Yeast She2p is an RNA- and membrane-binding protein

Association of the yeast RNA binding protein She2p with tubular endoplasmic reticulum depends on membrane curvature

Christian Genz¹, Julia Fundakowski¹, Orit Hermesh¹, Maria Schmid²,³, Ralf-Peter Jansen¹

¹ Interfaculty Institute of Biochemistry, Eberhard-Karls-Universität Tübingen, 72076 Tübingen, Germany
² GeneCenterLMU, Ludwig-Maximilians-Universität München, 81377 Munich, Germany
³ Current address: Roche Diagnostics GmbH, Penzberg, Germany

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Background: Transport of localized mRNAs encoding yeast membrane proteins to the bud is coordinated with segregation of endoplasmic reticulum.

Results: The localized RNA-binding protein She2p associates with membranes of endoplasmic reticulum in a curvature-dependent manner.

Conclusion: She2p is an RNA- and lipid-binding protein.

Significance: Direct targeting of mRNAs via membrane-associated RNA-binding proteins might facilitate their local translation.

ABSTRACT

Localization of mRNAs contributes to generation and maintenance of cellular asymmetry in a wide range of organisms. In Saccharomyces cerevisiae, the so-called locasome complex with its core components Myo4p, She2p, and She3p localizes >30 mRNAs to the yeast bud tip. A significant fraction of these mRNAs encodes membrane or secreted proteins. Their localization requires besides the locasome a functional segregation apparatus of the cortical endoplasmic reticulum (cER) including the machinery that is involved in the movement of ER tubules into the bud. Co-localization of RNA-containing particles with these tubules suggests a coordinated transport of localized mRNAs and cER to the bud. Association of localized mRNAs to ER requires the presence of the locasome component She2p. Here we report that She2p is not only an RNA-binding protein but can specifically bind to ER-derived membranes in a membrane curvature dependent manner in vitro. Although it does not contain any known curvature recognizing motifs, the protein shows a binding preference for liposomes with a diameter resembling that of yeast ER tubules. In addition, membrane binding depends on tetramerization of She2p. In an in vivo membrane-tethering assay, She2p can target a viral peptide GFP fusion protein to cortical ER, indicating that a fraction of She2p associates with ER in vivo. Combining RNA- and membrane-binding features makes She2p an ideal coordinator of ER tubule and mRNA co-transport.

INTRODUCTION

Localization of mRNA is widely used to spatially control gene expression and to generate cellular asymmetry (1, 2). Generally, messenger ribonuleoprotein (mRNP) particles containing
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translationally silent transcripts are transported from perinuclear areas to their peripheral destination where their translation is activated (2, 3).

In budding yeast, mRNA localization has been mainly studied using ASH1 mRNA as a model RNA. ASH1 encodes a transcriptional repressor and cell fate determinant and is localized to the tip of the mature bud (the daughter cell). Its local translation at the tip of the daughter cell ensures the repression of the HO endonuclease after cell division only in the daughter cell (4).

The RNA-binding protein She2p is a core component of mRNPs containing ASH1 (5, 6). She2p binds to ASH1 co-transcriptionally (7) and escorts it to the cytoplasm (8, 9). After nuclear export, this primary mRNA complex binds to the myosin motor protein Myo4p via the associated She3p protein (10, 11). Myo4p is required for the active translocation of localized mRNPs to the bud tip (4). In addition to the Myo4p-She3p-She2p core complex, three additional RNA-binding proteins (Loc1p, Puf6p, and Khd1p) are needed for efficient ASH1 mRNA localization and translational silencing of the mRNA (12-14). Besides ASH1 more than 30 additional mRNAs are localized in a Myo4p and She2p dependent way to the bud tip (15-17). Many of these mRNAs encode membrane or secreted proteins that are translocated into the ER. In contrast to ASH1, the localization of these mRNAs depends on additional proteins like reticulons, Aux1p, Srp101, or Sec3p that are also required for proper segregation of cortical endoplasmic reticulum (cER, also called plasma membrane attached ER) to the bud (15, 18, 19).

Cortical ER segregation begins during S phase when tubular structures emanate from the perinuclear ER and move in a Myo4p/She3p-dependent manner into the growing bud (20, 21). Shaping of tubular ER involves two protein families, the reticulons (Rtns) and DP1/Yop1p (22) that help to generate the high curvature required to form tubular membrane structures of 30 - 40 nm diameter in yeast or 50 nm in mammals (21, 23). These tubular structures move in an actin-dependent manner into the bud, followed by spreading of cER along the bud cortex (20). Movement but not spreading of cER along the plasma membrane is required for the localization of a subset of mRNAs to the bud tip (18). These mRNAs encode membrane proteins and are expressed at the time when ER tubules move into the bud. Co-transport of mRNAs with ER might therefore support an efficient synthesis at the cortical ER.

Although the RNA-binding protein She2p is not essential for cER segregation (24), it is necessary for association of some of its target mRNAs with ER tubules (19). She2p has been shown to co-fractionate with ER but the nature of the association remained obscure. Here we demonstrate that She2p can specifically bind to ER-derived microsomes in vitro, even in the absence of other components of the locosome or associated mRNAs. Furthermore, it can bind to protein-free liposomes with a preference for highly curved membranes. In vivo, it can direct a viral peptide tag to cortical ER, consistent with the idea that She2p is not only an RNA- but also a membrane-binding protein that could directly link its target mRNAs to ER membranes with high curvature as present in cER.

