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Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression

Jeremy D.Brown, Byron C.Hann[1], Katalin F.Medzihradszky[2], Maho Niwa, A.L.Burlingame[2] and Peter Walter

Departments of Biochemistry and Biophysics and [2]Pharmaceutical Chemistry, University of California at San Francisco, San Francisco, CA 94143-0448, USA

[1]Present address: CRC Laboratories, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK

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The signal recognition particle (SRP) is an evolutionarily conserved ribonucleoprotein (RNP) complex that functions in protein targeting to the endoplasmic reticulum (ER) membrane. Only two protein subunits of the SRP, Srp54p and Sec65p, and the RNA subunit, scR1, were previously known in the yeast *Saccharomyces cerevisiae*. Purification of yeast SRP by immunoaffinity chromatography revealed five additional proteins. Amino acid sequencing and cloning of the genes encoding four of these proteins demonstrated that the yeast SRP contains homologs (termed Srp14p, Srp68p and Srp72p) of the SRP14, SRP68 and SRP72 subunits found in mammalian SRP. The yeast SRP also contains a 21 kDa protein (termed Srp21p) that is not homologous to any protein in mammalian SRP. An additional 7 kDa protein may correspond to the mammalian SRP9. Disruption of any one of the four genes encoding the newly identified SRP proteins results in slow cell growth and inefficient protein translocation across the ER membrane. These phenotypes are indistinguishable from those resulting from the disruption of genes encoding SRP components identified previously. These data indicate that a lack of any of the analyzed SRP components results in loss of SRP function. ScR1 RNA and SRP proteins are at reduced levels in cells lacking any one of the newly identified proteins. In contrast, SRP components are present at near wild type levels and SRP subparticles are present in cells lacking either Srp54p or Sec65p. Thus Srp14p, Srp21p, Srp68p and Srp72p, but not Sec65p or Srp54p, are required for stable expression of the yeast SRP.

Key words: endoplasmic reticulum/ribonucleoprotein/secretion/signal sequence

Introduction

Mammalian signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein (RNP) particle that couples the synthesis of membrane and secretory proteins to their translocation across the endoplasmic reticulum (ER) membrane. *In vitro* studies have shown that SRP binds to hydrophobic signal sequences at the N-terminal end of nascent preproteins and causes a slowing of protein synthesis termed elongation arrest. SRP then targets the nascent chain–ribosome complex to the ER membrane via interaction with a membrane-bound SRP receptor (SR). After this docking event, the signal sequence is displaced from SRP in a GTP-dependent reaction, and translocation of the polypeptide into the ER then takes place co-translationally through a protein pore, the translocon (reviewed in Walter and Lingappa, 1986; Nunnari and Walter, 1992; Gilmore, 1993).

SRP purified from canine pancreas is composed of a 7S RNA (7SL RNA, here referred to as SRP RNA) and six proteins (Walter and Blobel, 1980, 1982). The SRP proteins (designated by size in kDa) are associated with the RNA as either monomers (SRP19 and SRP54) or heterodimers (SRP9/14 and SRP68/72), and cDNAs encoding them have been cloned (Lingelbach *et al.*, 1988; Bernstein *et al.*, 1989; Herz *et al.*, 1990; Römisch *et al.*, 1989; Strub and Walter, 1989, 1990; Lütcke *et al.*, 1993). SRP54 is the best characterized component of SRP. It has been demonstrated to interact with both the SRP RNA and signal sequences, and to bind GTP (Römisch *et al.*, 1990; Zopf *et al.*, 1990; High and Dobberstein, 1991; Miller *et al.*, 1993).

Less is known about the other protein subunits of SRP, and their sequences give few clues as to their functions. Most information on the roles of these proteins has come from studies carried out using SRP reconstituted with either a subset of the SRP proteins or components modified with *N*-ethyl maleimide (NEM). These studies indicated that SRP19 is required for the association of SRP54 with SRP RNA, that SRP9/14 is required for elongation arrest and that modification of SRP68/72 by NEM prevents strong interaction of SRP with SR and inhibits translocation-promoting activity (reviewed in Siegel and Walter, 1988a).

Homologs of mammalian SRP components have been identified in both *Escherichia coli* and yeast species. The homolog of mammalian SRP54 in *Saccharomyces cerevisiae*, Srp54p, was identified using the PCR with degenerate oligonucleotides based on conserved regions between the mammalian and *E.coli* proteins (Hann *et al.*, 1989). This protein has been demonstrated to be in a complex with a major yeast cytoplasmic RNA, scR1, and to be important for targeting proteins to the ER (Hann and Walter, 1991). In addition to Srp54p, homologs of SRP19 (Sec65p) and of both subunits of SR have been identified in yeast (Ogg *et al.*, 1992; Stirling and Hewitt, 1992; S.C.Ogg, J.D.Miller and P.Walter, unpublished results). Sec65p was identified through a temperature-sensitive mutation (*sec65-1*) isolated in a genetic screen

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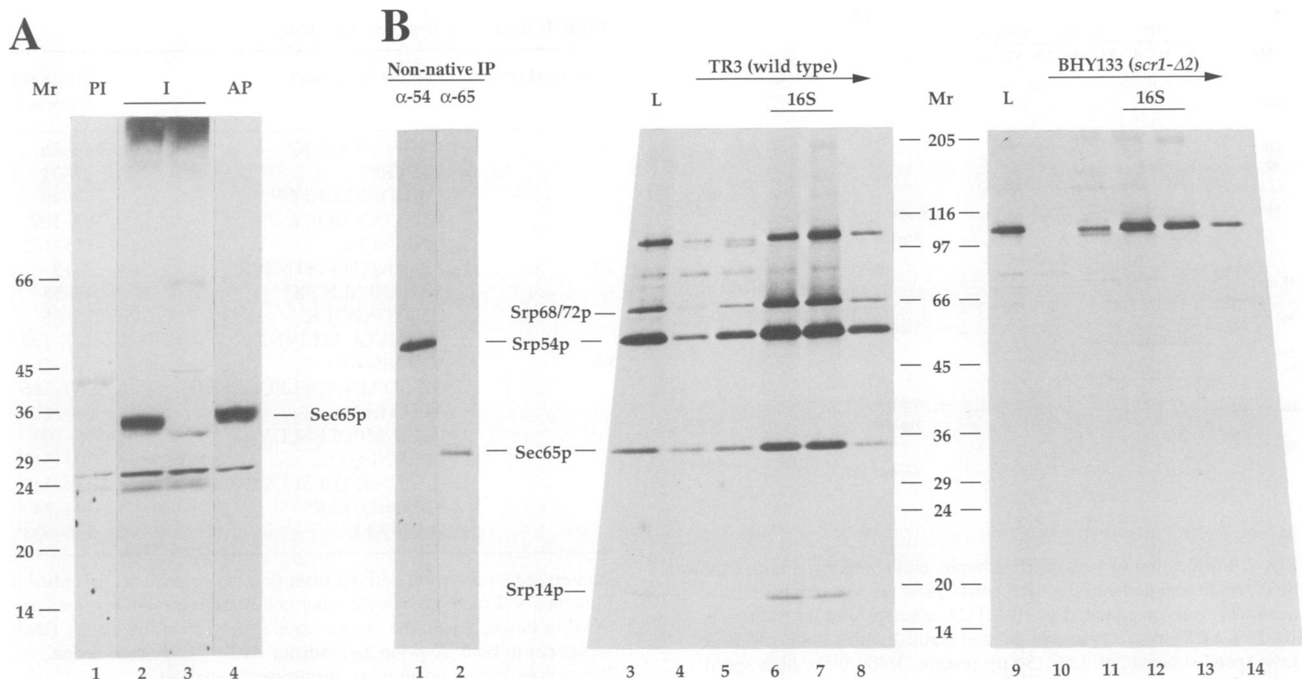

Fig. 1. Characterization of anti-Sec65p antibodies and immunoprecipitation of SRP proteins. (**A**) Western blot. Yeast whole cell extracts were electrophoresed through a 10–15% SDS–polyacrylamide gel, blotted to nitrocellulose and probed with antibodies as indicated. Lanes 1, 2 and 4 contain extract from strain TR3 (wild type); lane 3 contains extract from strain CSY186 (*sec65Δ*). Bound antibodies were visualized by enhanced chemiluminescence (Amersham Corporation). PI, pre-immune; I, immune; AP, affinity-purified. (**B**) [^{35}S]Methionine-labeled extracts from either strain TR3 (lanes 3–8) or strain BHY133 (*scr1-Δ2*; lanes 9–14) were fractionated by DEAE–Sepharose chromatography and sucrose gradient sedimentation. Native immunoprecipitations with affinity-purified anti-Sec65p antibodies were then carried out on either the sucrose gradient load fractions (lanes 3 and 9) or fractions from the 16S region of the gradient (lanes 4–8 and 10–14), as described in Materials and methods. Lanes 1 and 2 are immunoprecipitations with affinity-purified anti-Srp54p and anti-Sec65p antibodies of SDS-denatured proteins from the TR3 sucrose gradient load fraction. Immunoprecipitated material was electrophoresed through a 10–15% SDS–polyacrylamide gel and visualized by autoradiography. SRP proteins are labeled by their homology to mammalian SRP components except for Sec65p, the yeast SRP19 homolog. Sizes of molecular weight markers (in kDa), the direction of sedimentation in the sucrose gradients and the position of SRP (16S) are indicated.

for mutants defective in protein transport (Stirling *et al.*, 1992). To date, Sec65p is the only SRP component identified in such a genetic screen. Yeast SRP has a 16S sedimentation coefficient (Hann and Walter, 1991). 16S is greater than would be expected for a particle containing only Srp54p, Sec65p and the scR1 RNA, making it likely that yeast SRP contains other protein components.

