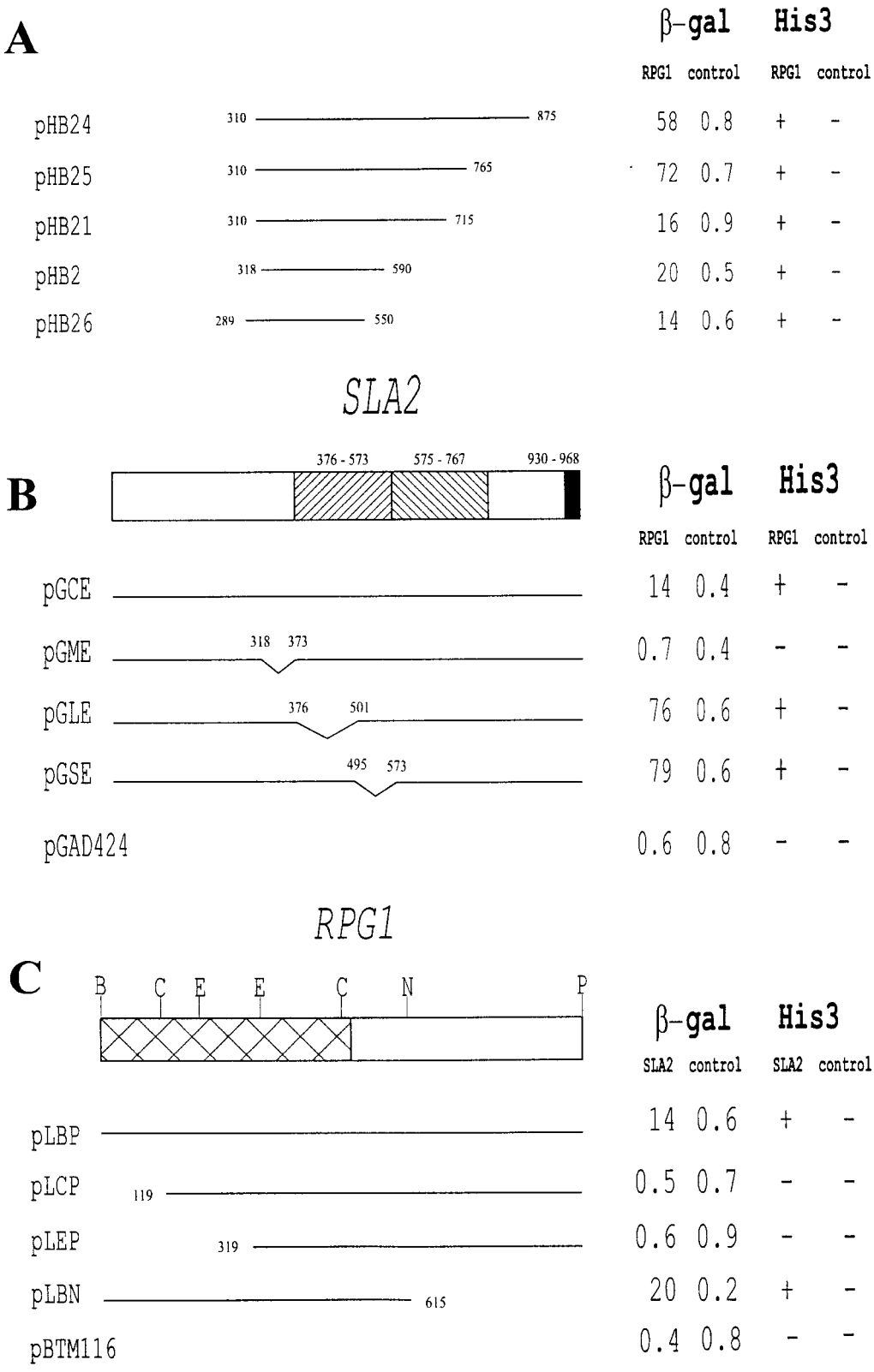

FIG. 1. Domains required for the Rpg1p-Sla2p interaction. The *SLA2* sequences (A, genomic library clones; B, the entire coding region and/or deletion mutated versions) expressed as fusion proteins with Gal4 transcription activation domain are indicated. Both central (aa 376–573) and second (aa 575–767) coiled-coil domains (hatched), and talin-like domain (black) is indicated in the *SLA2* coding region. Plasmids indicated on the left were introduced into L40 cells harboring either pLBP (wt *RPG1*) or pBTM116 (control). (C) The *RPG1* sequences expressed as lexA fusion proteins are represented by lines below the map of *RPG1*. The evolutionarily conserved domain (crosshatched) is indicated. Plasmids indicated on the left were introduced into L40 cells harboring either pGCE (wt *SLA2*) or pGAD424 (control). The activities of β-galactosidase (units/mg protein) and the His3 phenotypes were assayed. +, growth. −, no growth. B, *Bam*HI. C, *Cla*I. E, *Eco*RI. N, *Nde*I. P, *Pst*I.

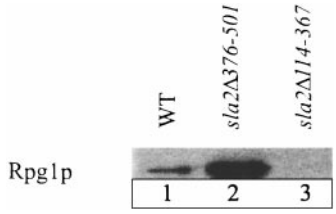

FIG. 2. Rpg1p/Tif32p can coprecipitate with the Sla2p immune complex. The Sla2p and associated proteins were precipitated by antiserum directed against the extreme C-terminal peptide of the Sla2p protein, from extracts prepared from wild-type (lane 1), *end4Δ376-501* (lane 2), and *end4Δ114-367* (lane 3) strains, respectively. Rpg1 protein was detected by specific antibody on Western blots and quantified by Image QuaNT software.

hypersensitivity to the translational inhibitor cycloheximide. A phenotype similar to that observed for *mop2* mutants was followed with *sla2Δ* deletion mutants defective in actin-related function(s) but not with a *sla2Δ318-373* strain (J. Palecek, unpublished results). These