EXPERIMENTAL PROCEDURES

Yeast strains and Plasmids

Plasmids for expression of glutathione-S-transferase (GST) fusions to wild type She2p and She2p N36→S/R63→K have been described (8, 25). To generate expression vectors for She2p mutants S120→Y and L130→Y, site-directed mutagenesis of pGEX-TEV-SHE2 was performed using oligonucleotide pair RJO2003 and RJO2004 (S120→Y), or RJO2035 and RJO2036 (L130→Y), respectively. Generation of GST-She2pΔhelix-E has been described elsewhere (26). The GFP-TGB3(25-52) plasmid allowing the expression of GFP fused to a peptide from the plant potexovirus TGB3 protein (27) was a gift from Dr. Chao-Wen Wang. In order to introduce the SHE2 or MS2 coat protein (MS2-CP) coding regions, they were amplified with primers RJO4320, RJO4321, RJO4405, and RJO4406 and cloned between GFP and the signal sequence of TGB3(25-52) to create the corresponding fusion proteins. The resulting plasmids RJP1848 and RJP1850 were transformed into BY4741 WT yeast cells. Further information on plasmids and oligonucleotides can be found as supplemental material.

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Yeast subcellular fractionation and sucrose gradient centrifugation

Cell lysis was performed from yeast spheroplasts generated from strains BY4742 (SHE2) or RJY2053 (she2Δ::kanMX4) to maximally preserve subcellular integrity. Velocity gradient centrifugation of cell lysates on 18-60% sucrose gradients was performed as described (19, 24, 29). In brief, cells corresponding to 400 OD600 units were harvested, spheroplasted, lysed with a needle and cleared from cell debris as described above. 1 ml of the homogenate (corresponding to 66 OD600 units) was then loaded onto a linear 18%-60% gradient of sucrose in 20 mM Hepes-KOH pH 6.8, 140 mM K-acetate, 1 mM Mg-acetate. Gradients were spun in a SW40 rotor for 2.3 h at 38000 g. 12 x 1 ml fractions were collected starting close to the bottom of the gradient. The remaining pellet was resuspended in 1 ml lysis buffer. Fractions were precipitated with trichloroacetic acid (TCA) and resuspended in 100 µl SDS sample buffer. 20 µl of these were used for western blot analysis except for the top three fractions where only 7 µl were used in order to avoid overloading of the gel. Western blots were performed using enhanced chemiluminescence (ECL), scanned, and quantified by using ImageJ. For in vitro binding experiments, yeast lysate was prepared from the she2Δ strain RJY2053. 1 ml of lysate (corresponding to 66 OD600) was pre-incubated with recombinant She2p or She2p mutant protein (50 pmol protein per binding reaction) for 30 min on ice. The suspension was loaded on an 18%-60% gradient and treated as described above.

Quick subcellular fraction of yeast cells to separate heavy membranes (nuclei and ER) from free polysomes and cytosolic proteins was performed according to Frey et al. (30). In short, yeast cells were lysed with glassbeads and three centrifugation steps at 4°C were performed (20 min at 6000 g to pellet heavy membranes, 20 min at 18000 g to pellet light membranes and heavy polysomes, and 20 min at 20000 g to pellet ribosomes).

In order to follow the distribution of She2p or marker proteins, commercial antibodies against GFP (Covance, Princeton), Dpm1 (Molecular Probes), Pgk1p (Invitrogen), GST (Novagen), maltose binding protein (MBP, Novagen), or custom antibodies against Sec61p, She2p, OM45p, or Mcr1p were used in western blots.

In vitro binding assay with flotation purified ER vesicles or mitochondria

Mitochondria of yeast cells were isolated by differential centrifugation as described previously (31). Further purification of mitochondria was achieved via a self-generated percoll or sucrose step gradients (32). Preparation of yeast microsomes from the she2Δ strains RJY2053 (she2Δ) or RJY2370 (she2Δ HMG1-GFP::URA3) was essentially performed as described previously (33, 34). In brief, yeast spheroplasts were lysed by dounce homogenization with a tight fitting pestle. To collect a membrane fraction enriched in microsomes (yeast rough membranes, YRM), 15 ml of lysate was loaded onto a 15 ml 1 M sucrose cushion, followed by a 10 min centrifugation at 3000 g and 4°C. The upper layer was recovered avoiding the interface and membranes were pelleted (21000 g, 10 min, 4°C). The membrane pellet was resuspended in buffer 88 (20 mM Hepes-KOH pH 6.8, 150 mM K-acetate pH 7.4, 250 mM sorbitol, 5 mM Mg-acetate), pelleted again and resuspended in 2 ml of buffer 88 to a final concentration of about OD260= 40/ml (corresponding to a protein concentration of 10-12 mg/ml). For flotation purification, membranes were mixed with 2.5 M sucrose in flotation buffer (50 mM Hepes-KOH pH 7.5, 5 mM Mg-acetate, 150 mM K-acetate, 1.5 mM DTT). This mix was layered at the bottom of an ultra clear SW55 tube and carefully covered by a cushion of 1.9 M sucrose in flotation buffer (cushion II) followed by a second cushion of flotation buffer only...
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Flotation assay with protein-free liposomes