In both yeast and *E.coli*, loss of SRP function affects the processing of individual preproteins to varying extents (Hann and Walter, 1991; Phillips and Silhavy, 1993). This, together with the unexpected observation that yeast SRP and SR components are not essential for cell viability (Hann and Walter, 1991; Stirling and Hewitt, 1992; Ogg *et al.*, 1992; S.C.Ogg and P.Walter, unpublished results), indicates that at least two distinct, but overlapping, targeting systems function in these organisms. In wild type cells, however, SRP may target most preproteins.

We have purified and characterized components of the yeast SRP to understand better the role of SRP in protein targeting in yeast, to enable a more detailed analysis of SRP protein functions and to allow a comparative analysis with the mammalian SRP. We have identified the genes encoding four novel yeast SRP proteins and our results demonstrate that the protein composition of SRP is mostly conserved from yeast to mammals. We also show that the function and/or stable assembly of the particle *in vivo* relies on the presence of all analyzed subunits.

Results

Antibodies against Sec65p immunoprecipitate novel SRP components

Yeast SRP contains Sec65p, a protein homologous to mammalian SRP19 (Stirling and Hewitt, 1992). We generated polyclonal antibodies directed against a glutathione-S-transferase (GST)–Sec65p fusion protein (see Materials and methods). On a Western blot of total yeast extract (Figure 1A) the immune serum (lane 2), but not the preimmune serum (lane 1), recognized a 34 kDa protein not present in extract from cells lacking the *SEC65* gene (lane 3). After affinity purification (lane 4) the antibodies decorated Sec65p, as well as one minor species which was also recognized by antibodies in the preimmune serum.

To identify previously uncharacterized protein components of yeast SRP, the affinity-purified anti-Sec65p antibodies were used in native immunoprecipitations from ^{35}S-labeled extracts of either wild type yeast cells (strain TR3) or cells lacking scR1 RNA because the *SCR1* gene was deleted (strain BHY133). To enrich for SRP, yeast cell extracts were subjected to fractionation on DEAE–Sepharose followed by sucrose gradient sedimentation (see Materials and methods). Yeast SRP sediments at 16S in sucrose gradients and scR1 RNA is required for integrity of the particle (Hann and Walter,

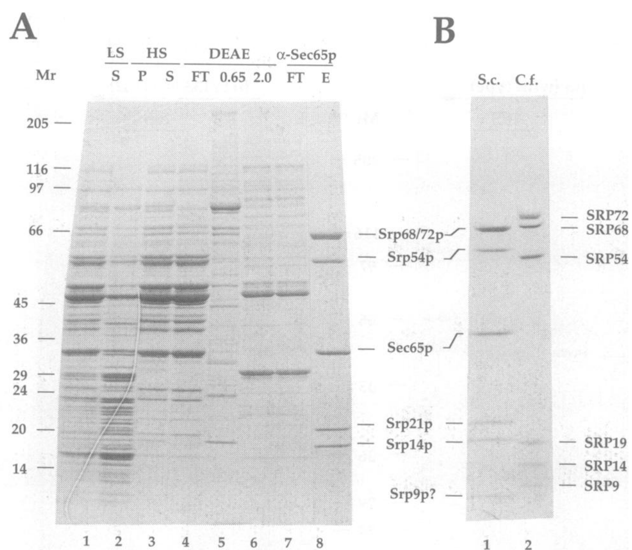

Fig. 2. Purification of yeast SRP subunits and comparison with canine SRP. **(A)** Fractions from the SRP purification (see Materials and methods) were precipitated in 10% TCA, electrophoresed through a 10–15% SDS–polyacrylamide gel and stained with Coomassie Blue. Low speed supernatant, LSS (50 μg protein, 1/400 000); high speed pellet, HSP (40 μg protein, 1/80 000); high speed supernatant, HSS (45 μg protein, 1/260 000); DEAE flow through fraction, FT (40 μg protein, 1/260 000); 0.65 M KOAc DEAE wash fraction, 0.65 (23 μg protein, 1/52 000); 2.0 M KOAc DEAE elution fraction, 2.0 (23 μg protein, 1/8000); anti-Sec65p immunoaffinity column flow through fraction, FT (23 μg protein, 1/8000); anti-Sec65p immunoaffinity column pH 2.0 elution fraction, E (3.5 μg protein, 1/75). Sizes of molecular weight markers (in kDa) are indicated. **(B)** Purified yeast (S.c.) and dog (C.f.) SRP subunits were electrophoresed through a 10–15% SDS–polyacrylamide gel and stained with Coomassie Blue. Samples prepared for analysis in this panel were not precipitated with TCA but were added directly to the SDS sample buffer.

1991). Thus, the 16S region of gradients on which BHY133 (*scR1*Δ) cell extract has been fractionated is devoid of SRP and provides a control for immunoprecipitation of non-specific proteins. In addition to Sec65p and Srp54p proteins (identified by comigration of bands in non-native immunoprecipitations), novel species of 19 and 66 kDa were immunoprecipitated from 16S fractions of a gradient through which wild type cell extract was sedimented (Figure 1B, compare lanes 1 and 2 with lanes 4–8). Consistent with these being SRP proteins, they were not observed in immunoprecipitations from 16S fractions of a gradient on which extract from BHY133 (*scr1*Δ) cells had been analyzed (lanes 10–14). The same specific protein bands immunoprecipitated by anti-Sec65p antibodies were also immunoprecipitated by antibodies against Srp54p, providing further evidence for the association of these proteins in a complex (not shown). As will be shown below, the 66 kDa band is a doublet corresponding to yeast Srp68p and Srp72p subunits, and the 19 kDa band corresponds to the yeast Srp14p homolog.

Purification and cloning of yeast SRP components

Sufficient amounts of yeast SRP proteins for peptide sequencing were purified by fractionating yeast post-ribosomal supernatant by DEAE–Sepharose chromatography followed by immunoaffinity chromatography using affinity-purified anti-Sec65p antibodies (see Materials and methods). A summary of the purification is shown in Figure 2A. The final fraction (lane 8) consisted of six proteins in approximately equimolar amounts: a doublet of bands migrating at 66 kDa (Srp68p and Srp72p) and single bands at 60 (Srp54p), 34 (Sec65p), 21 (Srp21p) and 19 kDa (Srp14p). The identification of an additional protein, Srp21p, in this purification, not seen in the immunoprecipitations of [^{35}S]methionine-labeled proteins, suggested that it lacked methionine residues and was therefore not visible in Figure 1B. This was confirmed by analysis of the protein sequence (see below).

The specificity of purification of SRP proteins was verified by subjecting aliquots of the immunoaffinity column load and flow through fractions to sucrose gradient sedimentation, and examining the gradient fractions around the 16S peak. Proteins corresponding in size to those eluted from the anti-Sec65p IgG column (including Srp21p) were observed in the 16S fraction of the load but not the flow through gradient, indicating that this set of proteins had been quantitatively depleted from the flow through fraction (data not shown). We concluded that we had succeeded in purifying six yeast SRP protein subunits.

Figure 2B shows a comparison of the purified yeast proteins with canine SRP proteins. Surprisingly, in this experiment an additional protein of ~7 kDa was observed in the yeast SRP sample (Figure 2B, lane 1) over those seen previously (Figure 2A, lane 8). This was subsequently found to be a species in the immunoaffinity column eluate that was trichloroacetic acid (TCA)-soluble (samples shown in Figure 2A were precipitated in 10% TCA prior to loading) and may correspond to the yeast homolog of mammalian SRP9 (see Discussion).

Peptide sequences were generated from four of the novel SRP proteins to allow identification of genes encoding them. Material eluted from the anti-Sec65p IgG

Table I. Results of peptide sequencing

Protein (kDa)	Peptide sequences	Position in sequence
19	VPEFFQTANEK[a]	16–26
	HITVR[a]	27–31
	LIEHDPVEGNLEF...	37–49
	ASELDQF**R**QEY...[b]	99–109
	QNMNLIK[a,c]	115–122
21	AcSVKPIDNYITNSVR[a]	2–15
	THNSHLSLNYK[a]	45–55
	GVSITPGKIEK[a]	75–85
	SIQDIVGLATLIVN...	107–120
66	VTTNINWR[a]	256–263[e]
	(**I**/S)IDAEVAQFLEQGLSIXP...	269–286[e]
	ALHYLK[a]	66–71[f]
	(S/**L**)NMPDEFN(**T**/V)L...	95–104[f]
	LNTFNLQTF...	303–311[f]
	(L/**I**)HS(K/**Q**)ESLLSR[a]	334–343[f]
	TFD(**K**/Q)YSK[a]	344–349[f]
	DIDI**F**KLI...[d]	526–533[f]

Peptide sequences were derived from isolated proteins as described in Materials and methods. Single-letter codes are used. Ambiguities are listed in brackets with the correct amino acid (according to the DNA sequence) in bold. X = no assignment; AcS = acetylated serine.
[a]Sequences from tandem mass spectrometry analysis.
Two of the peptide sequences derived from Srp14p differed from the amino acid sequence obtained by translating the DNA sequence, as did one of the peptides derived from Srp72p. Correct sequences are: [b]ASELDQFWQEY; [c]**GGM**QNLIK; [d]DIDI**D**KLI.
Assignments of peptide sequences from the two 66 kDa proteins are from the translated ORFs: [e]Srp68p; [f]Srp72p.

