the inhibitory peptide which does not affect CaMKII activity. The inhibitor (273–302) is a 30-amino-acid synthetic peptide that mimics the auto-inhibitory sequence of CaMKII and inhibits CaMKII activity[17,18]. Addition of 20 μM CaMKII inhibitory peptide (273–302) prevents the ATP-dependent inactivation of the calcium release channel (Fig. 3, middle). Its effect is reversible; removal of the inhibitory peptide restores ATP sensitivity to the patch. Note that in this experiment, channel P_o varied considerably after the addition of ATP. This was a common finding (never seen when ATP-γS was the substrate) and may indicate the presence of endogenous phosphatase activity[19]. A peptide inhibitor of protein kinase C (corresponding to residues (19–36) did not affect channel gating, suggesting that inactivation results specifically from the activity of CaMKII (Table 1).

Our results provide direct evidence that CaMKII modulates the activity of the SR ryanodine receptor protein/calcium release channel of skeletal muscle when studied in its native lipid environment. An analysis of the published sequence of the ryanodine receptor protein showed no homology with the consensus sequences for the active sites of CaMKII[20,21], suggesting that the channel protein is not capable of autophosphorylation. The calcium release channel is known to be a substrate for endogenous calmodulin-dependent protein kinase[16,20], but our results do not allow identification of the protein being phosphorylated in the membrane patch. It has been demonstrated that the cardiac form of the ryanodine receptor protein is more highly phosphorylated by CaMKII than the skeletal form and that phosphorylation increases, rather than decreases, activity of the cardiac receptor in lipid bilayers[22]. It is possible that phosphorylation of regulatory proteins that coexist with the skeletal release channel in our membrane patches could mediate inactivation[16,23]. Nonetheless, our results indicate that the calcium release channel from skeletal muscle is closely associated with the kinase and its cofactors. This conclusion follows from the observation that channels isolated in patch pipettes could be modulated by exposure to phosphatase or to ATP and its analogues without addition of exogenous kinase. A similar association between a channel protein and a modulating kinase has recently been proposed for a Ca^{2+}-activated K^+ channel[19]. Such associations may prove to be typical for enzymatically modulated ion channels. □

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Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex

Joen Luirink*, Stephen High, Heather Wood, Angelika Giner, David Tollervey & Bernhard Dobberstein

European Molecular Biology Laboratory, Postfach 102209, Meyerhofstrasse 1, 6900 Heidelberg, Germany

HYDROPHOBIC signal-sequences direct the transfer of secretory proteins across the inner membrane of prokaryotes and the endoplasmic reticulum membranes of eukaryotes[1]. In mammalian cells, signal-sequences are recognized by the 54K protein (M_r 54,000) of the signal recognition particle (SRP)[2,3] which is believed to hold the nascent chain in a translocation-competent conformation until it contacts the endoplasmic reticulum membrane[4]. The SRP consists of a 7S RNA and six different polypeptides. The 7S RNA and the 54K signal-sequence-binding protein (SRP54) of mammalian SRP exhibit strong sequence similarity to the 4.5S RNA and P48 protein (Ffh) of *Escherichia coli*[5–7] which form a ribonucleoprotein particle. Depletion of 4.5S RNA or overproduction of P48 causes the accumulation of the β-lactamase precursor, although not of other secretory proteins[8,9]. Whether 4.5S RNA and P48 are part of an SRP-like complex with a role in protein export is controversial. Here we show that the P48/4.5S RNA ribonucleoprotein complex interacts specifically with the signal sequence of a nascent secretory protein and therefore is a signal recognition particle.

* Present address: Department of Molecular Microbiology, Faculty of Biology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

SRP54 was identified as the component of the SRP that interacts with the signal sequence of nascent preprolactin and other secretory and membrane proteins by light-induced crosslinking[2,3]. We used a similar approach to identify proteins in an *E. coli* cell extract that interact with nascent polypeptides. A truncated messenger RNA, encoding the amino-terminal 86 amino-acid residues of preprolactin, was translated in a wheat-germ cell-free translation system, to generate nascent chain-ribosome complexes with an exposed signal sequence. As a control we used an mRNA encoding a mutant preprolactin with three amino-acid substitutions in the hydrophobic core of the signal sequence. This mutated nascent chain is unable to bind SRP54 and is not translocated across the endoplasmic reticulum membrane (not shown). Translation was done in the presence of [^{35}S]methionine to label the polypeptide, and in the presence of ε-TDBA 4-(3-trifluoromethyldiazirino)benzoic acid)-Lys-transfer RNA, to allow incorporation of photoreactive groups at positions 4 and 9 of the signal sequence. The nascent chain-ribosome complex was then purified and incubated with a crude *E. coli* cell extract.

