Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex

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SECRETORY-protein translocation into the endoplasmic reticulum (ER) is thought to be catalysed by integral membrane proteins. Genetic selections uncovered three Saccharomyces cerevisiae genes (Sec61, Sec62 and Sec63), mutations in which block import of precursor proteins into the ER lumen in vivo1 and in vitro2–4. The DNA sequences of SEC62 (ref. 4) and SEC63 (ref. 5) predict multispanning membrane proteins, and biochemical characterization of the SEC62 protein (Sec62) confirmed that it is an integral ER membrane protein5. Here we show that Sec61, Sec62 and Sec63 are assembled with two additional proteins into a multisubunit membrane-associated complex. These results confirm previous predictions, based upon genetic interactions between the SEC genes, that Sec61, Sec62 and Sec63 act together to facilitate protein translocation into the ER.

To test whether Sec62 is physically associated with other components of the ER membrane, we devised conditions for the efficient extraction of Sec62 from crude membrane fractions by monitoring the solubilization of invertase activity with a bifunctional Sec62–invertase hybrid protein (data not shown). Solubilization conditions that reproducibly extracted Sec62–invertase (see Fig. 1 legend) were also effective for wild-type Sec62. Detergent-solubilized membrane fractions prepared from radiolabelled cells were treated with the thiol-reversive cross-linking agent diitho-bis-(succinimidylpropionate) (DSP) and immunoprecipitated under denaturing conditions with either of two distinct anti-Sec62 IgG preparations, then the crosslinker was cleaved with dithiothreitol and the proteins evaluated by SDS-PAGE. As expected, samples treated with inactivated DSP contained a polypeptide of relative molecular mass 30,000 (M, 30K) corresponding to Sec62 (Fig. 1a). When active DSP was added, Sec62 and four additional polypeptides (73K, 31.5K, 23K and 41K) were reproducibly precipitated by either antibody. The 73K and 41K polypeptides comigrated exactly with Sec63 and Sec61 immunoprecipitated from non-crosslinked, SDS-denatured membranes. The 41K polypeptide was identified as Sec61 as it migrated as a ~39K species in complexes isolated from cells expressing a functional, truncated version of Sec61 lacking the C-terminal 17 amino acids (data not shown).

A similar cohort of polypeptides (minus Sec61) was co-immunoprecipitated with Sec62 under native, but not under denaturing, conditions (Fig. 1b). The absence of Sec61 from native immunoprecipitates suggests that its association with the complex is labile. As association of Sec61 with the complex was found to be variable, the stable 73K, 31.5K, 30K, 23K aggregate is referred to as the Sec62/Sec63 complex. A 50K species precipitated by anti-LacZ-Sec62 IgG (lane 10) was probably an irrelevant contaminant, as it was absent from immunoprecipitates prepared with anti-protein A–Sec62 IgG (lane 8), and was detected in precipitates prepared from sec62-1 membranes, even though the other components of the complex were absent (data not shown). The 73K species that co-immunoprecipitated with Sec62 was directly identified as Sec63 by sequential immunoprecipitation with anti-Sec62 IgG followed by anti-Sec63 serum (Fig. 1c).

The physical state of the Sec proteins in detergent-solubilized membrane extracts reflects the behaviour of these proteins in the ER membrane, as similar multisubunit complexes containing Sec61, Sec62 and Sec63 were detected by treating intact membranes with DSP followed by immunoprecipitation with either anti-Sec62 IgG (data not shown) or anti-Sec63 IgG (Fig. 2, lanes 1 and 2). Compared with results obtained with anti-Sec62 IgG, complexes immunoprecipitated with anti-Sec63 IgG contained more Sec63 and more of the 31.5K and 23K proteins. This

