

Functional Characterization of the 180-kD Ribosome Receptor In Vivo

Erich E. Wanker, Yin Sun, Adam J. Savitz, and David I. Meyer

Department of Biological Chemistry and the Molecular Biology Institute, UCLA School of Medicine, Los Angeles, California 90024

Abstract. A cDNA encoding the 180-kD canine ribosome receptor (RRp) was cloned and sequenced. The deduced primary structure indicates three distinct domains: an NH₂-terminal stretch of 28 uncharged amino acids representing the membrane anchor, a basic region (pI = 10.74) comprising the remainder of the NH₂-terminal half and an acidic COOH-terminal half (pI = 4.99). The most striking feature of the amino acid sequence is a 10-amino acid consensus motif, NQGK-*KAEGAP*, repeated 54 times in tandem without interruption in the NH₂-terminal positively charged region. We postulate that this repeated sequence represents a ribosome binding domain which mediates the interaction between the ribosome and the ER membrane. To substantiate this hypothesis, recombinant full-length ribosome receptor and two truncated versions of this protein, one lacking the potential ribosome binding domain, and one lacking the COOH terminus, were expressed in *Saccharomyces cerevisiae*. Morphological and biochemical analyses showed all proteins were targeted to, and oriented correctly in the ER membrane. In vitro ribosome binding assays demonstrated that yeast microsomes containing the full-length canine re-

ceptor or one lacking the COOH-terminal domain were able to bind two to four times as many human ribosomes as control membranes lacking a recombinant protein or microsomes containing a receptor lacking the NH₂-terminal basic domain. Electron micrographs of these cells revealed that the expression of all receptor constructs led to a proliferation of perinuclear ER membranes known as "karmellae." Strikingly, in those strains which expressed cDNAs encoding a receptor containing the putative ribosome binding domain, the induced ER membranes (examined in situ) were richly studded with ribosomes. In contrast, karmellae resulting from the expression of receptor cDNA lacking the putative ribosome binding domain were uniformly smooth and free of ribosomes. Cell fractionation and biochemical analyses corroborated the morphological characterization. Taken together these data provide further evidence that RRp functions as a ribosome receptor in vitro, provide new evidence indicating its functionality in vivo, and in both cases indicate that the NH₂-terminal basic domain is essential for ribosome binding.

THE cotranslational translocation of secretory proteins through the membrane of the rough ER is a complex process involving the sequential interactions of nascent chains with proteins of the translocation apparatus. Early in their synthesis, secretory or membrane proteins are recognized by the signal recognition particle (SRP;¹ Walter et al., 1981), and targeted to the membrane via an interaction between SRP and the docking protein (SRP receptor, SR α ; Meyer et al., 1982; Gilmore et al., 1982). Transport through the membrane into the ER lu-

men occurs via a channel or pore most likely composed of the Sec61p complex (Sanders et al., 1992; Görlich et al., 1992; Mothes et al., 1994).

Traditionally, models describing the process of cotranslational translocation have included an additional receptor protein, located in the ER membrane, which mediates ribosome binding (Dobberstein, 1994; Kalies et al., 1994). Prior to the isolation of receptor candidates, ribosome binding had been reported to be sensitive to proteases (Hortsch et al., 1986), puromycin, and high salt (Borgese et al., 1974). It was also shown that up to 40% of the ribosomes bound to rat liver rough microsomes can be released in high ionic strength medium without puromycin (Adelman et al., 1973), implying that a large proportion of ribosomes may bind to ER membranes independent of ongoing translocation. This raises the possibility that two functionally different ribosome-membrane interactions may be taking place. The purpose of this additional bind-

David Meyer, Dept. of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024-1737. Tel.: (310) 206-3122. Fax: (310) 206-5197.
Drs. Wanker and Sun contributed equally to this work.

1. *Abbreviations used in this paper:* RBD, ribosome binding domain; RR, ribosome receptor; SRP, signal recognition particle.

ing capacity is unknown, but may serve to accumulate ribosomes in an appropriate subcellular localization to enable a rapid synthetic response.

Different ER membrane proteins have been characterized as potential ribosome receptors by an *in vitro* ribosome binding assay (Borgese et al., 1974). Savitz and Meyer identified and purified a protein of 180 kD (p180) that was shown to be sufficient (Savitz and Meyer, 1990) and necessary (Savitz and Meyer, 1993) for the binding of ribosomes to ER membranes *in vitro*. The binding of Fab fragments of an anti-p180 monoclonal antibody to stripped microsomal membranes, as well as the immunodepletion of p180 from translocation-competent proteoliposomes resulted in a loss of both ribosome binding and translocation activity (Savitz and Meyer, 1993). A protein of 34 kD has also been postulated as a ribosome receptor using an *in vitro* assay (p34; Tazawa et al., 1991; Ichimura et al., 1992, 1993; Ohsumi et al., 1993), and it has been suggested that the putative translocation channel, the Sec61p complex, can also act as a ribosome receptor (Görllich et al., 1992; Kalies et al., 1994).

We report here the complete amino acid sequence of the 180-kD ribosome receptor (RRp). The most striking feature of the primary structure is a highly conserved motif, comprising 10 amino acids, that is repeated 54 times in tandem near the NH₂ terminus of the protein. We propose and present evidence to support the hypothesis that this region of the protein represents a ribosome binding domain, essential for the function of the protein *in vitro* as well as *in vivo*.

Materials and Methods

Strains and Microbiological Methods

Saccharomyces cerevisiae strain SEY6210 (MAT α *leu2-3,112 ura3-52 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9*) has been previously described (Wilsbach and Payne, 1993). Yeast media were prepared essentially as described in Sherman et al. (1986). Yeast transformations were performed using lithium acetate as described in Ito et al. (1983). Transformants were selected on synthetic complete medium lacking uracil. *Escherichia coli* cells were transformed by the method of Cohen et al. (1972) using HB101 or XL1-Blue. Yeast and *E. coli* genetic techniques were as described in Sherman et al. (1986) or Maniatis et al. (1982), respectively.

Isolation and Sequencing of cDNA Encoding RRp

The original cDNA clone (clone 1) was obtained from an MDCK cell cDNA library cloned into the pTEX vector (Herz et al., 1990), and screened with a polyclonal antibody against canine RRp (Savitz and Meyer, 1990). It encoded a 3.7-kb 3' fragment which included 3' polyadenylation. To obtain the full-length sequence of the cDNA, a primary oligo-d(T) and random-primed MDCK cell cDNA library was prepared using a cDNA synthesis kit (SuperScript_{tm}; Bethesda Research Laboratories, Gaithersburg, MD) and cloned into λ gt11. The new library was screened by hybridization with a probe generated by PCR which spanned the region between 2492–3327 bp. Positive clones (34 total) were isolated, and clones encoding 5' extensions beyond nucleotide 1638 were screened by the length of PCR products generated with primers corresponding the vector arm of λ gt11 and cDNA. Only one clone was identified; it encoded nucleotides 938–3796 (clone 2). Subsequently, the repeat region (nucleotides 1638–2468) was used as a probe to screen the same library by hybridization. 26 clones were identified as positive, and 14 encoded 5' extensions beyond nucleotide 938. Three of these clones encoded the 5' end of the full-length cDNA of the RR as determined by restriction analysis, and the sequence of one of them (clone 3) served as the basis for primer in extension studies on MDCK cell mRNA. This analysis revealed that clone 3 indeed encoded the 5' end of the mRNA. Both strands of clones 1–3 were

sequenced using Sequenase 2.0 (United States Biochemicals, Cleveland, OH) by generation of nested deletions with exonuclease III. A full-length clone (pRR-Yin) was reconstructed from these three clones by subcloning of the cDNA fragments into pBluescript II KS.

For the Northern analysis of the cDNA, poly(A)⁺-RNA was directly isolated from MDCK cells, separated on a 1% formaldehyde-agarose gel, transferred to nitrocellulose and probed as described by Clark and Meyer (1992). The RRp (p180) probe was the original 3.7-kb cDNA (clone 1).