Ultraviolet irradiation yielded a crosslinked product of M_r 56K (Fig. 1, lane 5) which was not seen in a control, nonirradiated sample (Fig. 1, lane 1). There is a 9K contribution from the nascent chain, and a crosslinked 'partner' of ~48K. The product was not visible when the nascent chain was derived from the mutant preprolactin (Fig. 1, lane 7), indicating that the ~48K protein interacts specifically with functional signal-sequences. The amount of the 56K product increased when the *E. coli* extract was prepared from a strain that overproduces P48 (Fig. 1, lane 6). As 4.5S RNA is synthesized in excess of P48 (ref. 10), it is likely that some of the overproduced P48 interacts with the free 4.5S RNA thereby increasing the amount of P48/4.5S RNA ribonucleoprotein complex. An antiserum directed against P48 immunoprecipitated the crosslinked

product (Fig. 1, lane 13), whereas a control antiserum did not (Fig. 1, lane 9). The amount of the immunoprecipitated product was increased when the P48-enriched *E. coli* extract was used (Fig. 1, lane 14). Similar results were obtained when the truncated nascent chain, including the N-terminal signal-sequence, was derived from *E. coli* murein lipoprotein (not shown). These results indicate that *E. coli* P48 binds specifically to signal sequences.

A small amount of a 64K product was found on irradiation of nascent mutant preprolactin, incubated in the presence of wild-type and P48-enriched *E. coli* extract (Fig. 1, lanes 7 and 8). This unidentified product was not immunoprecipitated with the anti-P48 serum (Fig. 1, lanes 15 and 16). It is possible that the nonfunctional signal sequence misfolds and binds to a cytosolic chaperone.

When purified canine SRP and *E. coli* cell extract were added simultaneously to nascent preprolactin-ribosome complexes before crosslinking, SRP54 was crosslinked in place of P48 (Fig. 2*a*, lane 2), indicating that the two proteins bind to the same site in nascent preprolactin.

SRP54 only interacts with nascent proteins[11]. To test whether this is also true for P48, we disrupted the nascent chain–ribosome complexes by puromycin before adding *E. coli* cell extract. A large reduction in P48 crosslinking was observed (Fig. 2*b*, lanes 1 and 2). Thus P48 also binds inefficiently, or not at all, to the signal sequences of proteins that have been released from the ribosome.

We then investigated the stability of the interaction between P48 and the preprolactin signal-sequence. P48 in wild-type *E. coli* extract was allowed to bind to nascent chains. Ribosome-nascent chain complexes were treated with high salt and/or puromycin, and then crosslinking was induced. Puromycin treatment virtually abolished P48 crosslinking (Fig. 2*c*, lanes 3 and 4), suggesting that P48 does not remain associated with polypeptides that have been released from the ribosome. Under these conditions, P48 may be displaced by other cytosolic or membrane components in the *E. coli* extract, although no new crosslinking partners were detected (Fig. 2*c*, lanes 3 and 4). Alternatively, the nascent chain could undergo conformational changes when released from the ribosome, thereby losing its affinity for P48. The interaction between P48 and the nascent chain–ribosome complex was substantially resistant to high salt alone (Fig. 2*c*, lane 2). Similar properties have been reported for SRP54 (ref. 12).