Liposomes were prepared from lipid mixes as described (35). For most binding studies including analysis of liposome size dependence, a phospholipid extract from soybean was used (Sigma) that contains as main phospholipids 55% phosphatidylcholine (PC) and 25% phosphatidylethanolamine (PE). To investigate the influence of lipid headgroup packing, increasing amounts of DOPC (1,2-di-(9Z-octadecenoyl)-sn-glycerol-3-phosphocholine) were added to yield final concentrations of 20% or 40% DOPC in the lipid mixture. To compare protein binding to liposomes with or without phosphatidylserine (PS) and phosphatidylinositol (PI), a lipid mix resembling lipid composition of yeast ER (36, 37) (40% PC, 24% PE, 10% PS, 10% PI, 16% ergosterol) or a mix containing 55% PC, 36% PE, and 20% ergosterol was used. Lipids were dissolved in chloroform and organic solvent was removed by evaporation under N₂ atmosphere. Lipids were completely dissolved to a final total lipid concentration of 10 mg/ml in degassed liposome buffer (20 mM HEPES pH 7.4, 100 mM NaCl) and the emulsion passed 21 times through a polycarbonate filter membrane mounted in a mini extruder (Avanti Polar Lipids) in order to create unilamellar liposomes. Liposomes with different diameter were generated using filter membranes with 30 nm, 80 nm, 200 nm, or 400 nm pore size. Liposome size distribution was verified by dynamic light scattering in a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany).

Liposomes were used directly for flotation assays or stored at 4°C for a maximum of one week. 50 µl liposomes were mixed with 50 pmol She2p, GST, MBP-Mim1p, or OM45p in 190 µl binding buffer (50 mM Hepes/KOH, 150 mM K-acetate, 1 mM Mg-acetate, 1 mM EDTA, 1 mM DTT) and incubated for 15 minutes on ice. 40 µl of the sample were kept as input control. 200 µl were mixed with 3 ml binding buffer containing 70% sucrose and added to the bottom of a SW40 polycarbonate tube. The sample was then covered with three cushions of 3 ml binding buffer containing 50%, 40% and 0% sucrose. After centrifugation to equilibrium (70000 g for 4 h at 4°C) the liposome-containing interface between the 40% and 0% sucrose cushions was harvested, precipitated by TCA and dissolved in 45 µl SDS sample buffer. Flotation samples and input controls were analyzed as described above. She2p signals were analyzed densitometrically using ImageJ. For the RNA competition assay, She2p was pre-incubated with 500 pmol (10x excess) or 1 nmol (20x excess) of in vitro transcribed ASHI E3 localization element (28) for 15 min at RT and 10 min on ice in binding buffer containing RNAsin. Subsequently, liposomes were added and the mix treated as described above. Input control and flotation samples were either processed for western blot or phenol/chloroform extracted for RNA recovery. After ethanol precipitation, RNA was resuspended in 25 µl of TE and applied to 2% agarose gel electrophoresis and ethidium bromide staining.

Microscopy

Yeast cells were scraped from a fresh selective plate, resuspended in 2 ml SC-complete medium,
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grown for 3 - 4 hours at 30°C, dropped onto a thin agarose layer of SC-complete medium, and covered with a cover slip. Images were captured as image stacks on a ZEISS AxioObserver.Z1 fluorescence microscope operated by AxioVision software (ZEISS) and deconvoluted using the same software package. Imaging data was analyzed using AxioVision Rel 4.8.

RESULTS
Oligomerization but not RNA-binding of She2p is required for ER association.
Since localization of mRNAs such as WSC2 depends on functional cER segregation but is independent of co-translational targeting (18), localization might require mRNA attachment to ER via RNA binding proteins like its cognate partner She2p. We have previously demonstrated that a fraction of She2p co-migrates with ER-derived membranes (microsomes) in sucrose gradient centrifugation (19). However, a further analysis of e.g. the amino acids of She2p that mediate this association has been hampered by the distinctive behavior of several She2p mutants since their expression in yeast often results in retention in the nucleus or nucleolus (8, 9, 19). In order to identify potential binding sites in She2p we therefore devised an in vitro assay to study wild type and mutant She2p proteins for their association with ER.

When added to a lysate from a she2Δ yeast strain, bacterially expressed She2p co-fractionates with the ER marker protein Sec61p to dense fractions of a sucrose gradient (Fig. 1A), which is similar to the pattern seen of endogenous She2p (19). In contrast to Sec61p, She2p can also be detected in the top fraction (16 +/- 1.5% of total She2p present in all fractions), implying that not all She2p added to the lysate is associated with ER. This distribution can also be detected with endogenous She2p (19), suggesting that recombinant She2p retains the ability to associate with microsomes. We next tested for co-fractionation of a mutant protein, She2p L130->Y that is impaired in tetramerization of She2p. Tetramer formation is crucial for RNA target selection but does not diminish general RNA binding (28). Compared to wild type She2p where 63% of total She2p co-fractionates with Sec61p, the L130->Y mutant shows a reduced co-fractionation since only 20% of She2p L130->Y can be found in gradient fractions containing Sec61p ER marker (fractions 1-3). In contrast, about 30% of She2p L130->Y accumulates as free protein in the top gradient (compared to 14% in case of wild type She2p) and a large part of it is found in the pellet of the gradient, presumably since the protein is partially aggregating (28). A reduction of She2p co-fractionation with ER was also observed in case of the mutant protein She2p S120->Y (Fig. 2), which carries a mutation in the She2p homodimerization interface (25). However, this reduction was not as strong as in the L130->Y mutant. In contrast to these mutants, deletion of helix E that is involved in target mRNA selection (26) does not significantly affect She2p Sec61p co-migration (Fig. 2). Similar observations have been made for the She2p N36->S/R63->K mutant that is impaired in RNA-binding (19).