data suggest that the Rpg1p-Sla2p complex is not essential for the *SLA2*-related function in Pma1p specific processing. Recently, a *sla2* mutant was identified during a screen for mutants exhibiting defects in the decay of several mRNAs. A role of the actin cytoskeleton in the decay of specific mRNAs was proposed in this context (36).

Disruption of the Rpg1p interaction domain of Sla2p (*sla2Δ318-373*) is not lethal for cells. In addition, the *sla2Δ318-373* mutant cells exhibit a normal polarization of the actin cytoskeleton (16) and deletion of even longer Sla2p fragments did not result in a defective cellular localization of the protein (28). One can assume that the interaction between Sla2p and Rpg1p is neither required for the essential role of Rpg1p in the initiation of translation nor for actin-nucleation activity of Sla2p (nor for its localization). Our immunofluorescence data indicate that the transient contact of Rpg1p with Sla2p may be involved in the intracellular localization of the eIF3 complex.

Recent data suggest that *Saccharomyces cerevisiae* may employ an analogous mRNA localization mechanisms as higher eukaryotes (11). The actin cytoskeleton was proposed to play an active role in generation of the asymmetric distribution of specific proteins (9, 10) (e.g., Ash1 protein localizes preferentially to the presumptive daughter nucleus, where it inhibits mating-type switching (37)). An interaction of a yeast translation factor with the actin cytoskeleton (Tef1p–Bni1p) was hypothesized to assist in the localized translation of specific *ASH1* mRNA (14, 38). Interestingly, at the time of the revision of the manuscript Lin *et al.* (39) has demonstrated a direct physical interaction between a human Rpg1p homologue and the intermediate fila-