Construction of Expression Plasmids

To introduce a BamHI and NcoI site at the translation initiation ATG a 0.125-kb BamHI-NdeI fragment was amplified from pRR-Yin by PCR using the primers 5'-CAGGATCCATGGATATTTACGAC-3' and 5'-TTC-ATATGACGTCTC-3'. This fragment was subcloned together with a 4.8-kb NdeI-MunI RR fragment, isolated from pRR-Yin, into pBluescript II KS creating pRRFL-EW1. To check for the presence of missense mutations, the PCR fragment in pRRFL-EW1 was sequenced. pRR was created by subcloning a 5.0-kb BamHI-HindIII RR fragment derived from pRRFL-EW1 into pEMBLyex4 (Baldari and Cesareni, 1985), downstream of the *GAL10-CYC1* promoter. pBSRR Δ CT was generated by deleting a 2.5-kb BstEII-HindIII fragment from pRRFL-EW1 and ligation of the vector with the oligonucleotides 5'-GTGACCCCTGAATTGA-3' and 5'-AGC-TTCAATTCAGGG-3'. From this plasmid a 2.5-kb BamHI-HindIII RR fragment was removed and cloned into pEMBLyex4 to generate pACT. For the construction of p Δ NT pRRFL-EW1 was cut with KpnI and BstEII and a 1.9-kb fragment encoding the putative RBD was removed. The vector was religated with the oligonucleotides 5'-CCCAGCCAAGG-3' and 5'-GTCACCCCTGGCTGGGGTAC-3' to generate pBSRR Δ NT. From this plasmid a 3.1-kb BamHI-HindIII RR fragment was isolated and cloned into pEMBLyex4 to create p Δ NT.

Preparation and Characterization of Microsomes

Rough microsomes were prepared from yeast by the method of Rothblatt and Meyer (1986). The nature of membrane association of RRp in yeast microsomes was determined as described in Feldheim et al. (1992), except that microsomes were suspended in MS buffer (0.25 M sucrose, 50 mM KOAc, 1 mM DTT, 20 mM Hepes-KOH, pH 7.4). Protease treatment was performed as follows: rough microsomes were adjusted to a concentration of 20 A₂₈₀/ml in MS buffer. Samples were separated in aliquots and treated at 0°C with 10 μ g/ml trypsin in the absence or presence of 1% Triton X-100. After a 1-h incubation, the reaction was terminated by the addition of 0.01 vol of 100 mM PMSF followed by a further incubation of the samples for 5 min on ice. Microsomes were solubilized with sample buffer and analyzed by SDS-PAGE and immunoblotting.

Immunological Techniques

The antiserum to RRp has been previously described (Savitz and Meyer, 1990). The antiserum against Kar2p was kindly provided by M. Rose (Princeton University, Princeton, NJ), and anti-Sec61p and anti-Sec63p antisera were provided by R. Schekman (University of California, Berkeley, CA). Immunofluorescent staining of yeast cells was performed by a modification of the methods of Adams and Pringle (1984) and Pringle et al. (1991). FITC-conjugated goat anti-rabbit antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

For immunoblots, yeast cells were extracted by the method of Payne et al. (1987) and separated by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979). Filters were incubated with antisera against RRp, Sec61p, and Sec63p at dilutions of 1:2,000, 1:1,000, and 1:1,000, respectively, followed by anti-rabbit or anti-mouse secondary antibodies conjugated to alkaline phosphatase.

Ribosome Binding

Ribosomes were removed from rough microsomes by two rounds of treatment with 1 mM puromycin, 15 U/ml micrococcal nuclease, 500 mM KOAc, 50 mM Tris-HCl, pH 7.5, 5 mM Mg(OAc)₂, and 1 mM CaCl₂ at 24°C for 30 min (Adelman et al., 1973; Savitz and Meyer, 1993). Ribosome binding assays were carried out in a constant volume of 50 μ l as previously described by Savitz and Meyer (1990). Tritiated ribosomes were prepared from [5,6-³H]uridine-labeled HeLa cells according to the method of Kreibich et al. (1983).

Electron Microscopy

Yeast cells were cross-linked with 2% glutaraldehyde in PBS and post-fixed with 1% OsO₄ in PBS, dehydrated with ethanol and embedded in Spurr. Approximately 60-nm thick sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100CX electron microscope (JEOL, Tokyo, Japan).

Fractionation of Microsomes

Microsomes prepared from yeast by the method of Rothblatt and Meyer (1986) were layered on top of a linear sucrose gradient ranging from 30–70% (wt/wt) sucrose in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 25 mM KCl. Gradient centrifugation was carried out to equilibrium in an SW-41 swinging bucket rotor (Beckman Instruments, Palo Alto, CA) at 40,000 rpm for 18 h. Fractions of light and heavy microsomes were withdrawn from the gradient with a needle at equilibrium densities 1.2025 and 1.2296 g/ml, respectively. The microsomes in both fractions were pelleted by centrifugation in a 80-Ti rotor (Beckman Instruments; 40,000 rpm for 30 min) and resuspended in 100 μl 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 25 mM KCl. In the light and heavy microsomal fractions total RNA was determined by a modification of the method of Fleck and Munro (1962). For phospholipid determinations, lipids were extracted from membrane suspensions with 20 vol chloroform/methanol (2:1) and purified according to Folch et al. (1957). In appropriate fractions of extracts phosphate was assayed as described by Ames (1966). Values for phosphate were converted to phospholipid using a factor of 25 (Borgese et al., 1974).

Results

The Primary Structure of the Ribosome Receptor Protein (RRp) Contains a Highly Repetitive Unique Motif

A combination of strategies was used to isolate and assemble a full-length clone encoding RRp. A 3.7-kb clone, representing the 3' end of the mRNA, was isolated from an MDCK cell cDNA expression library using a rabbit anti-RRp antiserum (Savitz and Meyer, 1990). The primary structure deduced from sequencing this clone contained a perfect overlap with the NH₂-terminal 16 amino acids of a cyanogen bromide fragment of RRp as indicated in Fig. 1. Northern analysis of MDCK cell poly(A)⁺ RNA using this clone as a probe revealed a single mRNA species of 5.5–6.0 kb in length (Fig. 2), sufficient to encode a 180-kD protein, that was at least 2-kb longer than our longest clone. All efforts at isolating larger cDNAs using a variety of primer-extension and PCR techniques were unsuccessful, as were those aimed at screening or rescreening oligo-(d)T-primed libraries. The highly repetitive nature of the 5' end of the mRNA (see Fig. 1) appears to have impeded primer extension-based reactions through this region. The problem was overcome by the construction of a new library of randomly primed cDNA derived from MDCK cell mRNA. From this library, the overlapping clones were obtained which enabled the deduction of the complete amino acid sequence of RRp (Fig. 1).

There are several noteworthy features of the primary structure of RRp. An uncharged stretch of 28 amino acids (underlined in Fig. 1) is present at the NH₂ terminus commencing with the sixth amino acid. This sequence most likely represents an insertion anchor (High and Dobberstein, 1992), making the cytosolic topology of RRp reminiscent of docking protein (SRP receptor, α subunit; Lauffer et al., 1985; Hortsch et al., 1988). A remarkable and exciting aspect of the primary structure of RRp is the 10-amino acid-long motif whose consensus is NQGK-