P48 is associated with the 4.5S RNA in *E. coli* lysates[8,9], so to determine whether P48 binds to signal sequences as part of a ribonucleoprotein particle, we compared the sucrose gradient mobility of P48, 4.5S RNA and the 56K crosslinked product (see Fig. 1). Nascent preprolactin-ribosome complexes were purified and incubated with wild-type *E. coli* extract. After crosslinking, the products were released from the ribosome by a puromycin/high-salt treatment and separated on a 5–20% sucrose gradient. The fractions were analysed by fluorography, western blotting, and northern blotting (Fig. 3). P48 and 4.5S RNA sedimented primarily in fractions 6 and 7 (Fig. 3*b*, *c*), whereas the 56K crosslinked product was most abundant in fraction 7 (Fig. 3*a*), possibly owing to the contribution of the nascent chain. Under these conditions, most free P48 from a strain that overproduces P48 shows a different distribution. It is found from fraction 3 onwards, probably because of aggregation (not shown), suggesting that all P48 present in a wild-type *E. coli* extract which interacts with nascent preprolactin is present in a complex with 4.5S RNA. To investigate whether P48 also binds to signal sequences as a free protein, an *E. coli* lysate depleted of 4.5S RNA[13] was used in the crosslinking assay. The efficiency of crosslinking to P48 was greatly reduced (Fig. 2*d*, lanes 1 and 2), although P48 was present in the lysate as determined by western blotting (not shown). The 4.5S RNA in an *E. coli* extract is thus essential for the signal recognition function of P48.

Taken together, these data indicate that an *E. coli* ribonucleoprotein particle containing P48 and 4.5S RNA interacts specifically with the signal sequence of nascent presecretory proteins, and therefore can be considered as an SRP. This interpretation is supported by recent data (H. D. Bernstein and P. Walter, manuscript in preparation) showing that P48 can be reconstituted into mammalian SRP to replace SRP54 in signal-sequence recognition. Furthermore, depletion of

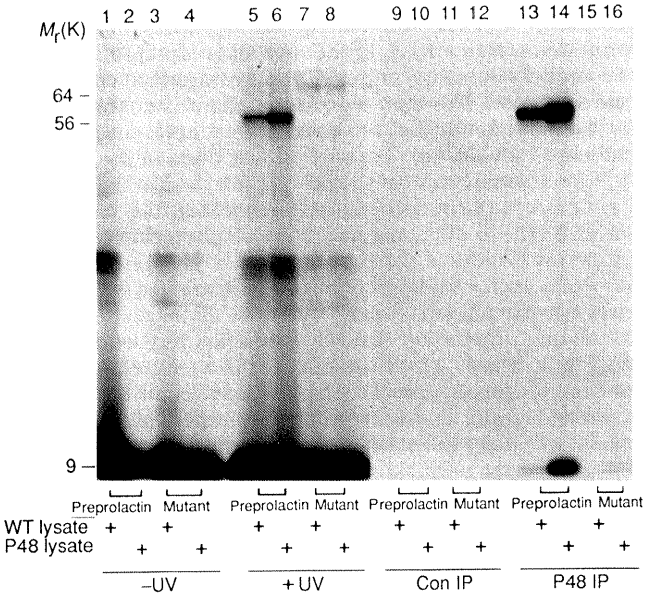

FIG. 1 Specific crosslinking of *E. coli* P48 to nascent preprolactin. A truncated transcript encoding the 86 N-terminal amino-acids of preprolactin or mutant preprolactin was translated in a wheat-germ extract containing [^{35}S]methionine and ε-TDBA-Lys-tRNA. The mutant preprolactin carries three amino-acid substitutions in the signal peptide (Leu 15 → Pro, Val 19 → Glu and Leu 23 → Arg) and serves as a control for signal-sequence specificity. Purified ribosome-nascent chain complexes (15 μl) were incubated with *E. coli* cell extract (10 μl) in the presence of 2 mM ATP, 40 μM GTP and an ATP-regenerating system for 5 min at 25 °C. The extracts were prepared from a wild-type *E. coli* strain (WT lysate) or from a strain that overproduces P48 (P48 lysate). The samples were chilled on ice for 5 min and subjected to ultraviolet irradiation (+UV; lanes 5–16). Subsequently, the samples were TCA precipitated (lanes 1–8) or immunoprecipitated under denaturing conditions using an antiserum directed against P48 (P48IP, lanes 13–16) or a nonrelated control antiserum (ConIP, lanes 9–12). The samples were analysed on 10–15% SDS–polyacrylamide gels and subjected to fluorography. METHODS. The generation of nascent preprolactin by *in vitro* transcription and translation was as described[12]. Ribosome-nascent chain complexes were purified by centrifugation through a 0.5 M sucrose cushion[17] and resuspended in 15 μl buffer (100 mM potassium acetate, 5 mM magnesium acetate, 50 mM HEPES, pH 7.9, and 2 mM cycloheximide) per 20 μl translation mixture loaded. *E. coli* extracts were prepared from strain MC4100 harbouring either the expression vector pDS12 (WT) or pDS12-48 (P48) which contains the cloned P48 gene under transcriptional control of the T5 promoter[9]. The cells were grown in Luria broth plus 0.4% glucose to an absorbance at 600 nm of 0.3, and P48 overexpression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG). The cells were collected after 2 h of induction and lysed by vortexing 40 A_{600} units of cells in 0.5 ml lysis buffer (150 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM dithiothreitol) with an equal volume of glass beads (Sigma) for 5 min at 4 °C. Immunoprecipitation was done as described[18].