A Srp21p

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Srp21p  MSVKPIDNYITNSVRLFEVNPSQTLFSIRYKPPTQKTDTKVSFRTHNSHLSLNYKFTTNKSKDVSRLLSA   70
Srp21p  LGPRGVSITPGKIEKIAQSKKKNNKIKESSKKIKGKSIQDIVGLATLIVNTDVEKSDPAAKKTTAEPKQK  140
Srp21p  AHAVQNNNGNSAASKKKKNKNKGKKKR                                              167
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B Srp14p

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S.c.  MANTGCLSPGAFLSKVPEFFQTANEKHITVRLTAKRLIEHDPVEGNLEFDSTNHPDYDVSKKASEISVSSRSD  73
M.m.     MVLLESEQFLTELTRLFQKCRSSG-SVFITLKK------------YDGRTKP---IPRKSSVEGLEPAEN  54
S.c.  REYPLLIRMSYGSHDKKTKCSTVVKASELDQFWQEYSSVFKGGMQNLIKK-KKKKSKNGTISKTGKKNKVAKKN 146
M.m.  KC---LLRAT----DGKRKISTVVSSKEVNKFQMAYSNLLRANMDGLKKRDKKNKSKKSKPAQ           110
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C Srp68p

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S.c.                                                MVAYSPIIATYGNRAEQFLETDSDFA  26
C.f.  MAAEKQVPGGGGGGGGSGGGRGAGGEENKENERPSAGSKANREFGDSLSLEILQIIKESQQQHGLRHGDFQ  72
S.c.  KYHAKLNKKLQHLRSRCHLVTKDTKKYSSKNKYGEINSEDYDNKTKLIGVLILLHAERDLALAETLKLRARQ  98
C.f.  RYRGYCSRRQRRLRKTLNFKMGNRHKFTGKKVTEDLLTDN------RYLLLVLMDAERAWSYANQLKQEANT 138
S.c.  RGKLKKSEEKVLSTRLKKACKTADKLVNVTQNEQQWITRAQYLAFAKLVHSEYLINGKRFKRKD-----NAK 167
C.f.  EPRKRFH----LLSRLRKAVKHAEELERLCESNRVDAKTK----LEAQAYTAYLSGMLRFEHQEWKAAIEAF 202
S.c.  ISNNLALVFAALEHLKNLSLLAEEVVDNIVNKYQYSLKQYAGNLITTPEINNFIVEKVQSDENKDDELVKLL 237
C.f.  NKCKTIYEKLASAFTEEQAVLYNQRVEEISPNIRYCAYN-IGDQSAINELMQMRLRSGGTEGLLAEKLEALI 273
S.c.  LDNGFNNKKITTSTEDQKVTTNINW---RSFNAKIIDAEVAQFLEQGLSIHPTQITQYTQRLSKLEKALD-R 305
C.f.  TQTRAKQAATMSEVEWRGRTVPVKIDKVRIFLLGLADNEAA--IAQAESEETKERLFESMLSEC-RDAIQAV 342
S.c.  HEFFIANHDDQDDIDEMVENSSENNQIILAYIKYNILLTSISRERDLFTHLWNQWLKLNTSLPSKLTKYKEM 377
C.f.  REELKPDQKQRDYTLDGESGKVSNLQYLHSYLTYIKLSTAIRRNENMAKGLQKALQQQPEDESKRSPRPQDL 414
S.c.  ERIVKNLTKYLSDIMELPGVYSDDELLSQLDLCKLYFQLFLNTGCLSVLYQSKGRYNEALALYVDAYRRLEN 449
C.f.  IRLYDIILQNLVELLQLPGLEEDRAFQKEIGLKTLVFKAY-RCFFIAQSYVLVKKWSEALVLYDRVLKYANE 485
S.c.  KLSEIESLDEILLPANLLSLNSVRSLQKRIENGGNSVITLAEYEKRN-HGGSLGKYDLTVIEKLDSKKILPT 520
C.f.  VNSDAGAFRNSL--KDLPDVQELITQVRSEKCSLQAAAILDASDSHQPETSSQVKDNKPLVERFETFCLDPS 555
S.c.  DI-QLKNLFPLKPKNLPIPSKPTLFDLAFNYITYDKQEPSASQVKDSVTETESISQTPISNEQTEGEPKKHR 591
C.f.  LVTKQANLVHFPPGFQPIPCKPLFFDLALNHVAFPPLEDKLEQKTKSGLTGYIKGIFGFRS            616
S.c.  GFLGLFGR                                                                 599
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D Srp72p

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S.c.  MKESKKMAKDNLTNLLSQLNIQLSQDEHSQVEQTCVKLLDSGCENPADVFRRC-LVAVIQQDKYQKALHYLK  71
C.f    MASGGSGGVSVPALWSEVNRYGQNGDFTRALKTVNKILQINKDDVTAL--HCKVVCLIQNGSFKEALNVIN  69
S.c.  KFKHIDDKYGRKFALEKLYIFYKLNMPDEFNTLYTAIITDDLDTVLKKDIESLRGILHVRAQYCYKNGLYQE 141
C.f.  THTKVLANNSLSF--EKAYCEYRLNRIE--NALKTIESANQQTDKLK----------ELYGQVLYRLERYDE 127
S.c.  AFKIYQHLASHNEKDQDSQIELSCNERVPLSVATELMNRSPLVTPMDESSYDLLFNESFIMASVGKYDKAIE 215
C.f.  CLAVYRDLVRNSQDDYDE--ERKTNLSAVVAAQSNWEKVVPENLGLQEGTHELCYNAACALIGQGQLSQAMK 197
S.c   LLEKALQ----------GATNEGYQNDINTIKLQLSFVLQMVGKTAQSKEILKGLLQELKADSPFSLICQNN 277
C.f   ILQKAEDLCRRSLSEDSDGTEEDPQAELAIIHGQMAYILQLQGRTEEALQLYNQIIK-LKP-TDVGLLAVIA 267
S.c.  LNAFVDFSKYNTNFNLLLRELNVEKLNTFNLQTFTHEQWSNIQRNVLFLRLFNNVKIHSQE---SLLSRTFD 346
C.f.  -NNIITINKDQNVFDSK-KKVKLTNAEGVEFK-LSKKQLQAIEFNKALLAMYTNQAEQCRKISASLQSQSPE 336
S.c.  KY-SKLVDNVTLESYKTQAK--KLYHHTTKTILSGTDGSTIGILLLTIQLLIIEKEWENAIRIGELFLNESW 415
C.f.  HLLPVLIQAAQLCREKQHTKAIELLQEFSDQHPENAAEIKLTMAQLKISQGNISKACLILRSIEELKHKPGM 408
S.c.  KSSF-EKFNDSQAIVCYI-----LFELYKIKGRNNSKSV-LLKKLGSVRVQLSGKIQENIPFWKHVGFELLS 480
C.f   VSALVTMYSHEEDIDSAIEVFTQAIQWYQNHQPKSSAHLSLIREAANFKLKY-GRKKEAIS-----DLEQLW 474
S.c.  MGNAKESKALLREISNFSKGDADVLVDRVVSSDSLDIAQGIDLVRDIDIDKLIQLGVKPLESSAKRSKNTAV 552
C.f.  KQNPKDIHTLAQLISAYSLVDPE--KAKALSKH-LPSSDSMSL--KVDVEAL-ENSPGATYIRKKGGKVAGD 540
S.c.  SKVQKRKVLELKKKRKIKRLEKFLQGRDTSKLPDPERWLPLRDRSTYRPKKKQQGAK------QTQGGAMNK 618
C.f.  SQPKEQGQGDLKKKKKKKK-GKLPKNYDPKVTPDPERWLPMRERSYYRGRKK--GKKKDQIGKGTQGATAGA 609
S.c.  KSEQALDISK-------------------------------KGKPTVNKKPKNKKKGRK              646
C.f.  SSE--LDASKTVSSPPTSPRPGSAATASASTSNIIPPRHQKPAGAPATKKKQQQKKKKGGKGGW         671
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Fig. 3. Sequences of novel yeast SRP proteins and alignment with mammalian homologs. (**A**) Sequence of yeast Srp21p from the YKL527 ORF (Colleaux *et al.*, 1992). (**B–D**) Alignments of the deduced yeast Srp14p, Srp68p and Srp72p protein sequences with their mammalian homologs, respectively. One-letter codes are used throughout; identical residues are indicated by a double dot; amino acids of similar chemical properties (Dayhoff *et al.*, 1972) are indicated by a single dot. Regions of higher homology in Srp68p and Srp72p mentioned in the text are boxed. Amino acid numbers are indicated on the right. Mammalian sequences used are: mouse Srp14p (Strub and Walter, 1989); canine Srp68p and Srp72p (Herz *et al.*, 1990; Lütcke *et al.*, 1993). GenBank accession numbers for the sequences reported in this paper are L35155 (*SRP14*), L35177 (*SRP68*) and L35178 (*SRP72*). In addition to homology to mammalian SRP72, the yeast Srp72p is identical over part of its length to a putative human partial cDNA (GenBank accession number Z15198) in the −3 frame with respect to the proposed ORF. This identifies the sequence as a yeast contaminant in the library from which the clone was sequenced. Also, *SRP72* lies 3′ to the *IPL1* gene (GenBank accession number U07163).

column was precipitated in TCA and electrophoresed through SDS−polyacrylamide gels. Bands were excised from the gels and the proteins then digested *in situ* with trypsin. Eluted peptides were separated by HPLC and their masses determined by electrospray mass spectrometry. Peptides were then sequenced by either Edman degradation or tandem mass spectrometry (see Materials and methods). The results of this analysis are listed in Table I. Note that peptide sequences generated from the two 66 kDa proteins (Srp68p and Srp72p) were from a pool of the two proteins as they were not resolved from each other in the gels used.