MDIYDT	QTLGVMVFGG	FMVVSAGIF	LVSTFSMKET	SYEELANQR	46
KEMAKTHHQ	VEKKKKEKTV	EKKGKTKKKE	EKPNGKIPDH	EPAPNVITILL	96
KDPVRAVAVP	VAPTPVQPPV	VIAPVATVPA	MPQEKLAPSP	KDKKKKERKV	146
AKVEPAVSSV	VNSVQVLASK	AAILLETAPKE	VPMMVVPEVG	AKAGTPATST	196
AQGKKAEGAP	<u>NQSRKAEGAP</u>	<u>NQGKKAEGAL</u>	<u>NQGKKAEGAP</u>	<u>NQGKRVVAP</u>	246
NQGKKAEGGQ	<u>NQGKVEGAP</u>	<u>NQGKKAEGTP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKTGAP</u>	296
NQGKKEGAP	<u>NQGKKAEGAP</u>	<u>NQGKKEVAP</u>	<u>NQGKKAEGGQ</u>	<u>NQGKVEGAP</u>	346
NQGKKAEGTP	<u>NQGKKAEGAP</u>	<u>NQGKKTGAP</u>	<u>NQGKKEGAP</u>	<u>NQGKVEGAP</u>	396
NQGKKEGAP	<u>NQGKKAEGAP</u>	<u>NQGKKEGTP</u>	<u>NQGKKEGTP</u>	<u>NLGGKKAEGSP</u>	446
NQGKKEGAP	<u>NQGKKEGAP</u>	<u>NQGKKEGAP</u>	<u>NQGKKEGAP</u>	<u>NQGKKTGAP</u>	496
NQGKKAEGAP	<u>NQGKVEGAP</u>	<u>NQGKKAEGTP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	546
NQGKKAEGAP	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	596
NQGKKAEGAP	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	646
NQGKKAEGAP	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	<u>NQGKKAEGAP</u>	696
NQGKKEGTP	<u>NQGKKEGTP</u>	<u>NQGKKEGTP</u>	<u>NQGKKEGTP</u>	<u>NQGKKAEGAP</u>	746
SQEAQKQAP	<u>AKKSGSKKK</u>	<u>GEPGPPDSDS</u>	<u>PLYLPYKTLV</u>	<u>STVGSVMFNE</u>	796
GEAQRLIEL	<u>SEKAGVIQDT</u>	<u>WHKATQKQDP</u>	<u>VAILKRLQLEE</u>	<u>KEKLLATEQE</u>	846
DAAVAKSKLR	<u>EVNKELAAEK</u>	<u>AKAAAGEAKV</u>	<u>KKQLVAREQE</u>	<u>ITAVQARIEA</u>	896
SYREHVKEVQ	<u>QLQKIRTLLQ</u>	<u>EQLNGPNTQ</u>	<u>LARLQENSI</u>	<u>LRDALNQATS</u>	946
QVESKQNTL	<u>AKLRQELSKV</u>	<u>SKELVKESEA</u>	<u>ARQEEQQRKA</u>	<u>LETKTAALEK</u>	996
QVLQLQASHK	<u>ESEALQKRL</u>	<u>DEVSRELCRS</u>	<u>QTSHASLRAD</u>	<u>AEKAQEQQQQ</u>	1046
MAELHSLQS	<u>SEAEVSKSE</u>	<u>ELSGLHGQLK</u>	<u>EARAENSQML</u>	<u>ERIRSEALL</u>	1096
EAGQARDTQD	<u>AQASRAEHQA</u>	<u>RLKELESQVW</u>	<u>CLEKEATELK</u>	<u>EAVEQQKVKV</u>	1146
NDLREKNWKA	<u>MEALASAERA</u>	<u>CEEKLRSLTQ</u>	<u>AKEESEKQLS</u>	<u>LTEAQKKEAL</u>	1196
LALLPALSSS	<u>APQSYTEWLQ</u>	<u>ELREKGPPELL</u>	<u>KQRPADTQPS</u>	<u>SDLASKLREA</u>	1246
EETQNNLQAE	<u>CDQYRTILAE</u>	<u>TEGMLKDLQK</u>	<u>SVEEBEQVWK</u>	<u>AKVSATEEEL</u>	1296
QKSRVTVKHL	<u>EDIVEKLGKE</u>	<u>LESSEQVREH</u>	<u>TSHLEAELEK</u>	<u>HMAAASAEQC</u>	1346
SYAKEVAGLR	<u>QLLLESQSQL</u>	<u>DAAKSEAQKQ</u>	<u>SNELALVRQQ</u>	<u>LSEMKSHVED</u>	1396
GDVAGSPAP	<u>PAEQDPVELK</u>	<u>AQLERTEATL</u>	<u>EDQALRRKL</u>	<u>TAEFQEAQSS</u>	1446
ACRLQAELEK	<u>LRSTGPLESS</u>	<u>AAEEATQLKE</u>	<u>RLEKEKLLTS</u>	<u>DLGHAATKLG</u>	1496
ELLKTTQEQ	<u>AKERDVTVKL</u>	<u>QEQLDKTDDS</u>	<u>SSKEGTSV</u>		1534

Figure 1. The amino acid sequence of canine RRp reveals a decapeptide motif repeated 54 times in tandem. Amino acids were deduced from the sequences of overlapping cDNA clones. The nucleotide sequence has been submitted to Genbank and is available from the authors. The decapeptide repeats region is boxed. An uncharged stretch of 28 amino acids that could serve as a membrane insertion/anchor is underlined. Methionine and NH₂-terminal amino acid sequence of a cyanogen bromide fragment of RRp is indicated in boldface type. These sequence data are available from Genbank/EMBL/DBJ database accession number x87224.

KAEGAP. This motif is repeated, in tandem and without interruption, 54 times. The slight but definitive differences within each of the repeats allows the precise assignment of the number of repeats and precludes either cloning or se-

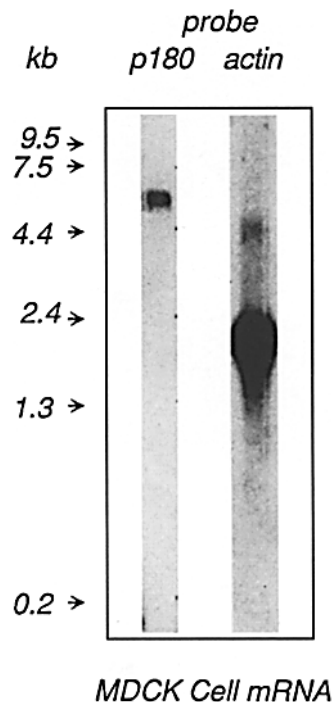


Figure 2. Northern analysis of RRp (p180) mRNA. Poly(A)⁺ RNA was isolated from MDCK cells and separated on a 1% formaldehyde gel. After transfer to nitrocellulose, identical lanes were probed with cDNA encoding either p180 (lane 1) or mouse β-actin (lane 2). The p180 cDNA hybridized to a mRNA species of approximately 5,800 nucleotides.

quencing artifacts. The high abundance of lysine residues is also striking. Of the 1,534 amino acids 215 are lysines, comprising 15% of the protein. Within the repeat section this value rises to 20%. Accordingly, RRp has an overall isoelectric pH of 9.95, and a net positive charge of 34. At the COOH terminus of the protein, the charge balance changes dramatically. Amino acid 766, only one residue away from the precise midpoint of the protein's primary sequence, marks the end of a very basic NH₂-terminal region (pI = 10.74) and the beginning of an acidic COOH terminus (pI = 4.99).

Shown in Fig. 3 is a plot of the frequencies of amino acids found within each position of each repeat. Of the 54 repeats, about 90% begin with NQGKK. The E and G at positions 7 and 8 are slightly less conserved. Even the least conserved positions, those at 6, 9, and 10 of the repeated motif, conform to the consensus over half of the time. The bulk of the deviations from the consensus are conservative substitutions, such as V for A at position 6, D for E at position 7, and Q for P at position 10.

The unique nature of the amino acid sequence provokes speculation as to its function. The preponderance of basic amino acids, together with arginines, glutamines, and glycines is reminiscent of the composition of known RNA binding motifs, particularly within ribosomal protein subunits (Wool et al., 1990). Accordingly, we propose that the repeat domain comprises a ribosome binding domain (RBD). Our studies designed to test this hypothesis are presented below. The possibility that the consensus repeat represents an RNA binding motif is the basis of a separate investigation.

Recombinant Ribosome Receptor Proteins Are Efficiently Synthesized in Yeast

The efficacy of the RRp as a ribosome receptor could be directly tested using an *in vivo* approach. ER membranes, derived from yeast expressing full-length or mutated versions of RRp, could be assessed for their ability to bind mammalian ribosomes. Since yeast microsomes have a di-

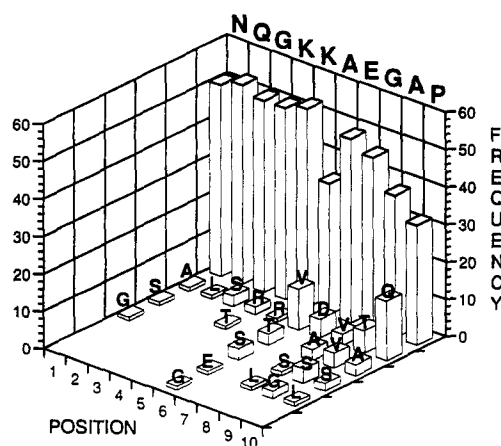


Figure 3. The decapeptide motif is highly-conserved. The frequency is indicative of the number of times (out of 54 possible) that a specified amino acid appears in a given position. The amino acids shown in bold across the top are those most frequently found in that location within the motif.

minished capacity to bind mammalian ribosomes compared to mammalian microsomes (Sanderson, C. M., A. J. Savitz, and D. I. Meyer, unpublished observations), integration of a protein exhibiting a high affinity for mammalian ribosomes into yeast membranes should result in an increased binding capacity for mammalian ribosomes. For the detailed functional analysis of recombinant RRp, different yeast expression plasmids (see Fig. 4 A) encoding the full-length 180-kD RRp, and two shorter versions of this protein were constructed. A truncated version lacks the COOH terminus of RRp (a deletion of amino acids 826-1534) and is referred to as Δ CT, while the other lacks the NH₂-terminal repeat region (amino acids 193-823) and is referred to as Δ NT. Retention of amino acids 1-193 in all three constructs guarantees the presence of the membrane anchor sequence (amino acids 6-33). These three ribosome receptor constructs were expressed in *S. cerevisiae* under control of the hybrid *GAL10-CYC1* promoter (Balardi and Cesareni, 1985), which is induced by galactose and repressed by glucose.