We conclude form these studies that the correct tertiary structure of She2p but not its RNA binding ability is required for proper association with ER.

Recombinant She2p binds to ER membranes but not to mitochondria
Next, we wanted to address if the co-migration of She2p and ER marker proteins reflect an association of She2p with ER and if She2p could also bind to other organelles. To test this, we implemented a second in vitro binding assay. Membrane vesicles were purified from yeast lysates via a two-step procedure, yielding a membrane fraction enriched in ER microsomes (see Experimental procedures and (33)). Purified She2p or glutathione S-transferase (GST) as control was added to these microsomes and the mixture subjected to centrifugation through a 1.2 M sucrose cushion, thus separating unbound She2p in the supernatant from membrane-bound She2p in the pellet (Fig. 3A). In the presence of microsomal membranes, She2p but not GST penetrates the cushion. When adding increasing amounts of She2p to purified membranes we observed that binding was saturable and thus might involve a binding partner on the membrane (Fig. 3B).

In order to test if this binding is specific for ER, we isolated microsomes or mitochondria from yeast (see Experimental procedures) and added She2p to each before subjecting them to membrane pelleting as described above. As described above,
addition of ER derived membranes leads to pelleting of She2p as indicated by the presence of both She2p and the ER maker Sec61p in the pellet (Fig. 3C, middle panel). In contrast, if microsomes are replaced by purified mitochondria, only little pelleting of She2p is detected although the mitochondrial marker protein Mcr1p is efficiently pelleted (Fig. 3C, right panel). This indicates that ER derived membranes carry a component that allows a specific association of She2p.

Protease-treated microsomes retain She2p binding properties.

In order to obtain additional evidence for a specific binding partner on the ER membrane, we treated the membrane fraction with protease before addition of She2p. Protease treatment results in removal of the cytoplasmic epitope recognized by the anti-Sec61p antibody, revealing that proteolysis was successful (Fig. 4A). Increasing amounts of protease for membrane treatment reduces but does not abrogate co-pelleting of She2p with membranes (Fig. 4B). Even after maximal protease treatment, more than 40% of added She2p can be detected in the membrane pellet fraction. Since She2p can potentially form large multimers in vitro (28) this pellet fraction could correspond to pelleted multimers. In order to rule out this possibility, She2p was mixed with a membrane-deprived 100,000 g supernatant of a cell lysate (S100). In this case, only very little She2p could be detected in the pellet (Fig. 4A), suggesting that its presence in the pellet fraction is not due to formation of large multimers.

She2p binds to protein-free liposomes

Our previous results suggest that binding of She2p to membranes requires proteins for optimal binding but that protease-free membranes could also serve as binding interface for She2p. We therefore prepared artificial liposomes from soybean lipids containing as major lipids 55% phosphatidylcholine (PC) and 25% phosphatidylethanolamine (PE). These artificial liposomes were equilibrated with 70% sucrose, covered with cushions of 40% sucrose and buffer lacking sucrose, and used in a flotation experiment adapted from Kanai et al. (38) (see Experimental procedures). The rationale of this experiment is that due to their density liposomes will float to the interphase between the 0% and 40% sucrose cushion together with proteins binding to their lipids. Figure 5 shows that She2p, if mixed with liposomes before flotation co-floats to sucrose cushion interphase (Fig. 5A, top). In contrast, GST does neither float in the presence nor absence of liposomes (Fig. 5A, bottom). Similar results were obtained when purified glycerophospholipids reflecting ER-like lipid composition (36, 37) (40% PC, 24% PE, 10% PS, 10% PI, 16% ergosterol) were used (not shown). In order to show that flotation assay results reflect membrane association, we used the mitochondrial outer membrane protein OM45p (encoded by the ORF YIL136w), which can bind to mitochondrial membranes and liposomes in vitro (39). The detection of OM45p in the floated fraction indicates that the assay reflects binding of the added protein to liposomes. In summary, She2p can directly bind to liposomes and therefore shares features with peripheral membrane proteins.

To address the question whether the lipid content of liposomes influences She2p binding, we prepared liposomes with varying lipid content. Since She2p carries a large number of positively charged amino acids at the surface (25) we were specifically interested if phospholipids with a negative net charge like phosphatidylserine (PS) and phosphatidylinositol (PI) increase She2p binding. Compared to liposomes with an ER-like lipid content of PI and PS (see above), no difference of She2p binding was detectable (Fig. 5B). We conclude that the negatively charged phospholipids PS and PI are dispensable for She2p binding to liposomes.