Comparison with sequence databases showed that all peptide sequences generated from the 21 kDa protein were identical to sequences within a previously identified open reading frame (ORF) YKL527 on yeast chromosome XI (Colleaux *et al.*, 1992). This protein has no previously ascribed function and no notable sequence motifs except a match to a short motif found in histone H1 proteins and an overall very basic amino acid composition (pI = 11.2). The sequence of this protein is shown in Figure 3A. One of the peptide sequences obtained from tandem mass spectrometry contained an acetylated serine residue at the N-terminus (Table I). This peptide aligns with the N-terminus of the YKL527 ORF minus the initiator methionine suggesting that the protein is N-terminally modified *in vivo*. The YKL527 ORF encodes no other methionine residues and this, in addition to the proposed N-terminal modification of the protein, explains why it was not detected in immunoprecipitations from ^{35}S-radiolabeled extracts (Figure 1B). We have not found any significant homology of the 21 kDa protein to other database entries, and we propose to call it Srp21p, after its apparent size in SDS−polyacrylamide gels, and its gene *SRP21*.

Peptide sequences from the three other proteins did not match database sequences. We therefore used PCR to amplify fragments of the corresponding genes from yeast genomic DNA using degenerate oligonucleotide primers designed from a number of the peptide sequences. PCR products were cloned and sequenced, and those containing the sequences of the oligonucleotides in appropriate ORFs were used as probes to isolate genomic clones for the three genes from yeast genomic DNA libraries (Carlson and Botstein, 1982; see Materials and methods). Sequencing of selected clones identified the complete ORFs and surrounding sequences for all three genes. The ORFs of the three proteins contained all the original peptide sequences (Table I) with few differences, and encoded proteins whose predicted sizes agree well with their apparent sizes in SDS−polyacrylamide gels (Srp14p, 146 amino acids, 16.4 kDa; Srp68p, 599 amino acids, 69 kDa; Srp72p, 646 amino acids, 74.2 kDa, Figure 3B−D). *SRP72* contains two methionine codons at its 5′ end, six amino acids apart. While the second of these has a much better fit to the yeast initiation codon consensus (Hamilton *et al.*, 1987), the first methionine codon has been used for the translation shown in Figure 3D.

We identified the three cloned yeast genes as coding

for homologs of the mammalian SRP14, SRP68 and SRP72 proteins by comparison of the translated ORFs with sequence databases (see Figure 3B–D). By convention, we named the genes and their encoded proteins after the established nomenclature of their mammalian counterparts. Srp14p is 31% identical and 56% similar to the mammalian SRP14 (over the length of the mammalian protein). The degree of conservation rises towards the C-terminus of the proteins where there are a number of conserved lysine residues (48% identity, 67% similarity between amino acids 88 and 129 of the yeast sequence). Srp68p and Srp72p are not as highly conserved, with 18 and 23% identity to the mammalian homologs, respectively. The homology, however, extends over the whole length of the proteins (except for the N-terminal region of mammalian SRP68 which is absent in the yeast protein). Within the Srp68p and Srp72p alignments there are short regions of higher identity (boxed in Figure 3C and D). The higher degree of conservation of these regions suggests functional importance (see Discussion).

From the above results we concluded that we had cloned the genes encoding yeast homologs of three mammalian SRP components and identified a fourth gene encoding a yeast SRP protein, Srp21p, with no known mammalian counterpart.

Yeast Srp14p, Srp21p, Srp68p and Srp72p are required for SRP function and integrity

We investigated the importance of the newly identified SRP proteins for cell growth by performing disruptions of their respective genes. Previous work has shown that the yeast SRP, while important for cell growth and protein translocation into the ER, is not essential for cell viability (Hann and Walter, 1991; Stirling and Hewitt, 1992). We examined four different diploid yeast strains, each of which had one chromosomal copy of one of the newly identified yeast SRP genes deleted (see Materials and methods). Tetrad dissection of these strains yielded results identical to those seen previously for diploid strains heterozygous for SRP component deletions, i.e. of the four meiotic progeny, two were slow-growing and two fast-growing (for example Figure 4A). Analysis of the resulting haploid strains showed that in every case the slower-growing haploids contained the SRP gene deletion (data not shown). The doubling time of strains lacking any one of the four genes was 250–300 min, similar to those of strains lacking SRP components identified previously (Srp54p, Sec65p and scR1). This is ~3-fold slower than the doubling time of an otherwise isogenic wild type strain (Figure 4B).

Lack of SRP function affects processing of different translocation substrates to various extents (Hann and Walter, 1991). To examine the effects of absence of the newly identified SRP proteins on targeting to the ER, we monitored processing of several proteins that enter the secretory pathway through the ER. Strains lacking individual SRP components, a wild type strain and control strains carrying the *sec63-1* and *sec65-1* mutations (after shift to the non-permissive temperature) were pulse-labeled with [^{35}S]methionine and proteins immunoprecipitated from cell extracts. Strains lacking the novel SRP components (Figure 4C, lanes 1, 2, 5 and 6) showed defects similar to those seen in strains deficient in SRP

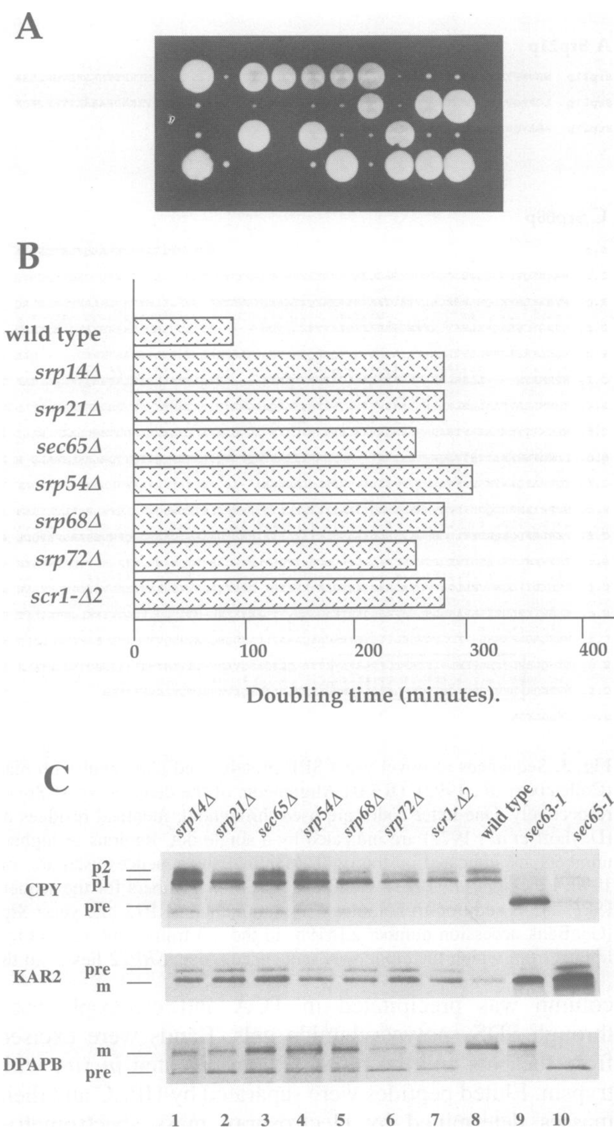

Fig. 4. SRP components are important for cell growth and protein transport. (**A**) Tetrads of yeast strain JDY40 (*SRP21, srp21Δ::HIS3*) dissected onto YEPD medium and incubated at 30°C. (**B**) Growth rates of yeast strains lacking SRP components. Cultures of the strains indicated in YEPD were diluted to maintain logarithmic growth (monitored as relative OD_{600}). (**C**) Effect of lack of SRP components on protein transport. Extracts were prepared from [^{35}S]methionine-labeled cells and immunoprecipitations carried out with antibodies against the indicated proteins as described in Materials and methods. Immunoprecipitated proteins were electrophoresed through 10–15% SDS–polyacrylamide gels and visualized by autoradiography. Strains were: JDY66 (*srp14Δ*), JDY105 (*srp21Δ*), CSY186 (*sec65Δ*), BHY116 (*srp54Δ*), JDY98 (*srp68Δ*), JDY100 (*srp72Δ*), BHY133 (*scr1-Δ2*), TR3 (wild type), RSY151 (*sec63-1*) and RSY458 (*sec65-1*). Precursor forms of proteins (pre), ER and Golgi glycosylated forms of CPY (p1 and p2, respectively) and mature KAR2 and DPAPB (m) are indicated.