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Fig. 1. Identification of a membrane-associated multisubunit Sec protein complex. a. Detergent-solubilized membranes from radiolabelled cells were incubated for 20 min with the crosslinking reagent DSP. Lane 1, samples were treated with previously inactivated DSP at 4°C; lanes 2 and 3, crosslinking reactions were conducted with active DSP at 4°C and 20°C, respectively. Crosslinking reactions were quenched, subjected to immunoprecipitation with affinity-purified anti-Sec62 IgG and analysed by SDS-PAGE as described below. Lanes 4 and 5, Sec63 and Sec61 immunoprecipitated from SDS-denatured membranes with anti-Sec63 (D.F., J. Rothblatt and R.S., submitted) and anti-Sec61 (prepared by immunization with the C-terminal peptide CLVPFSDF; provided by C. Stirling) serum. b. The same material used in part a was either denatured with SDS before immunoprecipitation (+SDS, lanes 6 and 7), or immunoprecipitated under native conditions (lanes 8 and 10) with affinity-purified IgG elicited against a protein A–Sec62 (lanes 6 and 8) or a LacZ–Sec62 (lanes 7 and 10) hybrid protein5. Lanes 9 and 11, aliquots of the same samples as lanes 4 and 5. Asterisk refers to a contaminant precipitated by the anti-LacZ-Sec62 IgG. c. Immunocomplexes formed under native conditions with anti-protein A–Sec62 antibodies were directly analysed by SDS-PAGE (lane 12), or subjected to secondary precipitation with anti-sec62 (lane 13) and anti-sec63 (lane 14) sera. METHODS. Wild-type strain BF-1 (ref. 6) was radiolabelled with Tran-35S-label (30 μCi per 107 cells) for 60 min at 30°C and spheroplast homogenates were prepared by agitation with glass beads in lysis buffer (0.2 M mannitol, 0.1 M NaCl, 25 mM sodium phosphates, pH 7.4, 1 mM MgCl2, 1 mM PMSF, 10 μM leupeptin and pepstatin (as described7,8). The homogenate was centrifuged for 4 min at 10,000 g followed by 12 min at 50,000 g. The final pellet was washed with lysis buffer, resuspended in lysis buffer (minus leupeptin and pepstatin) containing 10% glycerol (QLB), and adjusted to 1% Triton X-100. After 25 min at 4°C, unsoibilized material was sedimented for 30 min at 14,800g. For crosslinking, the 14,800g supernatant was supplemented with 0.02 volumes 10 mg ml−1 DSP (Pierce) in dimethylsulfoxide. Reactions were terminated by adding 1 volume 0.2 M ammonium acetate, incubated on ice for 10 min, adjusted to 1% SDS, heated at 65°C for 10 min and processed for immunoprecipitation with anti-LacZ-Sec62 IgG (ref. 6) as described9. For native immunoprecipitation, 6–10 × 106 c.p.m. of the 14,800g supernatant was diluted tenfold with GLB (GLB containing 1% Triton X-100), supplemented with 40 μl of a 10% suspension of fixed Saccharomyces cerevisiae cells and centrifuged 5 min at 1,000g. The supernatant was treated with antibodies, incubated 2–4 h at 4°C and centrifuged for 5 min at 10,000g to remove denaturated proteins. Immunocomplexes were collected on protein A-Sepharose CL-4B beads (1.5 h at 4°C) and washed six times with ice-cold GLB. Washed precipitates (of crosslinked and native samples) were resuspended in SDS-PAGE sample buffer supplemented with 50 mM DTT, heated for 10 min at 65°C and subjected to SDS-PAGE on 12.6% polyacrylamide gels followed by autoradiography.
difference may arise from the relative abundance of the Sec proteins. Quantitative immunoprecipitation of Sec61, Sec62 and Sec63 from radioactively labelled membranes indicated that Sec62, Sec63 and Sec61 exist in the membrane in the molar ratio of 1:5:14 (Fig. 2, lanes 3–5). Quantitation of radioactivity precipitated by anti-Sec62 antibodies indicated that Sec62 comprises −0.015% of newly synthesized membrane protein. The relative paucity of Sec62 in complexes detected with anti-Sec63 IgG, and the presence of Sec61 in solubilized preparations treated with either antibody suggest that Sec63 and the 31.5K and 23K proteins can exist as a stable complex lacking both Sec61 and Sec62. The dynamic assembly states of these Sec proteins may reflect cycles of subunit association and dissociation during the actual translocation event.

Digestion of immunopurified complexes with endoglycosidase H indicated that the 31.5K subunit is N-glycosylated, suggesting that it is either a transmembrane or luminal polypeptide of the ER (Fig. 3). The molecular weights of gp31.5 and the 23K polypeptide (p23) are reminiscent of those reported for the two subunits (34K and 23K (refs 7–9)) of the dog pancreas microsomal membrane signal-sequence receptor. Like glycoprotein 31.5, the 34K subunit of this receptor contains asparagine-linked oligosaccharide.

The observation that yeast Sec proteins required for translocation are assembled into a complex is consistent with the view that a multisubunit protein ‘machine’ catalyses the transfer of secretory precursor proteins across the ER membrane. Complexes containing the Sec proteins may constitute the bulk of the membrane-associated portion of the translocon, or may be subcomplexes of the translocon holoenzyme. We previously suggested that SEC61, SEC62 and SEC63 encode interacting proteins on the basis of genetic interactions observed between these loci. The observations documented here validate the usefulness of synthetic lethality and high copy-number suppression in identifying genes encoding affiliated polypeptides.

A subdomain of Sec63 is homologous to an amino-terminal segment of the Escherichia coli DnaJ protein, which collaborates with the Hsp70-like DnaK protein to promote activation of bacteriophage lambda DNA replication. Certain alleles of the yeast KAR2 gene, which encodes an ER luminal Hsp70 homolog (known as BiP) involved in translocation, display synthetic lethality with sec63-1 (M. Rose, personal communication). We propose that Kar2 acts reversibly through Sec63 to modify the composition of the Sec62/Sec63 complex, or to catalyse the association/dissociation of the complex with other factors, including Sec61, cytoplasmic Hsp70 (refs 18, 19) and components of a putative cytoplasmic signal sequence receptor. The identification of a complex of Sec proteins involved in translocation provides a focus for future biochemical reconstitution.

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