Specific interaction of peripheral proteins with membranes can also be mediated by recognition of the membrane curvature (50). In order to test this idea, we generated protein-free liposomes of different diameter ranging from 30 nm to 400 nm (see Experimental procedure). The rationale was that smaller liposomes generated by this method have higher membrane curvature than larger ones. Equal amounts of phospholipids were used for the preparation of differently sized liposomes, which were then mixed with She2p and used in parallel flotation experiments. As control proteins, we used two mitochondrial membrane proteins, OM45p and Mim1p (40). Western blot analysis and quantification of the She2p signals in the floated fraction and the input revealed an increase of bound She2p with decreasing liposome size. If
binding of She2p to liposomes with 30 nm diameter was arbitrarily set to 100% (Fig. 5C), 80 nm liposomes bound 73% (+/- 9%, n=3) of She2p, 200 nm liposomes only 50% (+/- 13%), and liposomes with an average diameter of 400 nm only 30% (+/- 14.5%). No such preference could be detected for OM45p that showed similar binding to liposomes of various size, whereas Mim1p binding was strongest to larger liposomes but did not significantly vary between 30 nm and 200 nm liposomes. These data suggest that binding of She2p to membranes, in contrast to OM45p or Mim1p depends on liposome curvature or phospholipid headgroup packing, which is also altered by liposome size. To distinguish between these possibilities we prepared liposomes of 80 nm diameter containing 20% and 40% Dioleoylphosphatidylcholine (DOPC), an inverted cone-shaped phospholipid that allows higher packing in curved membranes (41). If lipid packing were important, one would expect an increase in membrane interaction of She2p due to incorporation of DOPC in liposomes. However, although quantification of the She2p western blot signals in the floated fraction and the input revealed a modest increase in average binding (9%) compared to 80 nm liposomes without DOPC, this indicating that the increased interaction of She2p with smaller liposomes might indeed mainly be due to curvature increase (Fig. 5D).

**Liposome association of an RNA localization element occurs via She2p**

A previous analysis demonstrated that co-pelleting of localized mRNAs with ER is altered in cells lacking She2p, indicating that She2p is a crucial factor for their association with membranes (15). Our flotation assay allowed us to address if She2p is not only required but also sufficient for this association. If binding of She2p to RNA targets and lipids could occur simultaneously they should show no competition. We therefore tested if a cognate RNA, the *ASH1* E3 localization element competes with liposomes for She2p binding. In two independent experiments, a 10fold or 20fold molar excess of an *in vitro* transcribed E3 localization element was added to a mixture of liposomes and She2p before flotation. In parallel controls, the E3 RNA was omitted (‘mock’). Flotation of She2p remained unperturbed even in the presence of 20fold excess of E3 (Fig. 6), indicating that an RNA localization element does not compete with liposomes for She2p binding. Analysis of RNA extracted from floated fractions by agarose gel electrophoresis and ethidium bromide staining revealed the presence of E3 RNA in the floated fraction. These results demonstrate that She2p can simultaneously bind to liposomes and a localization element and suggest that She2p mediates the observed association of localized mRNAs with ER (15, 19).

**She2p targets a viral peptide to ER**

Our *in vitro* analysis indicates that She2p can bind to ER-derived membranes. These results are supported by previous observations that mRNPs of mRNAs associated with She2p co-localize with ER tubules during budding (19). In order to address whether She2p can bind ER *in vivo* we implemented a new assay based on the observation that expression of an N-terminal peptide from Bamboo mosaic potexovirus protein Tgb3 results in its targeting to ER if fused to a membrane protein (27). This assay would allow us to detect membrane binding even if this is transient or if only a subfraction of She2p is membrane associated. If expressed in yeast, the potexovirus Tgb3 protein is targeted to cortical ER. The targeting is due to a signal at its N-terminus (comprising amino acids 25-52). The signal peptide, when expressed alone results in cytoplasmic localization, but a fusion of the signal peptide with a membrane protein results in targeting to the cortical ER (27). We therefore wondered if She2p can adopt the role of the membrane protein and target a Tgbp3(aa25-52)-GFP fusion to the cell cortex by providing the missing membrane targeting information. As a control to test if this is a general feature of other RNA-binding proteins, we used the coat protein from bacteriophage MS2 (42). This fusion protein showed the same diffuse cytoplasmic distribution as Tgbp3(aa25-52)-GFP (Fig. 7A). In contrast, fusion of She2p results in re-distribution of Tgbp3(aa25-52)-GFP to foci in the bud and mother cell (Fig. 7A, left panel). A majority of these foci (73%, n = 55) co-localize or overlap with endoplasmic reticulum in the mother cell and at the bud tip (Fig. 7B), suggesting that She2p might target the viral peptide to ER membranes. Co-localization of similar foci with ER structures has
been reported from other fusions proteins with Tgb3p (27). In order to independently test for ER targeting, we performed subcellular fractionation by differential centrifugation of cell lysates (30) containing either Tgbp3(aa25-52)-GFP or She2p-Tgbp3(aa25-52)-GFP (Fig. 7B). The majority of Tgbp3(aa25-52)-GFP is detected in fraction S200 containing cytosolic proteins like Pgk1p (30). In contrast, She2p-Tgbp3(aa25-52)-GFP is enriched in fraction P6 that contains heavy membranes including ER as seen by the ER marker Dpm1p (30). These results indicate that She2p has the potential to target a reporter peptide to ER membranes in vivo and therefore strongly supports our in vitro analysis.