Immunoblot analysis of total cell lysates indicated that all three constructs were efficiently expressed in yeast. RRp, Δ CTp and Δ NTp were identified in each case with apparent molecular weights of 185, 109, and 117 kD, respectively (Fig. 4 B). This is in good agreement with the predicted molecular weights based on the amino acid sequence. In cell extracts prepared from the yeast strain harboring the control plasmid pEMBLyex4 (Balardi and Cesareni, 1985), no recombinant RRp was detected on immunoblots, whereas Sec61p, an integral ER membrane protein (Stirling et al., 1992), was present in all analyzed cell extracts. Expression of these constructs did not appear to have any deleterious effects on cell growth compared to vector-only controls when assayed in selective liquid medium with galactose as the sole carbon source.

Recombinant RR Proteins Are Inserted into Yeast ER Membranes with the Correct Topology

The initial determination of the intracellular localization of RRp, Δ CTp, and Δ NTp within the yeast cells was also accomplished by immunoblotting. Both the wild-type and mutant forms of RRp were present in rough microsomes prepared as described previously (Rothblatt and Meyer, 1986), indicating that the recombinant proteins are localized in a fraction which contains rough membranes. This was confirmed morphologically by indirect immunofluorescence microscopy (Adams and Pringle, 1984). Wild type cells harboring the control plasmid and cells expressing RRp were fixed, permeabilized and probed with anti-RRp antibodies, and then labeled with FITC-conjugated goat anti-rabbit antibodies. Nuclei were labeled with the DNA-binding dye DAPI. Yeast cells synthesizing RRp showed bright perinuclear staining, typical of the endoplasmic reticulum (Fig. 5). An identical result was obtained with anti-Kar2p antibodies (Rose et al., 1989; data not shown), whereas in the control cells no perinuclear immunoreactivity was found (Fig. 5). When cells were analyzed in which RRp expression was repressed by glucose, no perinuclear staining pattern were detected, showing that the observed immunoreactivity and localization is due to the synthesis of the recombinant protein.

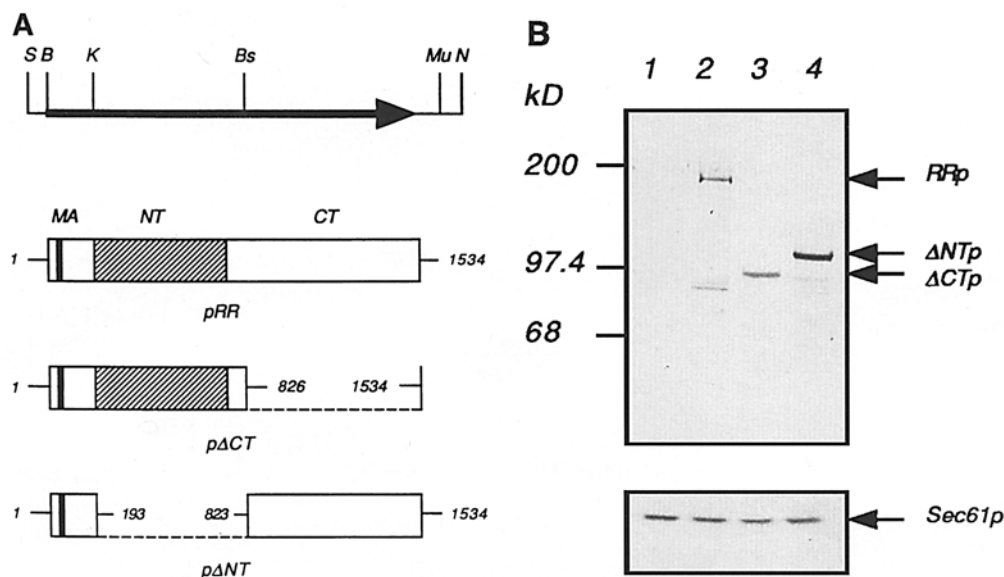


Figure 4. Expression of RRp in yeast. (A) Restriction map of the 5.4 kb RR gene fragment. The closed arrow represents the RRp open reading frame. The map below shows the RR DNA fragments cloned downstream of the *GAL10-CYC1* promoter into the yeast expression vector pEMBLyex4 (Baldari and Cesareni, 1985). MA, membrane anchor; NT, ribosome binding domain; CT, COOH-terminal region. Restriction enzymes are as follows: S, SalI; B, BamHI; K, KpnI; Bs, BstEII; Mu, MunI; N, NotI. (B) Immunoblot of total protein prepared from yeast transformants expressing different RR gene

fragments. Cells were grown in synthetic complete medium with galactose to induce the *GAL10-CYC1* promoter. Lane 1, SEY6210 (pEMBLyex4); lane 2, SEY6210(pRR); lane 3, SEY6210 (pΔCT); lane 4, SEY6210 (pΔNT).

RRp behaves as an integral membrane protein as evidenced by extraction of isolated yeast microsomes with a variety of chaotropic agents. Fig. 6 A shows that RRp remained with the insoluble fraction after treatment with 0.5 M NaCl, 1.0 M NaCl, 0.1 M Na₂CO₃ (pH 11), or 1.6 M urea, but was released into the soluble fraction upon treatment with 1% Triton X-100. As a positive control, it was found that Sec61p, an integral ER membrane protein (Stirling et al., 1992), fractionated identically with RRp, whereas Kar2p, a luminal ER protein (Rose et al., 1989), was partially solubilized with 0.1 M Na₂CO₃ (pH 11) and 1.6 M urea and totally solubilized with 1% Triton X-100.

For RRp located in the yeast ER membrane to function as a ribosome receptor, it must be oriented toward the cytosol. Such a topology predicts that RRp should be sensi-

tive to proteolysis, whereas RRp in the alternative orientation should be resistant to exogenous protease unless the membrane is first solubilized with detergent. Samples of yeast microsomes containing RRp were subjected to digestion with trypsin in the presence and absence of Triton X-100 (Fig. 6 B). In the absence of detergent RRp was found to be sensitive to protease, resulting in three smaller protease-resistant fragments with sizes of about 79, 76, and 65 kD. In the presence of detergent, protease-resistant fragments were not observed. As a control, the digestion pattern of Sec63p, a 73-kD integral ER membrane protein with three transmembrane domains (Feldheim et al., 1992), was examined. Sec63p was found accessible to protease digestion with the pattern of digestion products in good agreement with the results of Feldheim et al. (1992).

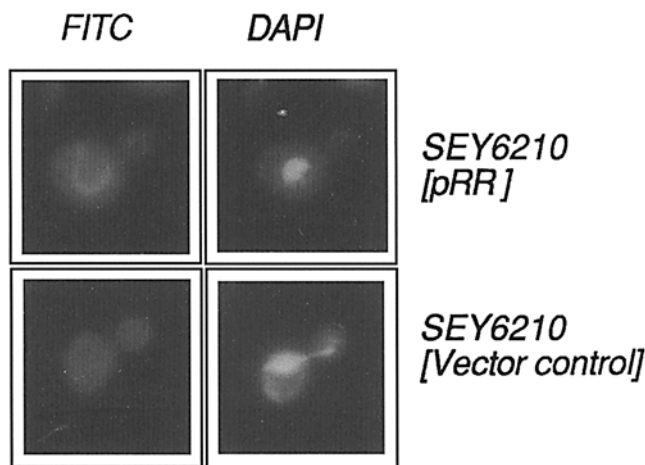


Figure 5. Immunofluorescent localization of RRp. SEY6210 (pEMBLyex4) and SEY6210(pRR) were grown in selective medium with galactose to induce RRp expression. (Left) The RRp specific antibody stains a bright perinuclear ring, with occasional thin filaments extending into cytoplasm. (Right) DAPI staining of DNA to localize the nuclei.