components identified previously (lanes 3, 4 and 7). Partial defects were seen in the processing of both KAR2 and dipeptidyl aminopeptidase B (DPAPB) precursors, whereas no defect was seen for carboxypeptidase Y (CPY). These results, combined with the similar growth rates of all the SRP deletion mutants, suggest that yeast SRP function is completely lost when any of these subunits are lacking. The *sec63-1* control strain showed a strong defect in the processing of CPY, no defect in DPAPB processing and

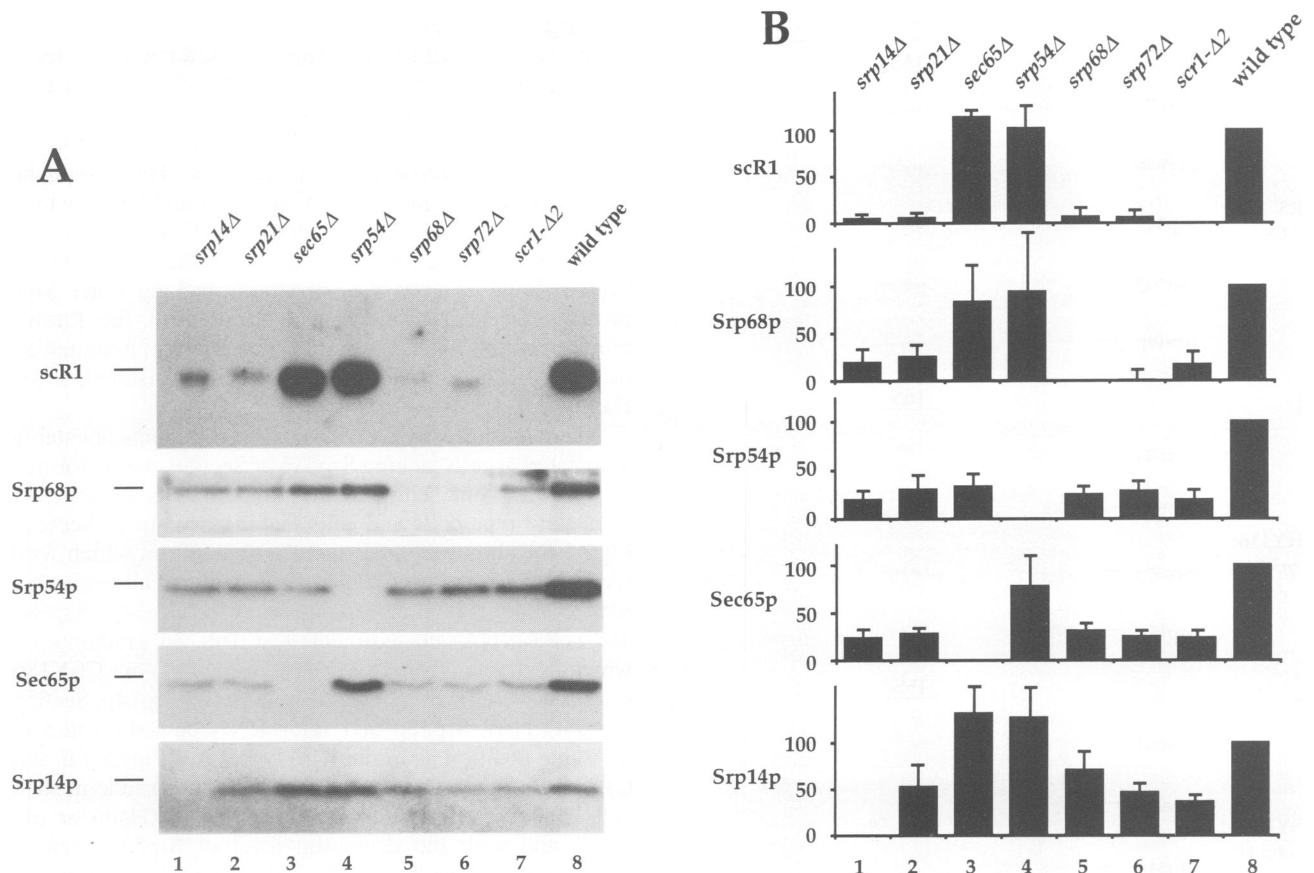

Fig. 5. Levels of SRP subunits in strains lacking individual SRP components. RNA or protein samples were prepared from logarithmically growing cells as described in Materials and methods. To examine scR1 levels, equal amounts of total RNA (assessed by measuring OD_{260}) were denatured, electrophoresed through an agarose gel, blotted to Hybond-N (Amersham) and hybridized with a ^{32}P-labeled DNA fragment of the *scR1* gene. SRP protein levels were assessed by electrophoresing equal amounts of the protein samples from each strain (assessed by Biorad protein assay) through a 10–15% SDS–polyacrylamide gel, blotting to nitrocellulose, cutting the blot into strips and probing the strips (according to the size of the protein being examined) with the antibody indicated. Bound antibodies were visualized by incubating with ^{125}I-labeled secondary antibody and autoradiography. (**A**) Autoradiographs of results of a single experiment. (**B**) Averaged quantitated results of either two experiments (for scR1 levels) or four experiments (for SRP protein levels). Amounts of RNA and proteins are shown relative to wild type as 100% ± 1 standard deviation. Quantitation of both RNA and proteins was carried out by PhosphorImager analysis. Linearity of measurements was ensured by measuring the signal obtained from serial dilutions of the wild type samples. Strains are as in the legend to Figure 4. To verify loading accuracy, RNA blots were hybridized with a probe to U5 snRNA, and protein blots were incubated with an anti-β-tubulin antibody (not shown). SRP component levels were normalized to the U5 or tubulin signals.

only a slight defect in KAR2 processing. These results are consistent with previous observations (Rothbatt *et al.*, 1989; Stirling *et al.*, 1992) and with the proposal that the Sec63p complex may target primarily precursors which use an SRP-independent pathway to the translocation pore (Feldheim *et al.*, 1993).

In vitro analysis of canine SRP indicated that particles reconstituted without SRP9/14 target precursor proteins to ER-derived vesicles, although these particles lack the elongation arrest function (Siegel and Walter, 1985). Since a defect in elongation arrest might also be expected to cause less functional impairment of SRP *in vivo* than loss of other functions, we were surprised to find that deletion of the *SRP14* gene resulted in growth and secretion defects identical to those seen for other deletions. One possible explanation is that loss of individual yeast SRP components affects the stability of the particle as a whole. Thus, we assessed the effect of deficiency of individual SRP proteins on the integrity of SRP by assaying the levels of other SRP components. Analysis of scR1 RNA levels in a wild type strain or in strains lacking SRP components showed that the individual absence of most SRP proteins had a dramatic effect, scR1 RNA appearing to be almost absent (Figure 5A, top panel, lanes 1, 2, 5 and 6). Quantitation (Figure 5B) revealed that the amount of scR1 RNA in cells in which the *SRP14*, *SRP21*, *SRP68* or *SRP72* gene was deleted was ~10-fold less than that in wild type cells. In marked contrast, scR1 RNA was present at approximately wild type levels in strains in which the *SRP54* or *SEC65* gene was deleted (Figure 5A and B, lanes 3 and 4).

We tested if the levels of SRP proteins in wild type yeast and strains lacking individual SRP components showed similar effects using antibodies against Srp14p, Sec65p, Srp54p and Srp68p (see Materials and methods for preparation of the anti-Srp14p and anti-Srp68p antisera). The antibodies against Srp14p and Srp68p recognized proteins in wild type cell extract of the expected size and sedimentation velocity (see Figure 6A). Western blots of cell extracts were probed with the SRP subunit-specific antibodies and the proteins were visualized using ^{125}I-labeled secondary antibodies (Figure 5A). We found