**DISCUSSION**

In budding yeast, more than 30 mRNAs localized to the growing bud encode membrane or secreted proteins. Localization of these mRNAs does not only require the locosome components Myo4p, She3p, and She2p (17), but also proteins that are crucial for early steps during the segregation of cortical ER (15, 18, 19). Cortical ER segregation is initiated by the movement of tubular ER structures into the bud, which among other factors requires Myo4p and She3p but not She2p (24). However, localizing mRNPs co-migrate with ER tubules during movement, (19), and since mRNAs in these mRNPs are bound by She2p, it might nevertheless be present on these tubules. Consistently, localized mRNAs only co-fractionate with membranes in subcellular fractionation studies if She2p is present in these cells (15), suggesting that She2p might mediate the binding of these mRNAs with ER membranes.

Although association of mRNAs to ER independent of SRP-mediated targeting is a widespread phenomenon (43-45), the role of RNA-binding proteins in targeting mRNA to the ER is not well understood. Targeting could involve RNA-binding membrane proteins like the ER protein p180 (46) or cytoplasmic RNA-binding proteins that direct mRNAs to ER (47). In budding yeast, the RNA-binding proteins Sep160p (30), Whi3p (48), Bfr1p (49), and She2p at least partially co-localize or co-fractionate with ER. Whereas the exact mechanism of ER association of Whi3p is unknown, co-localization of Sep160p and Bfr1p with ER depends on translation and might therefore be indirectly mediated via nascent peptide chains (30, 49). In contrast, She2p co-fractionates with ER even after disruption of polysomes or degradation of mRNA (15, 19) and therefore represents a likely candidate for a protein mediating the association of specific mRNAs to ER in vivo. The observations that She2p can simultaneously bind to an RNA localization element and to membranes (Fig. 6) and that it is required for mRNA-ER co-fractionation (15) are consistent with this idea. So far, She2p has been visualized in cells only in a crescent at the bud tip (5), as diffuse signal in the cytoplasm (8) or in the nucleus (8, 9) but co-localization with ER has not been reported. However, it can target a viral peptide from the Bamboo potexvirus Tgb3 protein to ER, a feature that has so far been demonstrated for membrane or membrane-associated proteins (27). This suggests that at least a fraction of She2p is able to temporarily associate with membranes in vivo, which allows the peptide to be transferred to the ER.

How does She2p bind to ER membranes? Two lines of evidence suggest that binding occurs both via lipids and a protein component. Since She2p can bind to protein-free liposomes, it is able to directly interact with lipids. However, She2p binding to protease-treated ER membranes is significantly reduced and binding occurs to ER membranes but not to mitochondrial membranes with a different protein composition. ER-specific versus mitochondrial binding cannot be explained by smaller size of ER-derived vesicles as compared to vesicles from mitochondrial preparations as dynamic light scattering indicated that mitochondrial vesicles are on average half the size as microsomal vesicles (data not shown). Binding also does not require membranes with a specific lipid composition since omission of phospholipids with a negative net charge like phosphatidylycerine or -inositol does not change the binding behavior. Instead, we have observed a preferential binding of She2p for liposomes of small diameter as compared to larger ones. This increase could result from a change in curvature, which suggests that She2p recognizes membrane shape. Alternatively, it might result from an overall larger surface area of the sum of all small liposomes, or as the density of lipid headgroup changes with liposome size, She2p binding might also depend on lipid headgroup density. Since She2p binding to 80 nm liposomes drops by about
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30% as compared to 30 nm but surface area of the outer leaflet decreases by only 10%, a simple difference in total binding area cannot account for the observed difference. Our results that incorporation into liposomes of DOPC, a cone-shaped lipid with a small headgroup does not significantly alter binding efficiency argue against lipid density recognition. Consistent with curvature recognition, mRNPs containing She2p co-localize with tubular ER structures (19) that in yeast have an average diameter of 30 - 40 nm (21), which reflects the observed binding preference in our flotation assays. Furthermore, Δyop1Arnl1 mutant yeast cells that contain bloated ER tubules with an increased diameter (21) are defective in localization of mRNAs whose localization depends on ER segregation (18). Interestingly, these bloated tubules are still segregated into the bud (21), but might no more associate with RNPs containing She2p.

According to both sequence and protein structure analysis, She2p does not contain known motifs present in membrane-shape recognizing peripheral membrane proteins (50, 51) or other membrane-binding motifs. Since we used a bacterially expressed She2p in our binding assays, it is also less likely that other posttranslational modifications like phosphorylation are required for association of She2p with ER. It thus remains open how the protein associates with ER or liposomes.

Tetramerization of She2p seems to be involved in membrane attachment since the fraction of She2p that co-migrates with ER in sucrose gradients is reduced in a She2p mutant carrying the Leu(130)→Tyr mutation that disrupts tetramer formation (28). Although this mutation also diminishes RNA-binding, other mutations that disturb RNA binding like the deletion of helix E (26) do not alter She2p-ER co-migration. Tetramerization therefore seems to serve RNA and membrane binding, in the latter case possibly by providing a larger interaction interface of She2p with components of the cytoplasmic face of ER. In addition, not only ER tubules but other ER structures like central cisternal ER and plasma membrane attached ER (21, 52) as well as small vesicles exhibit high membrane curvature, suggesting an additional mechanism to specifically direct She2p and bound mRNAs to ER tubules. This might involve yet unknown protein adapters on the ER and is corroborated by our finding that protease treatment of microsomes reduces the fraction of She2p interacting with microsomal membranes.