RRp Enhances the Ability of Yeast ER to Bind Ribosomes In Vitro and In Vivo

The functionality of recombinant RRp, ΔCTp, and ΔNTp, located in yeast microsomes, was tested using the in vitro ribosome binding assay of Borgese et al. (1974). Microsomes were stripped of ribosomes by treatment with puromycin and high salt (Savitz and Meyer, 1990). Stripped microsomes were incubated with an excess of radiolabeled HeLa cell ribosomes to obtain saturation levels and submitted to flotation in a sucrose gradient to separate bound from unbound ribosomes (Borgese et al., 1974). In this assay, microsomes containing RRp bound twice the number of ribosomes bound by the control (vector-only) microsomes (Fig. 7). Microsomes containing the NH₂-terminal domain (ΔCTp) bound four times the number of ribosomes as the control. In contrast, microsomes containing the COOH-terminal domain (ΔNTp) did not differ significantly from the control in their ribosome binding activity. These results indicate that within the context of this assay, RRp and ΔCTp function to bind ribosomes, and that the NH₂-terminal repeat-containing domain is essential for ribosome binding activity.

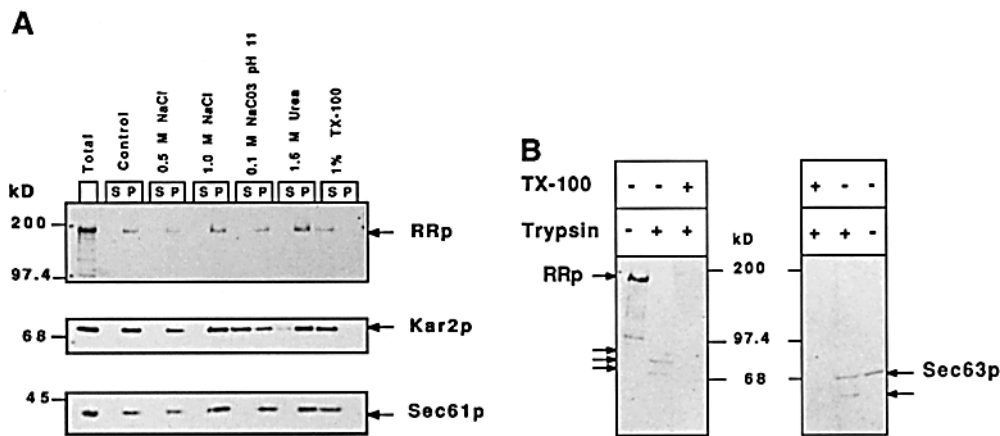


Figure 6. Characterization of membrane-bound RRp. (A) RRp is an integral membrane protein. Membrane fractions were prepared and treated with either 0.5 M NaCl, 1.0 M NaCl, 0.1 M Na₂CO₃ (pH 11), 1.6 M urea, or 1% Triton X-100. After incubation on ice for 20 min, all samples were separated into supernatant (S) and pellet (P) fractions by centrifugation (96,000 g), subjected to SDS-PAGE and immunoblotted with anti-RRp, anti-Kar2p, and anti-Sec61p antibodies. (B) RRp is accessible to exogenous protease. Rough microsomes containing RRp were digested with 10 μg/ml trypsin for one hour on ice in the presence or absence of Triton X-100. The digests were terminated by addition of 0.01 vol of 100 mM PMSF. Microsomes were suspended in sample buffer and analyzed by immunoblotting with an anti-RRp and anti-Sec63p antibody.

All experiments on ribosome binding carried out to date in this and other laboratories have used *in vitro* assays under a variety of conditions and reconstituted from a number of sources (Borgese et al., 1974; Connolly and Gilmore, 1986; Tazawa et al., 1991; Savitz and Meyer, 1990; Kalies et al., 1994). The expression of the RRp and its derivatives in yeast, provided an opportunity to examine ribosome binding *in situ*. Having established that yeast ribosomes can bind to mammalian microsomes (Wanker, E. E., and D. I. Meyer, unpublished observations), we speculated that the overexpression of RRp in yeast cells would lead to an increase in the number of ribosomes bound to ER membranes.

The electron micrographs of intact yeast cells expressing the various aforementioned constructs are shown in Fig. 8. Most striking is the fact that the expression of all constructs led to the proliferation of perinuclear membrane structures, previously identified and named "karmellae" (Fig. 8, A–C and E–G). Control strains (vector only) exhibited the subcellular morphology, and relative lack of discernible rough ER, seen in wild type yeast strains (Novick et al., 1980) (Fig. 8, D and H). Karmellae were first described in yeast cells overproducing the ER membrane protein HMG-CoA reductase as consisting of closely apposed pairs of membranes, morphologically identical to the normal double membrane of the nuclear envelope (Wright et al., 1988). Approximately 20–50% of all cells observed by Wright et al. (1988) exhibited karmellae.

We examined the ultrastructure of yeast cells that ex-

pressed RR cDNA and the various deletions. Karmellae were observed in 32–42% of all sections examined, except for vector-only controls (Table I). By comparison, nuclei were observed in 63–79% of all sections, including controls. The electron micrographs in Fig. 8, A and E illustrate an example of the karmellae found in the strain expressing the full-length RR. The organization of karmellae as pairs of membranes is apparent, as both leaflets of each membrane bilayer in the karmellar stacks are well resolved at higher magnification (Fig. 8 E). The spacing (72 nm) and organization of the membrane layers are typically very uniform and regular, although occasionally the layers exhibited discontinuity, gaps, and a tubular organization. The space in between the karmellae was typically elec-

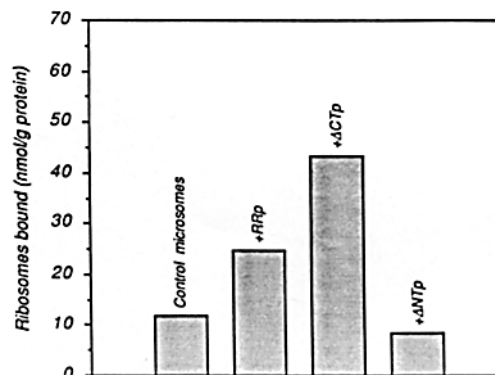


Figure 7. Binding of HeLa ribosomes to yeast microsomes. Control microsomes and microsomes containing RRp, ΔCTp, and ΔNTp, respectively, were treated with high salt and puromycin to remove bound ribosomes (Savitz and Meyer, 1993). Stripped microsomes (100 μg protein) were incubated for 15 min with 4.2 fmol of radiolabeled HeLa cell ribosomes and were then subjected to sucrose gradient centrifugation (Savitz and Meyer, 1990) to separate membrane bound from unbound ribosomes. The concentration of ribosomes added to microsomes was determined using a μM extinction coefficient of 60.8 at 260 nm (Collins and Gilmore, 1991). The extinction coefficient was calculated using 4.5×10^6 Da as the molecular mass for the 80S ribosome (Hamilton et al., 1971).

Table I. Appearance of Karmellae in Yeast Cells Expressing Recombinant Canine Ribosome Receptor Proteins

Construct	Percentage of sections with karmellae (n = 200)	Percentage of sections with nuclei (n = 200)
pEMBLyex4	0	63
pRR	38	69
pΔCT	42	79
pΔNT	32	75

Randomly chosen thin sections were examined for the presence of karmellae and of nuclei. Karmellae are defined as two or more flat cisternae arranged in a parallel array in the perinuclear region.