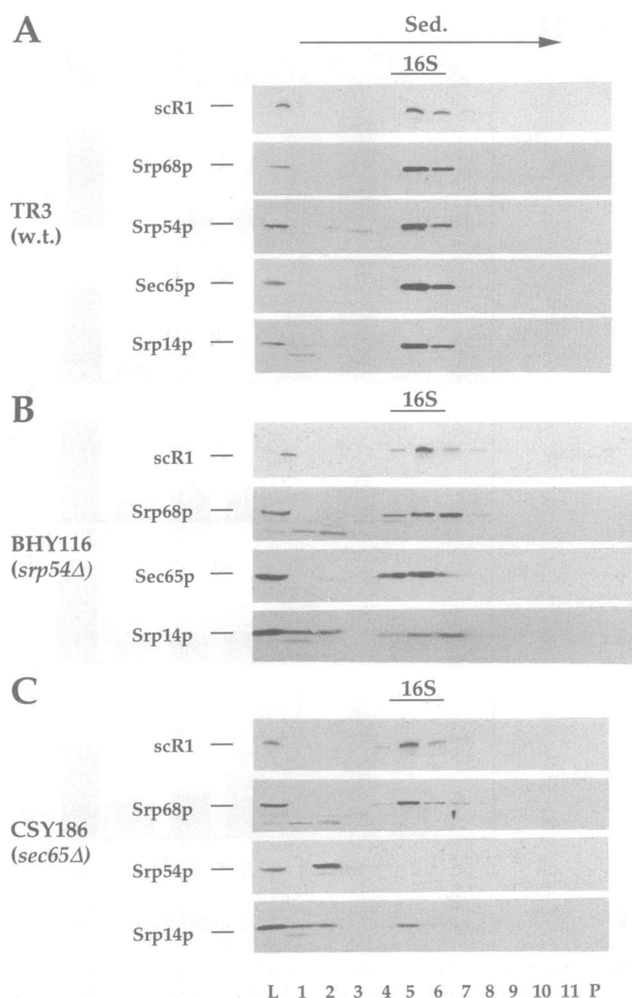

Fig. 6. An SRP subparticle in the absence of Srp54p or Sec65p. Whole cell extracts were prepared and fractionated on sucrose gradients as described in Materials and methods. A portion of each gradient fraction and an aliquot of the load fraction were analyzed for scR1 RNA (as in Figure 5, except that the RNA samples were electrophoresed on 6% acrylamide 50% urea gels; one eighth of each fraction, 1/40th of the load fraction) or for SRP proteins (as in Figure 5; one quarter of each fraction, 1/20th of the load fraction). Bound antibodies on the protein blots were visualized by Enhanced Chemiluminescence (Amersham Corporation). In this experiment more Srp14p sedimented as free protein in the gradients of extracts lacking Srp54p or Sec65p than in the extract from wild type cells. This, however, was not observed consistently.

that in a strain from which scR1 RNA was absent (lane 7), the levels of all the SRP proteins we detected were reduced by 3- to 6-fold (Figure 5B), indicating that the unassembled proteins are unstable or that transcription of the SRP genes or translation of their mRNAs is reduced. A similar reduction of protein amounts was seen in extracts of strains lacking Srp14p, Srp21p, Srp68p or Srp72p (lanes 1, 2, 5 and 6). An exception to this general decrease in protein levels was that in cells lacking Srp72p, Srp68p was virtually undetectable. Given the known close association of mammalian SRP68 and SRP72, this result suggests that in yeast these proteins may also tightly associate with each other, and that this interaction is required to stabilize the two proteins.

In contrast to the above results, cells lacking Srp54p or Sec65p (Figure 5, lanes 3 and 4) contained both Srp14p and Srp68p at near wild type levels. This, together with the presence of wild type amounts of scR1 RNA in these cells, suggests that they contain a stable SRP subparticle. Sec65p was also found at about wild type levels in cells lacking Srp54p (lane 4), indicating a probable association with any SRP subparticle in these cells. This would be consistent with the properties of mammalian SRP19, which binds to SRP RNA independent of SRP54. The amount of Srp54p, however, was as reduced in the strain lacking Srp65p (lane 3) as it was in strains lacking other SRP proteins or scR1. This is consistent with the known requirement of SRP19 for stable association of mammalian or yeast SRP54 with SRP RNA (Walter and Blobel, 1983; Hann *et al.*, 1992).

To address directly whether SRP components are stably associated in cells lacking Srp54p or Sec65p, we performed sucrose gradient sedimentation experiments on extracts from wild type cells and cells lacking Srp54p or Sec65p. Figure 6A shows an analysis of a gradient on which wild type extract was fractionated. The bulk of all measured SRP components (i.e. Srp14p, Sec65p, Srp54p, Srp68p and scR1 RNA) co-sedimented at 16S. In gradients on which extracts from BHY116 (*srp54Δ*) or CSY186 (*sec65Δ*) were fractionated, the majority of Srp14p, Sec65p (in BHY116), Srp68p and scR1 RNA co-sedimented in the same position as in the wild type case (Figure 6B and C), confirming the presence of an SRP subparticle in such cells. In agreement with previous results (Hann *et al.*, 1992) and with the decreased level of Srp54p seen in these cells (Figure 5, lane 3), lack of Sec65p activity led to Srp54p sedimenting as free protein and none of it associating with the remaining SRP subparticle (Figure 6C).

Discussion

Previous analysis of the yeast SRP indicated that there were likely to be protein components in addition to Srp54p and Sec65p (Hann and Walter, 1991). In the work reported in this paper we have demonstrated that the yeast SRP contains six or seven proteins. We have identified and characterized the genes encoding four previously unidentified yeast SRP proteins. Three of the novel yeast SRP components share significant sequence similarity to mammalian SRP proteins, demonstrating that the whole RNP is, to a large extent, conserved among divergent eukaryotes.

The approach we took to isolate yeast SRP components employed affinity-purified antibodies directed against a known SRP component, Sec65p. This allowed us to purify SRP subunits in two steps from a post-ribosomal supernatant of yeast cell extract (Figure 2A). Immunoaffinity approaches have been extremely powerful in the purification of multi-subunit complexes, for example in the purification of splicing snRNPs (Bach *et al.*, 1990). Elution from the anti-Sec65p immunoaffinity column was carried out under conditions that would be expected to destroy the integrity of SRP (pH 2.0) and therefore render it non-functional in activity assays. The protein products of the four genes we identified are all required for SRP integrity *in vivo*. We are confident, therefore, that the purification was specific for SRP proteins.

Sequence analysis of SRP components

The homology between yeast and mammalian SRP components shows a marked difference between that found for Srp54p and that found for the other components. Yeast Srp54p is almost 50% identical to its mammalian homolog (Hann *et al.*, 1989), whereas the other SRP components share only 18–31% identity (Stirling and Hewitt, 1992; this paper). The higher degree of conservation of Srp54p suggests more constraints on sequence and structure, consistent with the central role of Srp54p in SRP function.

For both Srp68p and Srp72p, alignment of mammalian and yeast sequences reveals short regions of increased similarity which may indicate functional importance (Figure 3C and D). In Srp68p from amino acids 526–550 (yeast amino acid number) the proteins are 50% identical. Using truncated versions of SRP68 and assessing binding of the truncated proteins to SRP RNA and SRP72, Lütcke *et al.* (1993) found that this region of SRP68 was required for interaction with SRP72. The higher conservation of this region of SRP68 is consistent with the proposal that this region of SRP68 may be important for interaction between SRP68 and SRP72. The alignment of Srp72p reveals a stretch of 20 amino acids (positions 585–604) that are 75% identical. Experiments similar to those described above for SRP68 using truncated versions of SRP72 indicated that this conserved region of SRP72 could be removed without affecting significantly binding to SRP68 and SRP RNA (Lütcke *et al.*, 1993). Thus, there is no evidence as to the function of this conserved region of SRP72. It may be involved in interaction of SRP72 with SRP components other than the SRP RNA and SRP68, or in interactions of SRP with other cellular factors.

Yeast SRP architecture

The components of yeast SRP identified from this and previous analyses include homologs of all but one of the mammalian SRP protein subunits, i.e. SRP9. Mammalian SRP9 dissociates from SRP as a heterodimer with SRP14 (Walter and Blobel, 1983) and, *in vitro*, association of these proteins is strictly required before they will bind SRP RNA (Strub and Walter, 1990). By analogy, therefore, yeast SRP probably contains an SRP9 homolog. The 7 kDa protein that we observed in the immunoaffinity column eluate (Figure 3B) could be the yeast Srp9p, but confirmation awaits sequencing of this protein or its gene.

Yeast SRP contains a protein (Srp21p) which is not significantly homologous to any protein found in the canine particle. Deletion of the *SRP21* gene leads to phenotypes quantitatively the same as deletion of the genes encoding several other SRP components (Figures 4–6). In addition, other SRP proteins can be co-immunoprecipitated from yeast post-ribosomal supernatant along with an epitope-tagged Srp21p using antibodies against the tag (data not shown). These results demonstrate that Srp21p is a bona fide SRP protein. In preliminary experiments we have found that antibodies against the epitope tag on Srp21p do not co-immunoprecipitate other SRP proteins from a fraction containing yeast SRP proteins disassembled on DE53 in a non-destructive fashion, analogous to disassembly of the canine SRP (Walter and Blobel, 1983). Thus, Srp21p does not dissociate from SRP RNA complexed with other SRP proteins. By this criterion, Srp21p is unlikely to be the yeast equivalent of mammalian SRP9, as it would be expected to remain assembled as a heterodimer with Srp14p. Like several of the other SRP proteins, Srp21p is basic and may, therefore, bind the scR1 RNA, possibly to yeast-specific sequences absent from the mammalian SRP RNA.