Our study strongly suggests that despite absence of recognizable lipid interaction domains, the RNA-binding protein She2p has an unexpected membrane binding property, which is consistent with its requirement for the association of localized mRNAs with ER. It not only raises the question about the molecular basis of this association but also if the proposed direct linking of specific mRNAs to membranes via She2p is a mechanism used by other RNA-binding proteins involved in targeting of specific mRNAs to ER like rice OsTudorSN (53) or Xenopus laevis Vg1RBP/Vera (54).

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We appreciate help of T.-G. Du in generating the She2p S120→Y mutant. We thank D. Niessing and M. Müller for providing plasmid pGEX-SHE2-AhE and C.-W. Wang for the GFP-TGB3(25-52) expression plasmid. We are grateful to E. Merklinger, R. Singhal, M. Sinzel, and D. Rapaport for providing purified OM45p, MBP-Mim1p and purified mitochondria. J. Bauer helped during dynamic light scattering. This work was supported by a grant to R.-P.J. (DFG JA696/7-1).

REFERENCES


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Yeast She2p is an RNA- and membrane-binding protein.


**FIGURE LEGENDS**

Figure 1: Recombinant She2p but not She2p(L130Y) co-fractionates with yeast microsomes in vitro. (A) Recombinant She2p co-migrates with ER in sucrose velocity gradient centrifugation. Upper panel: Bacterially expressed She2p was incubated with a cell extract from strain RJY2053 (she2Δ) before separation on a linear 18-60% sucrose gradient. Aliquots of 12 fractions (with fraction 1 as the bottom and 12 as top fraction) and the pellet were analyzed by western blotting against an ER integral membrane protein (Sec61p) and She2p. Lower panel: Relative amounts of She2p and Sec61p in the corresponding fractions (100% = total amount of She2p or Sec61p detected in all fractions). Average values and standard deviation from three experiments are displayed. (B) Reduced co-migration with ER of the tetramerization mutant She2p-L130Y. Upper panel shows western blots against recombinant She2p-L130Y and Sec61p after gradient centrifugation as described above. Lower panel displays quantification as described for (A).
Yeast She2p is an RNA- and membrane-binding protein

Figure 2: She2p-S120Y and She2p(ΔhelixE) mutant proteins that affect RNA-binding co-migrate with ER in sucrose velocity gradients.
(A) Recombinant She2p co-migrates with ER in sucrose velocity gradient centrifugation. Upper panels: Bacterially expressed She2p was incubated with a cell extract from strain RJY2053 (she2Δ) before separation on a linear 18-60% sucrose gradient. Relative amounts of She2p and Sec61p in the corresponding fractions (with fraction 1 as the top fraction) of a sucrose density gradient are displayed. Numbers are given in % of total She2p or Sec61p detected in all fractions. Average values and standard deviation from three experiments are shown. (B) Co-migration with ER of the mutant She2p-S120Y. Quantitative results are shown as described for (A). (C) Co-migration with ER of the mutant She2p-S120Y. Quantitative results are shown as described for (A).

Figure 3: Recombinant She2p binds to ER microsomes but not to mitochondria.
(A) Pelleting of purified microsomes with recombinant She2p and GST. She2p or GST were mixed with ER membranes or buffer before centrifugation through a 1.2 M sucrose cushion. Equivalent amounts of the pellet, supernatant or the input material were analyzed by western blotting against She2p, GST or Sec61p. She2p is detectable in the pellet only in the presence of membranes. Spurious amounts of GST can be seen in the pellet fraction. (B) Indicated amounts of She2p were incubated with 40µl microsomes (corresponding to 40 µg of membrane-associated protein). Input and bound She2p were analyzed by quantitative western blotting and the ratio of bound She2p signal versus input was quantified. Average ratio (in %) and standard deviation of n=3 experiments is displayed. (C) Purified microsomes or yeast mitochondria were mixed with She2p and pelleted through a sucrose cushion. She2p co-pellets only with microsomes. Sec61p served as an ER marker whereas Mcr1p indicated mitochondria. In the absence of membranes She2p can only be detected in the supernatant.

Figure 4: Protease treatment of membranes reduces but does not inhibit binding of She2p.
(A) Pelleting assay of recombinant She2p and protease-treated microsomes. She2p was either incubated with membranes, membranes treated with a combination of Pronase E and Proteinase K, or a supernatant depleted of membranes (S100). After the binding reaction, all three samples were analyzed as described above. (B) Protease treatment of microsomes results in only partial loss of She2p binding capacity. Identical quantities of microsomes were treated with increasing protease amounts before addition of She2p and membrane pelleting. Even at high protease concentration, >40% of She2p can be pelleted. Bars indicate average values from n=4 experiments.