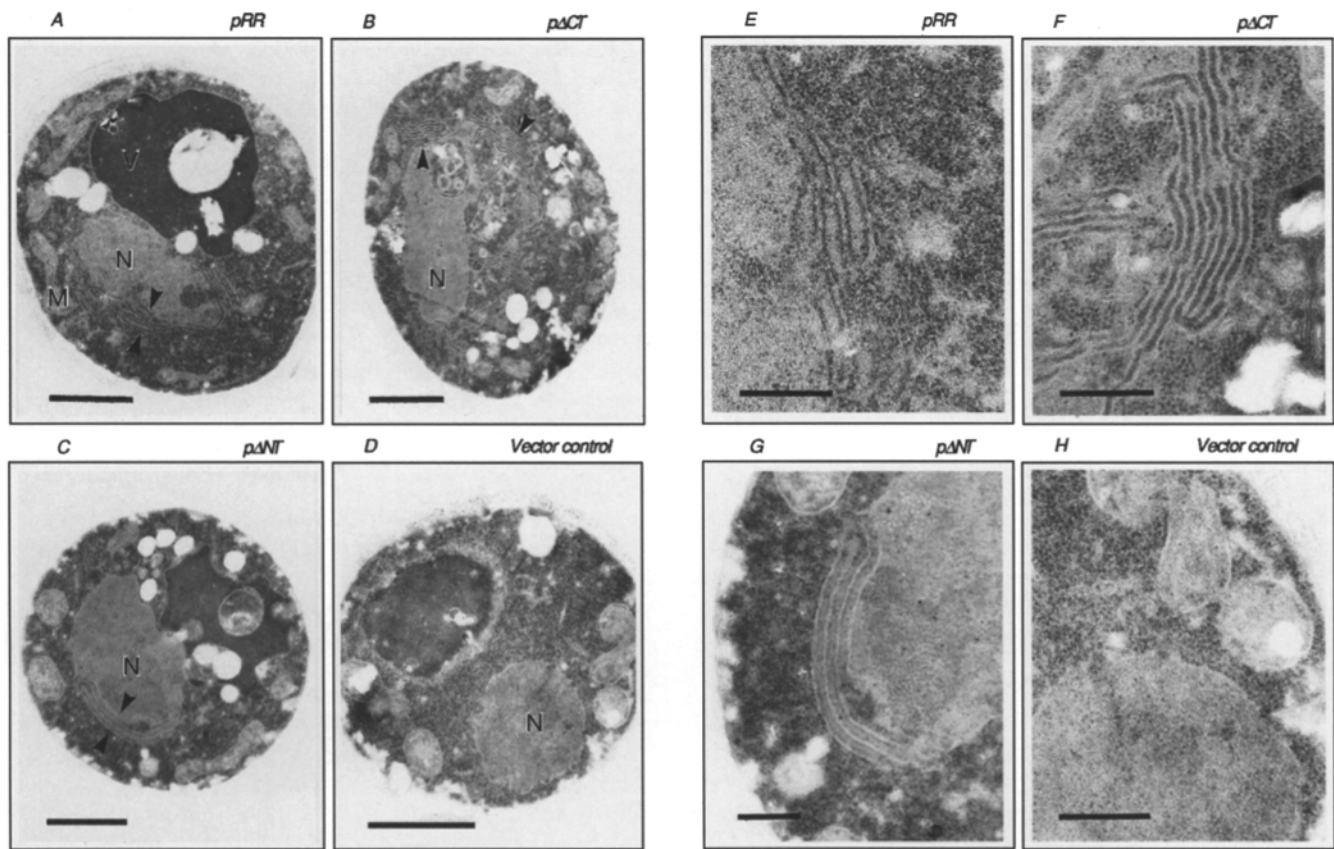


Figure 8. Thin-section electron micrographs of yeast cells. Cells were grown in selective complete media with galactose to induce the *GAL10-CYC1* promoter. (A and E) SEY6210 (*pRR*); (B and F) SEY6210 (*pΔCT*); (C and G) SEY6210 (*pΔNT*); (D and H) wild-type SEY6210 (*pEMBLyex4*). N, nucleus; V, vacuole; M, mitochondria; Arrows in A–C point to karmellae. Bars: (A–D) 1 μm ; (E–H) 0.3 μm .

tron-dense, owing to a high concentration of membrane-associated ribosomes, which are clearly visualized at higher magnification (Fig. 8 E). Expression of ΔCT cDNA also resulted in the formation of karmellae, with electron dense material in between membranes (Fig. 8, B and F). In this case, as the spacing between the layers was half as wide as was found between layers obtained by the expression of full-length RR cDNA, visualization of ribosomes as discrete particles was not as obvious.

In stark contrast was the visualization of cells expressing ΔNT , the RR protein lacking the putative ribosome binding domain. In these cells, karmellae were produced at levels similar to the cells harboring the RR construct. Most striking is the fact that the karmellae were almost uniformly smooth with no electron dense material observable between the membranes. Even though the spacing between karmellae was similar in size to RR-expressing cells, few if any ribosomes could be observed in these spaces.

To confirm and extend results obtained by electron microscopy (Fig. 8), and quantify ratios of smooth to rough membranes, a biochemical analysis was carried out. Microsomes were prepared from the yeast strains expressing the different RR constructs (Rothblatt and Meyer, 1986) and fractionated by sucrose gradient centrifugation into a light and heavy microsomal fraction. Sanderson and Meyer (1991) demonstrated that the light microsomal fraction is enriched in smooth ER membranes, whereas the heavy

microsomal fraction is composed of largely rough ER membranes which are competent for protein translocation. Electron microscopy suggested that the expression of full-length RR and ΔCT cDNA should lead to a microsomal fraction containing significantly more heavy than light microsomes, due to enhanced ribosome binding via the recombinant ribosome receptors. On the other hand, microsomes prepared from yeast expressing ΔNT cDNA should be enriched in light microsomes. The result of the fractionation of yeast microsomes is summarized in Table

Table II. Biochemical Characterization of Rough Microsome Content of Yeast Strains Expressing Recombinant Canine Ribosome Receptor Proteins

Construct	Ratio of heavy/light microsomes*	RNA content [†]	
		Light microsomes	Heavy microsomes
pEMBLyex4	1.16	0.34	1.31
pRR	1.75	0.48	1.76
p ΔCT	1.81	0.39	1.26
p ΔNT	0.36	0.30	1.87

Appropriate samples were taken in duplicate for RNA and phospholipid (PL) determinations. Each number represents an average value obtained by duplicate assays. The table gives values from two different membrane preparations and fractionations into heavy and light microsomes.

*Ratio heavy/light was calculated as $\mu\text{g PL}$ in the heavy fraction/ $\mu\text{g PL}$ in the light fraction.

[†]RNA content is defined as $\mu\text{g RNA}/\mu\text{g PL}$ in a given fraction.

II. Phospholipid content was used as a measure of membrane content of a given fraction. The ratio of rough/smooth microsomes in wild-type (vector-only) controls was about 1:1. In the case of RRp or Δ CTp expressing cells, the rough:smooth ratio was increased by an average of 75%. In stark contrast were strains expressing Δ NTp, where the proportion of smooth membranes was dramatically increased. As expected, the RNA content was greater in all heavy microsomal fractions as a result of enhanced ribosome binding. On the basis of the morphological and the biochemical analyses, we conclude that the RR protein confers the ability to bind ribosomes to membranes and does so via the repeat-rich domain at the NH₂ terminus.

Discussion

We have previously isolated and functionally characterized a 180-kD ER-specific integral membrane protein (p180) as a ribosome receptor (Savitz and Meyer, 1990, 1993) using an *in vitro* ribosome binding assay (Borgese et al., 1974). In this study we have isolated cDNAs and assembled a full-length clone encoding this protein which we now refer to as RRp. RRp contains a novel decapeptide motif tandemly repeated 54 times without interruption. Our working model describes this region as a RBD, and we have presented data here demonstrating that RRp has the ability to bind ribosomes via this region not only *in vitro*, but *in vivo* as well.

Structural Features of RRp

We synthesized full-length RRp, RRp without a COOH-terminal region (Δ CTp) and RRp without the repeat-rich domain (Δ NTp) in yeast. Even though this is a heterologous system for the production and characterization of RRp, it is not without precedent. Many mammalian proteins have been shown to be functionally expressed in yeast (Mellor et al., 1983; Tuite et al., 1982) and recent studies have demonstrated significant homology between the components of the translocation machinery in yeast and in mammalian cells. For example, homologues of the SRP particle (Hann et al., 1992; Stirling and Hewitt, 1992; Brown et al., 1994), the SRP receptor (Ogg et al., 1992), and Sec61p (Stirling et al., 1992) exist in yeast. *E. coli* also has many homologous components of the translocation machinery, and we considered expression of RRp in this organism. Unfortunately, synthesis of RRp resulted in a variety of degradation products (data not shown). In yeast, on the other hand, RRp and the truncated proteins, Δ CTp and Δ NTp, were produced with the expected sizes and only few, if any, degradation products were observed (Fig. 4 B).

The hydropathy profile of RRp revealed a single hydrophobic stretch of 28 residues close to the NH₂ terminus of sufficient length to span the lipid bilayer (Singer et al., 1987). Treatment of yeast microsomes containing RRp with high salt, urea or Na₂CO₃ (pH 11; Fujiki et al., 1982) demonstrated that RRp behaves exclusively as an integral membrane protein. Thus, the predicted membrane-spanning domain appears to be sufficient for the integration of RRp into the membrane. It is also necessary for ER insertion, since the expression of an RR construct lacking the NH₂-terminal hydrophobic amino acids resulted in an

RRp molecule that was not anchored in the membrane (data not shown). RRp was shown to be located in the yeast ER by both biochemical (cell fractionation and immunoblotting) and morphological (immunofluorescence microscopy) analysis. Taken together, these findings indicate that the predicted membrane anchor sequence at the NH₂ terminus of RRp is functional in yeast and that RRp contains a sequence that targets the protein to the expected location. Analysis of the amino acid sequence of RRp did not reveal the ER retention motif found in some membrane proteins (Jackson et al., 1990; Schultze et al., 1994), indicating that RRp may contain a retention signal that is different from the RR or KK motif. We have shown previously that the mammalian rough ER-specific membrane protein ribophorin I is also retained in the yeast rough ER despite a lack of the basic retention motif (Sanderson et al., 1990).