ScR1 was identified as the RNA component of yeast SRP by its association with Srp54p (Hann and Walter, 1991). It was suggested previously to be the yeast SRP RNA equivalent on the basis of its abundance and cytoplasmic disposition (Felici *et al.*, 1989). ScR1 is almost twice as long as mammalian SRP RNA, and secondary structure predictions have not satisfactorily identified important elements seen in other SRP RNA homologs, in particular the so-called domain IV stem–loop which is the binding site for SRP54 (Poritz *et al.*, 1990; Wood *et al.*, 1992; Selinger *et al.*, 1993). Binding sites for SRP9/14 and SRP68/72 have also been mapped on mammalian SRP RNA (Siegel and Walter, 1988b; Strub *et al.*, 1991). Since yeast SRP contains Srp14p, Srp68p and Srp72p subunits, their binding sites are probably conserved in scR1 RNA. Determination of these binding sites should provide valuable information for predictions of the secondary structure of the RNA. Interestingly, the sequence of a single-stranded region that forms part of the SRP9/14 binding site on mammalian SRP RNA is conserved in scR1. From this it was suggested that Srp9/14p might be present in yeast (Strub *et al.*, 1991).

Consequences of loss of SRP subunit function

The phenotypes of yeast strains lacking SRP proteins or the scR1 RNA, i.e. slow growth, and defects in the processing of pre-secretory proteins, are equivalent regardless of which SRP component is lacking (Hann and Walter, 1991; Stirling and Hewitt, 1992; Figure 4). Thus, *in vivo*, SRP-dependent targeting requires the presence of all the characterized subunits of SRP. Deletions of individual subunits, however, do not have equivalent effects on the integrity of SRP.

SRP RNA forms the structural backbone onto which the SRP proteins assemble. In the absence of scR1 RNA the levels of SRP protein components are decreased (Figure 5). This is not surprising since unassembled or inappropriately located proteins are often unstable *in vivo*; examples are the degradation of unassembled secretory immunoglobulin and T cell receptor subunits in a pre-Golgi compartment (Klausner and Sitia, 1990) and of excess spectrin and ankyrin components of the erythroid membrane skeleton (Lazarides and Moon, 1984). Strains lacking any one of Srp14p, Srp21p, Srp68p or Srp72p showed a large reduction in the level of scR1 RNA and other SRP proteins. This suggests that the Srp14p, Srp21p, Srp68p or Srp72p proteins are required for early stages of SRP biogenesis. Alternatively it is possible that in the absence of any one of these proteins the partially assembled SRP is unstable. Failure to assemble RNPs or destabilization of RNPs by the loss of particular protein components has been observed previously (e.g. Moritz *et al.*, 1990; Brown and Beggs, 1992; Deshmukh *et al.*, 1993).

In contrast, lack of Srp54p or Sec65p activity does not affect the level of scR1 RNA or Srp68p and Srp14p significantly. Presumably Srp21p, Srp72p and the putative Srp9p, which we did not assay, are also present at wild type levels. We found that these remaining SRP

Table II. Yeast strains used in this study

Strain	Genotype	Source
PS886	*trp1, leu2, ura3-52, pep4-3, prb, prc, bar1::LEU2/bar1*, MATa/α	P.Sorger
TR1	*trp1, lys2, his3, ura3, ade2*, MATa/α	Parker *et al.* (1988)
TR3	*trp1, lys2, his3, ura3, ade2*, MATα	T.Simmons
BHY116	*trp1, lys2, his3, ura3, ade2, srp54::LYS2, [rho⁻]*, MATα	Hann and Walter (1991)
BHY133	*trp1, lys2, his3, ura3, ade2, scr1::HIS3, [rho⁻]*, MATα	this study
CSY186	*trp1, lys2, his3, ura3, ade2, sec65::HIS3, [rho⁻]*, MATα	Stirling and Hewitt (1992)
JDY66	*trp1, lys2, his3, ura3, ade2, srp14::HIS3, [rho⁻]*, MATα	this study
JDY98	*trp1, lys2, his3, ura3, ade2, srp68::HIS3, [rho⁻]*, MATα	this study
JDY100	*trp1, lys2, his3, ura3, ade2, srp72::HIS3, [rho⁻]*, MATα	this study
JDY105	*trp1, lys2, his3, ura3, ade2, srp21::HIS3, [rho⁻]*, MATα	this study
RSY151	*sec63-1, leu2-3, -112, ura3-52, pep4-3*, MATα	Feldheim *et al.* (1993)
RSY458	*sec65-1, trp1-Δ, leu2-3, −112, his3, ura3-52, ade2*, MATα	R.Schekman

components were assembled into a subparticle. This indicates that Srp54p and Sec65p are not required during early steps in SRP assembly.

Srp68p was not detected in a strain lacking Srp72p. This suggests an additional dependence of these two proteins on each other for stability, possibly via the formation of a heterodimer. *In vitro*, mammalian SRP68 and SRP72 dissociate from SRP as a tightly associated heterodimer. Lütcke *et al.* (1993) found, however, that *in vitro* synthesized mammalian SRP68 and SRP72 assemble into heterodimers poorly, if at all, without SRP RNA, and thus may depend on RNA binding for association. Since yeast Srp68p was found in cells lacking scR1, the Srp68p−Srp72p complex is likely to form prior to assembly with scR1. Thus, SRP68 and SRP72 appear to behave differently in this respect in mammalian cell extracts and in yeast.

Our goal is to understand in detail the relationship between the components and functions of SRP. Taken together, the results presented in this paper reveal many similarities in the composition and organization of the mammalian and yeast SRP. Yeast affords a combination of *in vitro* biochemistry to analyze targeting and translocation, and *in vivo* analysis of protein function through genetic manipulation. The availability of clones encoding subunits of yeast SRP should, therefore, allow detailed dissection of the functions, interactions and assembly of this evolutionarily conserved RNP.

Materials and methods

Yeast strains, techniques and antibodies

S.cerevisiae strains used in this study are listed in Table II. Yeast growth media and genetic manipulations were carried out as described in Rose *et al.* (1990). Anti-Srp54p antibodies are described in Hann and Walter (1991). Antisera were kindly provided by T.Stevens, Institute of Molecular Biology, University of Oregon (anti-DPAPB), R.Schekman, Division of Biochemistry and Molecular Biology, University of California at Berkeley (anti-CPY), D.Ng, this laboratory (anti-KAR2) and C.Tran, this laboratory (anti-β-tubulin; YL1/2). Primary antibodies bound to antigens on Western blots were visualized by either incubating with ^{125}I-labeled secondary antibody (ICN) or Enhanced Chemiluminescence (Amersham Corporation) as indicated.

Preparation of antisera

GST fusion proteins were expressed and purified from *E.coli* using the following constructs.

GST−Sec65p: The *SEC65* gene was amplified by PCR from pCS52 (a gift from C.Stirling, University of Manchester, UK), introducing *Bam*HI and *Xba*I sites at the N- and C-terminal ends, respectively. This product was cloned into the *Bam*HI and *Xba*I sites of pGEM2 (Promega Biotec.). From this, a *Bam*HI−*Hinc*II fragment was subcloned into pGEX-2T (Smith and Johnson, 1988).

GST−Srp14p: The *SRP14* gene was amplified by PCR from pJDY37, introducing an *Xba*I site immediately after the methionine initiation codon and an *Eco*RI site C-terminal to the ORF, and cloned into pRS314 (Sikorski and Heiter, 1989). A 352 bp *Xba*I−*Sph*I fragment from the resulting plasmid coding for amino acids 2–111 of Srp14p was end-filled with T4 DNA polymerase and ligated into *Sma*I cut pGEXI (Smith and Johnson, 1988).

GST−Srp68p: A 624 bp *Bgl*II−*Eco*RI fragment from pJDY95 coding for amino acids 264–470 of Srp68p was cloned into the *Bam*HI and *Eco*RI sites of pGEX4T-1 (Promega Biotec.).

The GST−Sec65p and GST−Srp14p fusion proteins were soluble, and were purified on glutathione agarose resin as described (Smith and Johnson, 1988). The GST−Srp14p fusion protein was additionally purified by SDS−PAGE. The GST−Srp68p fusion protein was insoluble and was purified from inclusion bodies by SDS−PAGE. Polyclonal sera against the fusion proteins were prepared by Berkeley Antibody Company (Richmond, CA).

Antibodies against Sec65p were affinity-purified from serum by first removing anti-GST antibodies by adsorption on GST−Sepharose beads and then binding and eluting the anti-Sec65p antibodies to a column of GST−Sec65p−Sepharose. Proteins were coupled to the beads as recommended by the manufacturer (Pharmacia) and bound antibodies eluted with 0.1 M glycine pH 2.5 as described (Harlow and Lane, 1988). Affinity-purified antibodies were concentrated by precipitation with ammonium sulfate and stored at −20°C in phosphate-buffered saline containing 50% glycerol and 0.02% NaN_3 at a concentration of 1.5 mg/ml.