Figure 5: She2p binds to protein-free liposomes.
(A) Flotation assay of recombinant proteins added to artificial liposomes (200 nm average diameter). Like the outer mitochondrial membrane protein OM45p, She2p but not GST floats to the top of a sucrose gradient. Flotation of OM45p and She2p depends on the presence of liposomes. (B) She2p interacts with liposomes deprived of phosphatidylserine and phosphatidylinositol. She2p was floated with either ER-like liposomes as in (A) or with vesicles lacking the phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI). No difference in co-flotation is detectable. (C) She2p binding to liposomes inversely correlates to lipidosome size. Flotation was performed as described in experimental procedures with liposomes of indicated diameter. Bars displaying average ratio of floated protein versus input in artificial units (a.u.). Binding to liposomes with 30 nm diameter is set to 100 a.u. for She2p, OM45p, or Mim1p. Whereas She2p flotation decreases with increasing liposome size, relative binding of OM45p or Mim1p to liposomes is unchanged over a large liposome size range. Error bars indicate standard deviation in quantitation of three independent flotation experiments. (D) Lipid headgroup packing does not influence She2p binding to liposomes. Flotation assay with liposomes of 30 nm and 80 nm diameter and varying percentage of headgroup packing increasing phospholipid DOPC was performed as described.
Bars displaying average ratio of floated protein versus input as described in (C). Binding to 30 nm liposomes is set to 100%. Relative to 80 nm liposomes without DOPC, liposomes with 20% DOPC or 40% DOPC liposomes do not significantly bind more She2p. Error bars indicate standard deviation in quantitation of three independent flotation experiments.

**Figure 6: Simultaneous binding of She2p to liposomes and an RNA localization element.**
Recombinant She2p was incubated with only binding buffer (mock), a 10-fold and 20-fold molar excess of *in vitro* transcribed *ASH1* E3 element, followed by incubation with liposomes and flotation as described in figure 3. Upper panel: western blot against She2p, lower panel: ethidium bromide stained gel of RNA extracted from floated fractions (Flot.) and input.

**Figure 7: She2p mediates binding of a viral peptide to yeast cortical ER.**
(A) GFP fused to an aminoterminal peptide of Bamboo poteoxvirus protein TGB3 (Tgb3(aa25-52)) accumulates in the cytoplasm of yeast cells. Fusion of She2p but not of MS2 coat protein allows formation of distinct particles (left). (B) She2p-Tgb3(aa25-52) particles co-localize with ER. Left panel: differential interference contrast image (DIC), middle left: Sec63-mCherry, middle right: She2p-Tgb3(aa25-52)-GFP, right panel: overlay of DIC, mCherry, and GFP. Images represent single frames from a deconvolved Z-stack to ensure better resolution in the Z-axis. White bar corresponds to 2 µm. (C) In subcellular fractionation, She2p-Tgb3(25-52)-GFP accumulates in fraction P6 that contains ER as shown by the presence of the ER Marker Dpm1p. In contrast, Tgb3(25-52)-GFP is mainly detected in a cytosolic fraction (S200) as indicated by the presence of phosphoglycerokinase (Pgk1p).
Fig. 1 (Genz et al.)

A

60%

Sec61p

She2p

18%

B

60%

Sec61p

She2p

(L130Y)

18%
Fig. 2 (Genz et al.)

A

[Graph showing protein signal (% of total) for She2p and Sec61p across different pellets]

B

[Graph showing protein signal (% of total) for She2p(S120Y) and Sec61p across different pellets]

C

[Graph showing protein signal (% of total) for She2p(ΔhelixE) and Sec61 across different pellets]
Fig. 3 (Genz et al.)

A

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<tr>
<td>Pel</td>
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Binding Assay:
- Sec61p
- She2p
- GST

B

% bound She2p (of input)

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C

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<td>Mcr1p</td>
<td>Inp</td>
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Western Blot:
- She2p
- Sec61p
- Mcr1p
### A

**Binding Assay**

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### B

**She2p (%) of input**

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Fig. 5 (Genz et al.)

A

[Image: Gel electrophoresis results showing bands for She2p, OM45p, and GST with input samples and floated or non-floated liposomes.]

B

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C

[Bar graph showing liposome-bound protein (a.u.) for She2p, OM45p, and Mim1p at different average liposome diameters (nm).]

D

[Bar graph showing liposome-bound She2p (a.u.) at different DOPC content of lipid mix (%).]

- Fig. 5 (Genz et al.)
- Panel A: Gel electrophoresis results showing bands for She2p, OM45p, and GST with input samples and floated or non-floated liposomes.
- Panel B: Table showing protein expression under different conditions.
- Panel C: Bar graph showing protein binding at varying liposome diameters.
- Panel D: Bar graph showing protein binding at different DOPC content of lipid mix.

Legend:
- She2p
- OM45p
- Mim1p

Av. liposome diameter (nm): 30, 80, 200, 400

DOPC content of lipid mix (%): 0, 20, 40

Liposome size: 30 nm, 80 nm
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She2p

ASH1 E3

Fig. 6 (Genz et al.)
Fig. 7 (Genz et al.)

A

Tgb3(25-52)-GFP  MS2CP-Tgb3(25-52)-GFP  She2p-Tgb3(25-52)-GFP

B

DIC  Sec63p-mCherry  She2p-Tgb3(25-52)-GFP  overlay

C

She2p-Tgb3(25-52)-GFP  Tgb3(25-52)-GFP  Dpm1p  Pgk1p

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