A functional ribosome receptor must have a cytosolic disposition in the ER membrane. Savitz and Meyer (1990) demonstrated that treatment of stripped canine microsomes with low concentrations of thermolysin resulted in the release of a 160-kD ribosome receptor fragment with the capacity to inhibit ribosome binding *in vitro*. An antibody raised against this fragment aided in the identification of the corresponding full length protein, which had an apparent molecular weight of 180 kD. RRp integrated into yeast microsomes was also found to be sensitive to exogenously added protease (Fig. 6 B). Overall, the topology of the RRp most closely resembles that of a major component of the ER translocation apparatus, the SRP receptor α subunit, also known as the docking protein (Lauffer et al., 1985; Hortsch et al., 1988). In the case of this integral rough ER membrane protein, a largely hydrophobic membrane insertion/anchor sequence is found at its NH₂ terminus. Just as the docking protein does not contain a cleavable signal sequence, and does not require the SRP-mediated targeting mechanism (Hortsch and Meyer, 1988; Andrews et al., 1989), we imagine that RRp may be targeted and inserted into the ER in a similar fashion.

Functional Role of RRp in Binding Ribosomes to ER Membranes

Initially, we examined the ability of RRp, Δ CTp or Δ NTp expressed in yeast microsomes to bind mammalian ribosomes using the *in vitro* ribosome binding assay of Borgese et al. (1974). Under these conditions, microsomes containing RRp or Δ CTp were able to bind two to four times as many ribosomes as control membranes lacking a recombinant protein or microsomes containing Δ NTp. This indicates that RRp and Δ CTp present in yeast microsomes are functional, and that yeast represents a useful system to assemble and test membrane proteins *in vivo*.

The ability of the standard *in vitro* ribosome binding assay of Borgese et al. (1974) to unambiguously characterize a ribosome binding protein has to be questioned, as several different proteins including p180 (Savitz and Meyer, 1990, 1993), p34 (Tazawa et al., 1991; Ichimura et al., 1992, 1993; Ohsumi et al., 1993) and Sec61p (Görlich et al., 1992; Kalies et al., 1994) have been identified as ribosome receptors using essentially the same assay. The assay has several limitations: (a) it uses radiolabeled ribosomes which lack nascent chains, and are not organized into the polysomes

representative of the typical state of membrane bound ribosomes (Palade, 1975); (b) stripping of microsomes with high salt and puromycin is not physiological and may alter the true ribosome binding ability of membrane proteins; (c) the assay has been of greatest value under what may be lower than physiological salt concentrations; and (d) it is difficult to unequivocally rule out the possibility that binding activity is due to non-specific electrostatic interactions which could theoretically occur in vitro between ribosomes and microsomes. To overcome these limitations and to go beyond in vitro ribosome binding data we have developed experiments to study ribosome binding in situ, by examining the expression of RR-based constructs in yeast cells using electron microscopy.

Wright et al. (1988) demonstrated that the overexpression of the of HMG-CoA reductase gene, which encodes an ER membrane protein, induced the perinuclear proliferation of ER membranes termed "karmellae." Karmellae are morphologically similar to the normal double membrane of the nuclear envelope, but do not contain attached ribosomes. We found that expression of Δ NTP (Fig. 8, C and G) resulted in the proliferation of smooth membranes which most closely resembled the karmellae characterized by Wright et al. On the other hand, yeast synthesizing RRp or Δ CTp induced karmellae-like structures that were clearly studded with electron-dense material consistent with the appearance of bound ribosomes (Fig. 8, A and B and E and F). Thus, forms of RRp which contain the repeat-containing domain are capable of binding ribosomes in situ, while those lacking it are not. This is in good agreement with the ribosome binding data obtained with the in vitro ribosome binding assay and indicates that the use of this assay, despite its limitations, is reasonable at least as an initial screen for ribosome-binding proteins.

Preliminary experiments indicate that the RBD binds to ribosomes via an interaction with ribosomal RNA (Sun, Y., A. J. Savitz, and D. I. Meyer, unpublished results), but further investigations using the recombinant proteins described in this study will be carried out to address this question in detail. Ribosomal proteins containing motifs with amino acid compositions similar to the repeated decapeptide NQGKKAEGAP of RRp have been reported in the databases. These motifs range from 8 to 12 amino acids in length, but are repeated usually only two to four times. They are postulated to be involved in the recognition of ribosomal RNA by the individual ribosomal proteins (Wool et al., 1990). One could imagine that the sizeable number of uninterrupted repeats in RRp could provide an adequately sized surface for the stable binding of a large ribonucleoprotein complex (e.g., a ribosome) to the ER membrane.

The influence of ribosome binding mediated by RRp on the translocation process itself was not investigated in this study. Previous work in our laboratory (Savitz and Meyer, 1993), using immunodepletion and readdition of p180 from translocation-competent proteoliposomes (Nicchitta et al., 1991), has shown a requirement for p180 within the context of all of the proteins normally residing in the rough ER. However, Görlich and Rapoport (1993) showed that in artificial lipid vesicles, only five proteins are required to achieve the translocation of in vitro-synthesized presecretory proteins. As p180 was not present in these liposomes, it was concluded that it was not essential to the transloca-

tion process in general, and that the Sec61p complex has sufficient ribosome binding capacity to fulfill such a role (Kalies et al., 1994). Since a requirement for ribosome binding in this simplified translocation assay has never been demonstrated, concluding that p180 is dispensable is somewhat premature. Moreover, in vitro translocation into liposomes is generally inefficient (Nicchitta and Blobel, 1990), which raises the possibility that in vivo or in more representative in vitro systems (using intact membranes or proteoliposomes), additional factors such as RRp may be beneficial, or even essential (Savitz and Meyer, 1993).

However, in light of the finding that only five polypeptides are needed to translocate a protein through a lipid bilayer (Görlich and Rapoport, 1993), it may be especially appropriate to consider an alternative hypothesis to describe the ribosome binding role of RRp in vivo. We have clearly shown that the appearance of bound ribosomes is linked to the presence of an RBD-containing RRp in vivo. Is it possible that a great number of the ribosomes bound to the ER membrane are not actively involved in the translocation process? A regulated mechanism for the sequestration of protein synthetic capacity at a particular site within the cell could allow the cell to respond rapidly to secretory cues, or merely to guarantee a high and sustained secretory output. One observation consistent with this notion is the finding that virtually half of all bound ribosomes can be removed from rough microsomes with high salt alone (Adelman et al., 1973), in the absence of puromycin. This implies that a major portion of the population of bound ribosomes are "parked" on the membrane and not actively involved in protein synthesis and translocation. We are currently pursuing studies to investigate whether RRp is an integral component of a regulated ribosomal parking lot within the ER membrane. Relevant to regulation is the detection of an ATP binding site located in the C-terminal region of the protein (Wanker, E. E., and D. I. Meyer, unpublished data). This property could provide a regulatory function for the COOH terminus of RRp, since we have shown that it is not essential for ribosome binding per se.

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References

- Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934-945.