Preparation and fractionation of yeast cell extracts

Metabolic labeling of yeast for analysis of SRP (Figure 1B) was carried out by growing 60 OD_{600} uints of cells in SC-Met medium in the presence of 5 mCi Tran-^{35}S-label (ICN) for 4.5 h. Cells were pelleted, washed with TE (10 mM Tris−HCl pH 7.5, 1 mM EDTA), resuspended in 0.5 ml lysis buffer [20 mM HEPES−KOH pH 7.5, 0.1 M KOAc pH 7.5, 6 mM $Mg(OAc)_2$, 1 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 100 U/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml each of pepstatin A, antipain and leupeptin] and transferred to a pre-chilled 2 ml screw cap tube containing 1 ml zirconium beads (Biospec Products, Bartlesville, OK). Tubes were filled with lysis buffer and subjected to three 20 s bursts in a Mini-Bead Beater (Biospec Products), with cooling in ice between cycles. The supernatant was transferred to a clean tube and pooled, with 400 μl of lysis buffer used to wash the beads. This was then centrifuged at 4°C sequentially for 30 s in a microfuge at half speed and, after adjusting to 0.02% Nikkol detergent [octa-ethyleneglycol-mono-*n*-dodecyl ether (a non-ionic detergent that stabilizes SRP activity); Nikko Chemical Co. Ltd, Tokyo, Japan] and 0.5 M KOAc, for 1 h at 50 000 r.p.m. in a Beckman TLA 100.3 rotor. The supernatant was diluted 1:1 with lysis buffer without KOAc and applied to a 1 ml DEAE−Sepharose column equilibrated in lysis buffer at 0.25 M KOAc. After extensive washing with buffer A [20 mM HEPES−KOH pH 7.5, 6 mM $Mg(OAc)_2$, 0.2 mM DTT, 0.02% Nikkol] containing 0.25 M KOAc and then buffer A containing 0.4 M KOAc, SRP was eluted from the column in ~1.5 column volumes buffer A containing 0.65 M KOAc and the protease inhibitor combination used in the lysis buffer. 400 μl aliquots of this 0.40–0.65 M DEAE fraction were subjected to sucrose gradient

sedimentation as described (Hann and Walter, 1991). Fractions around the 16S marker were diluted 1:1 with immunoprecipitation buffer [IPB; 50 mM Tris–HCl pH 7.5, 0.25 M NH_4OAc, 6 mM $Mg(OAc)_2$, 0.1% Triton X-100, 0.02% Nikkol and the protease inhibitor combination used in lysis buffer]. 800 µl of each fraction were immunoprecipitated with 4.5 µg affinity-purified anti-Sec65p antibodies after pre-clearing with 50 µl protein A–Sepharose (PAS) beads (1:1 in IPB) and tumbling for 30 min. Immune complexes were collected by the addition of 40 µl PAS beads and tumbling for a further hour. The beads were washed four times with 1 ml IPB and prepared for protein analysis. Non-native immunoprecipitations were carried out on 50 µl of the 0.40–0.65 M KOAc fraction as described (Hann and Walter, 1991).

Large-scale purification of SRP was carried out on cells from 60 litres of strain PS886 grown in a fermentor at 30°C in YEPD to an OD_{600} of 7.0. Cells were pelleted and washed extensively with cold H_2O and then frozen by extrusion through a caulking gun into liquid N_2. The frozen cell 'spaghetti' was ground in a Sorvall blender (three or four bursts at high speed in 100 g batches adding liquid N_2 between bursts) to break the cells. Frozen cell powder was thawed by adding slowly to one cell mass in 700 ml of a modified lysis buffer [L; 20 mM Tris–HCl pH 7.5, 0.5 M KOAc, 6 mM $Mg(OAc)_2$, 0.5 mM EDTA, 1 mM DTT, 0.01% Nikkol with protease inhibitors], warming when necessary to maintain the temperature between 0 and 4°C. The thawed lysate was centrifuged for 10 min at 3000 r.p.m. in a Sorvall RC3B rotor. The supernatant and 150 ml of buffer L used to wash the pellet were combined, adjusted to 0.5 M KOAc and centrifuged for 150 min at 40 000 r.p.m. in a Beckman Ti45 rotor. This high speed supernatant was diluted with 1 vol of buffer L without KOAc and applied to a 140 ml DEAE–Sepharose Fast Flow column (5.0×7.0 cm) equilibrated with buffer L at 0.25 M KOAc. The column was developed with 0.25, 0.40, 0.65 and 2.00 M KOAc buffer L. Fractions containing SRP were identified by dot blotting and these fractions pooled and diluted to 0.5 M KOAc with buffer L lacking KOAc. 12 ml aliquots of this pool were applied at 0.3 ml/min to a 1.5 ml affinity column consisting of 2.5 mg affinity-purified anti-Sec65p antibodies coupled to Affinica Beads according to the manufacturer's instructions (Schleicher & Schuell). The column was washed with 10 column volumes of IPB and eluted with 0.1 M glycine pH 2.0. Peak fractions, as judged by Bio-Rad protein assay, were pooled and adjusted to pH 7.5 with 2 M Tris base.

Generation and analysis of peptides

Purified SRP components (100 pmol as assessed by Coomassie Blue staining compared with standards) were precipitated with TCA and electrophoresed through a 10–15% SDS–polyacrylamide gel. Protein bands were excised and digested with trypsin in the gel slice and peptides eluted as described by Rosenfeld *et al.* (1992) using trypsin at 2% (w/w) to digest the proteins. Peptides eluted from the gel slices were separated on an Aquapore 300 C18 microbore HPLC column (Applied Biosystems) using water/acetonitrile/trifluoroacetic acid as the mobile phase. Column effluent was monitored at 215 nm, then split at a ratio of 20:1, and peptide masses determined on 5% of the HPLC effluent introducing it directly onto a VG Biotech/Fisons Bio-Q mass spectrometer with an electrospray source. The remaining 95% of the peptide peaks was collected manually (Medzihradszki *et al.*, 1994).

Peptide sequences were generated either by Edman degradation (molecular weights ~2000 or higher from fractions containing single peptides) on an ABI 470A Gas Phase Sequencer, or by high energy collision–induced dissociation (CID) analysis. CID experiments were carried out on a Kratos Concept II HH four sector mass spectrometer equipped with a Cs source, a continuous flow probe and a charge-coupled device detector (Walls *et al.*, 1993). The collision gas was helium, its pressure adjusted to attenuate the precursor ion intensity by 70%; the collision energy was 4 keV.

Cloning and sequencing SRP genes

Degenerate oligonucleotides were designed based on the following peptide sequences in both the N–C and C–N orientation and containing 5′ extensions with restriction sites to aid in cloning: IEHDPV and DQFRQE (19 kDa), QFLEQG, NMPDEF, VTTNINW and FNLQTF (66 kDa). PCRs were carried out with single oligonucleotides or pairwise combinations of oligonucleotides from different peptides. PCR products were cloned using the restriction sites incorporated into the oligonucleotides, and sequenced to identify those that contained both peptide sequences and an ORF. The cloned PCR fragments of the *SRP14*, *SRP68* and *SRP72* genes were used to screen approximately five genome equivalents of two of the Carlson and Botstein YEP24-based yeast genomic libraries (Carlson and Botstein, 1982). Selected positive clones were sequenced on both strands with either the Sequenase kit (US Biochemical Corporation) or an Applied Biosystems model 373A automated sequencer and synthetic oligonucleotide walking primers.

Sequences were analyzed using the Blast and Fasta algorithms (Pearson and Lipman, 1988; Altschul *et al.*, 1990) on the NCBI Blast network server and the EMBL Fasta server.

Disruption of SRP genes

SRP genes were replaced with the *HIS3* gene in the diploid strain TR1 using the following method. DNA sequences (300–500 bases) flanking the gene to be replaced were amplified by PCR using oligonucleotides that incorporated *Bam*HI and *Eco*RI restriction sites at the ends of the fragments distant from the gene, and *Sma*I sites at the inside ends. These were then digested with the relevant restriction enzymes and cloned in a three-way ligation into *Eco*RI- and *Bam*HI-cut pRS316 (Sikorski and Heiter, 1989). The resulting plasmids were then linearized with *Sma*I, and a 1.6 kb *Ssp*I fragment containing the *HIS3* gene ligated into them. The fragment containing the *SRP* gene flanking regions and *HIS3* gene was then removed from the plasmid by digestion with *Eco*RI and *Bam*HI and used to transform yeast directly. Replacement of the desired gene was verified by PCR and/or Southern blotting.

Sucrose gradient sedimentation, Northern analysis and immunoprecipitation

Cell extracts for sucrose gradient sedimentation were made as described above for extracts from metabolically labeled cells, except that the lysis buffer contained 500 mM KOAc and additionally 2 mM vanadyl ribonucleoside complex (Gibco BRL) and 200 µg/ml *E.coli* MRE 600 tRNA (Boehringer Mannheim) (these reagents were added to quench ribonuclease activity in the extracts which, particularly in extracts from strains lacking Sec65p, degraded scR1 RNA). Extracts were spun for 10 min at full speed in a microfuge at 4°C. Sucrose gradient sedimentation and analysis of protein and RNA were carried out as described (Hann and Walter, 1991), except that the gradients contained the combination of RNase quenchers present in the lysis buffer and centrifugation was carried out in an SW60 rotor for 4.5 h at 55 000 r.p.m. Gradients were manually fractionated into 11 fractions of 0.4 ml.

Metabolic labeling and non-native immunoprecipitation of proteins were carried out as described (Hann and Walter, 1991). Extracts for analysis of SRP protein levels were made as for non-native immunoprecipitations except that 100–200 OD_{600} units of cells were used. For analysis of scR1 levels, RNA was extracted from yeast strains using hot phenol and SDS as described (Köhrer and Domdey, 1991) and analyzed on 1.5% agarose gels containing formaldehyde. Quantitation of bands on probed Western and Northern blots was carried out using a Molecular Dynamics PhosphorImager and Imagequant software.

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