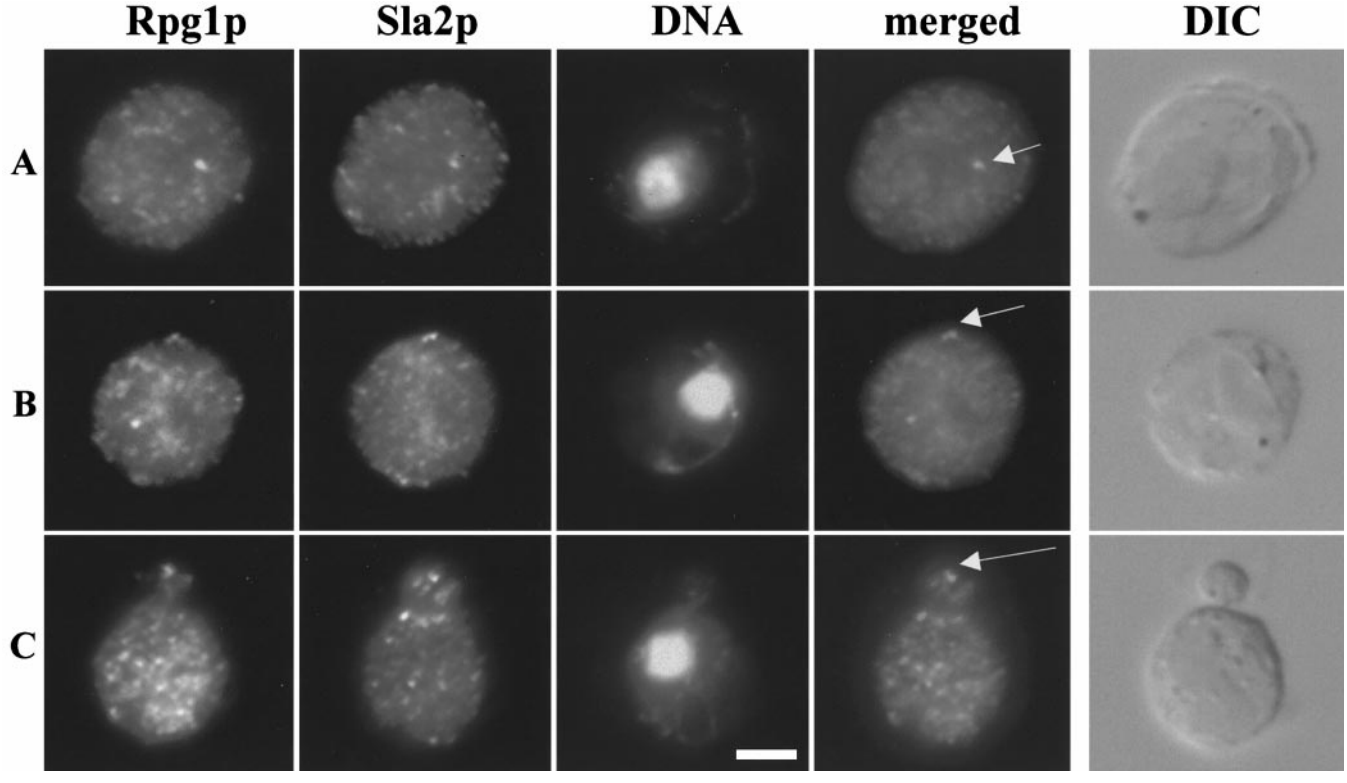

FIG. 3. Rpg1p/Tif32p partially colocalizes with Sla2p. The exponentially growing cells (strain FY1679) were fixed and double-stained with the monoclonal anti-Rpg1p antibody PK1/1 and the polyclonal antibody against C-terminal domain of Sla2p. DAPI was used to visualize DNA. Arrows point to sites on individual images where both proteins seem to colocalize. Bar, 5 μm.

ment protein cytokeratin 7. Their data support our idea that the largest subunit of the eIF3 could serve as a bridging molecule between eIF3 and cytoskeleton. To test a role of Sla2p–Rpg1p interaction in such processes (i.e., in a localized translation of *ASH1* and/or of mitochondria-specific mRNAs) additional studies are required.

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