- Adelman, M. R., D. D. Sabatini, and G. Blobel. 1973. Nondestructive disassembly of rat liver rough microsomes into ribosomal and membranous components. *J. Cell Biol.* 56:206-229.
- Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8:115-118.
- Andrews, D. W., L. Lauffer, P. Walter, and V. R. Lingappa. 1989. Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. *J. Cell Biol.* 108:797-810.
- Baldari, C., and G. Cesareni. 1985. Plasmids pEMBL Y: new single-stranded shuttle vectors for the recovery and analysis of yeast DNA sequences. *Gene (Amst.)* 35:27-32.
- Borgese, N., W. Mok, G. Kreibich, and D. D. Sabatini. 1974. Ribosome-membrane interaction: *in vitro* binding of ribosomes to microsomal membranes. *J. Mol. Biol.* 88:559-580.
- Brown, J. D., B. C. Hann, K. F. Medzihradsky, M. Niwa, A. L. Burlingame, and P. Walter. 1994. Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4390-4400.
- Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA.* 69:2110-2114.
- Clark, S. W., and D. I. Meyer. 1992. Contractin is an actin homologue associated with the centrosome. *Nature (Lond.)* 359:246-250.
- Collins, P. G., and R. Gilmore. 1991. Ribosome binding to the endoplasmic reticulum: a 180-kD protein identified by crosslinking to membrane bound ribosomes is not required for ribosome binding activity. *J. Cell Biol.* 114:639-649.
- Connolly, T., and R. Gilmore. 1986. Formation of a functional ribosome-membrane junction during translocation requires the participation of a GTP-binding protein. *J. Cell Biol.* 103:2253-2261.
- Dobberstein, B. 1994. On the beaten pathway. *Nature (Lond.)* 367:599-600.
- Feldheim, D., J. Rothblatt, and R. Schekman. 1992. Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Mol. Cell Biol.* 12:3288-3296.
- Fleck, A., and H. N. Munro. 1962. The precision of ultraviolet absorption measurement in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. Biophys. Acta.* 55:571-583.
- Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497-509.
- Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.
- Gilmore, R., P. Walter, and G. Blobel. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of a signal recognition particle receptor. *J. Cell Biol.* 95:470-477.
- Görlich, D., S. Prehn, E. Hartmann, K. U. Kalies, and T. A. Rapoport. 1992. A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. *Cell.* 71:489-503.
- Görlich, D., and T. A. Rapoport. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell.* 75:615-630.
- Hamilton, M. G., A. Pavlovic, and M. L. Petermann. 1971. Molecular weight, buoyant density and composition of active subunits of rat liver ribosomes. *Biochemistry.* 10:3424-3427.
- Hann, B. C., C. J. Stirling, and P. Walter. 1992. SEC65 gene product is a subunit of the yeast signal recognition particle required for its integrity. *Nature (Lond.)* 356:532-533.
- Herz, J., N. Flint, K. Stanley, R. Frank, and B. Dobberstein. 1990. The 68 kD protein of signal recognition particle contains a glycine-rich region also found in certain RNA-binding proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 276:103-107.
- High, S., and B. Dobberstein. 1992. Membrane protein insertion into the endoplasmic reticulum: signals, machinery and mechanism. In *New Comprehensive Biochemistry*. W. Neupert, and R. Lill, editors. Elsevier Science Publishers, Amsterdam. 105-118.
- Hortsch, M., and D. I. Meyer. 1988. The human docking protein does not associate with the membrane of the rough endoplasmic reticulum via a signal or insertion sequence-mediated mechanism. *Biochem. Biophys. Res.* 150:111-117.
- Hortsch, M., D. Avossa, and D. I. Meyer. 1986. Characterization of secretory protein translocation: ribosome-membrane interaction in endoplasmic reticulum. *J. Cell Biol.* 103:241-253.
- Hortsch, M., S. Labelit, and D. I. Meyer. 1988. Complete cDNA sequence from human docking protein. *Nucleic Acids Res.* 16:361-362.
- Ichimura, T., T. Ohsumi, Y. Shindo, T. Ohwada, H. Yagame, Y. Momose, S. Omata, and H. Sugano. 1992. Isolation and some properties of a 34-kD membrane protein that may be responsible for ribosome binding in rat liver rough microsomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 296:7-10.
- Ichimura, T., Y. Shindo, Y. Uda, T. Ohsumi, S. Omata, and H. Sugano. 1993. Anti-(p34 protein) antibodies inhibit ribosome binding to and protein translocation across the rough microsomal membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 326:241-245.
- Ito, H., Y. Fukada, F. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163-168.
- Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153-3162.
- Kalies, K.-U., D. Görlich, and T. A. Rapoport. 1994. Binding of ribosomes to the rough endoplasmic reticulum mediated by the Sec61p-complex. *J. Cell Biol.* 126:925-934.
- Kreibich, G., E. E. Marcantonio, and D. D. Sabatini. 1983. Ribophorin I and II: membrane proteins characteristic of the rough endoplasmic reticulum. *Methods Enzymol.* 96:520-530.
- Lauffer, L., P. D. Garcia, R. N. Harkins, L. Coussens, A. Ullrich, and P. Walter. 1985. Topology of signal recognition particle receptor in endoplasmic reticulum membrane. *Nature (Lond.)* 318:334-338.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Mellor, J., M. J. Dobson, N. A. Roberts, M. F. Tuite, J. S. Emtage, S. White, P. A. Lowe, T. Partel, A. J. Kingsman, and S. M. Kingsman. 1983. Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. *Gene (Amst.)* 24:1-14.
- Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes: the role of the "docking protein." *Nature (Lond.)* 297:647-650.
- Mothes, W., S. Prehn, and T. A. Rapoport. 1994. Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3973-3982.
- Nicchitta, C. V., and G. Blobel. 1990. Assembly of translocation competent proteoliposomes from detergent-solubilized rough microsomes. *Cell.* 60:259-269.
- Nicchitta, C. V., G. Mighaccio, and G. Blobel. 1991. Biochemical fractionation and assembly of the membrane components that mediate nascent chain targeting and translocation. *Cell.* 65:587-598.
- Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 21:205-215.
- Ogg, S. C., M. A. Poritz, and P. Walter. 1992. Signal recognition particle receptor is important for cell growth and protein secretion in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 3:895-911.
- Ohsumi, T., T. Ichimura, H. Sugano, S. Omata, T. Isob, and R. Kuwano. 1993. Ribosome-binding protein p34 is a member of the leucine-rich-repeat-protein superfamily. *Biochem. J.* 294:465-472.
- Palade, G. E. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. DC)* 189:347-358.
- Payne, G. S., T. B. Hasson, M. S. Hasson, and R. Schekman. 1987. Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 7:3888-3898.
- Pringle, J. R., A. E. M. Adams, D. G. Drubin, and B. K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565-602.
- Rose, M. D., L. M. Misra, and J. P. Vogel. 1989. KAR2, a karyogamy gene, is the yeast homolog of the mammalian BIP/GRP78 gene. *Cell.* 57:1211-1221.
- Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: Reconstitution of the translocation and glycosylation of a-factor and invertase in a homologous cell-free system. *Cell.* 44:619-628.
- Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell.* 69:353-366.
- Sanderson, C. M., J. S. Crowe, and D. I. Meyer. 1990. Protein retention in yeast rough endoplasmic reticulum: expression and assembly of human ribophorin I. *J. Cell Biol.* 111:2861-2870.
- Sanderson, C. M., and D. I. Meyer. 1991. Purification and functional characterization of membranes derived from rough endoplasmic reticulum of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 266:13423-13430.
- Savitz, A. J., and D. I. Meyer. 1990. Identification of a ribosome receptor in the rough endoplasmic reticulum. *Nature (Lond.)* 346:540-544.
- Savitz, A. J., and D. I. Meyer. 1993. 180-kD ribosome receptor is essential for both ribosome binding and protein translocation. *J. Cell Biol.* 120:853-863.
- Schultz, M.-P., P. A. Peterson, and M. R. Jackson. 1994. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1696-1705.
- Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Singer, S. J., P. A. Maher, and M. P. Yaffe. 1987. On the transfer of integral proteins into membranes. *Proc. Natl. Acad. Sci. USA.* 84:1960-1964.
- Stirling, C. J., and E. W. Hewitt. 1992. The *S. cerevisiae* SEC65 gene encodes a component of yeast signal recognition particle with homology to human SRP19. *Nature (Lond.)* 356:534-537.
- Stirling, C. J., J. Rothblatt, M. Hosobuchi, R. Deshaies, and R. Schekman. 1992. Protein translocation mutants defective in insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. Cell.* 3:129-142.
- Tazawa, S., M. Unuma, N. Tondokoro, Y. Asano, T. Ohsumi, T. Ichimura, and H. Sugano. 1991. Identification of a membrane protein responsible for ribosome binding in rough microsomal membranes. *J. Biochem.* 109:89-98.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Tuite, M. F., M. J. Dobson, N. A. Roberts, R. M. King, D. C. Burke, S. M. Kingsman, and A. J. Kingsman. 1982. Regulated high efficiency expression of

- human interferon-alpha in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:603-608.
- Walter, P., I. Ibrahimi, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition particle (SRP) binds to *in vitro* assembled polysomes synthesizing secretory proteins. *J. Cell Biol.* 91:545-550.
- Wilsbach, K., and G. S. Payne. 1993. Vps1p a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3049-3059
- Wool, I. G., Y. Endo, Y.-L. Chan, and A. Gluck. 1990. Structure, function, and evolution of mammalian ribosomes. In *The Ribosome: Structure, Function and Evolution*. W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner, editors. American Society for Microbiology, Washington, D. C. 203-214.
- Wright, R., M. Basson, L. D'Ari, and J. Rine. 1988. Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. *J. Cell Biol.* 107:101-114.