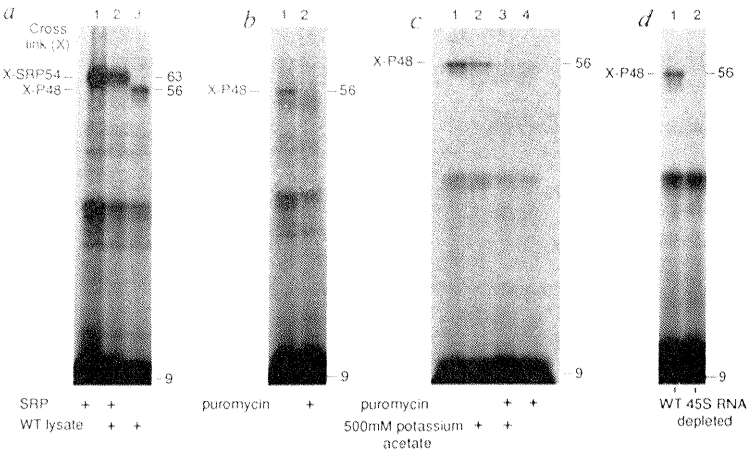

FIG. 2 Characteristics of the interaction between *E. coli* P48 and nascent preprolactin. *a*, SRP inhibits the interaction of P48 with nascent preprolactin. Purified canine SRP (2.5 pmol) (lane 1), 10 μl wild-type *E. coli* extract (lane 3) or both (lane 2) were added to 15 μl nascent preprolactin-ribosome complexes before crosslinking. *b*, P48 only interacts with ribosome-bound nascent preprolactin. Purified nascent preprolactin-ribosome complexes were incubated with 500 mM potassium acetate (final) in the presence (lane 2) or absence (lane 1) of 2 mM puromycin for 5 min at 25 °C, before the addition of wild-type *E. coli* lysate and crosslinking. *c*, P48 does not remain bound to nascent preprolactin after release of the P48-nascent chain complex from the ribosome. Nascent preprolactin-ribosome complexes were incubated with wild-type *E. coli* extract as described above and subsequently with H_2O (lane 1), 500 mM potassium acetate (lane 2), 2 mM puromycin (lane 4) or both puromycin and potassium acetate (lane 3) for 5 min at 25 °C followed by crosslinking. *d*, Expression of 4.5S RNA is essential for the interaction of P48 from an *E. coli* extract with nascent preprolactin. Nascent chain-ribosome complexes were incubated with an *E. coli* extract prepared from strain FF283 grown in the presence (lane 1) or absence (lane 2) of IPTG to induce 4.5S RNA expression, and then crosslinking was induced.
METHODS. The crosslinking assays were as described in the legend to Fig. 1. Canine SRP was purified as described[19]. An *E. coli* extract depleted for 4.5S RNA was prepared from strain FF283 in which 4.5S RNA synthesis is under control of the *Tac* promoter[1,3]. Cells were grown from fresh plates in M9 medium plus 0.4% glucose for 5 h at 37 °C in the absence of IPTG[9]. After this the culture turbidity of the 4.5S RNA depleted cells was similar to that of cells grown with 1 mM IPTG, indicating that cell death had not occurred.

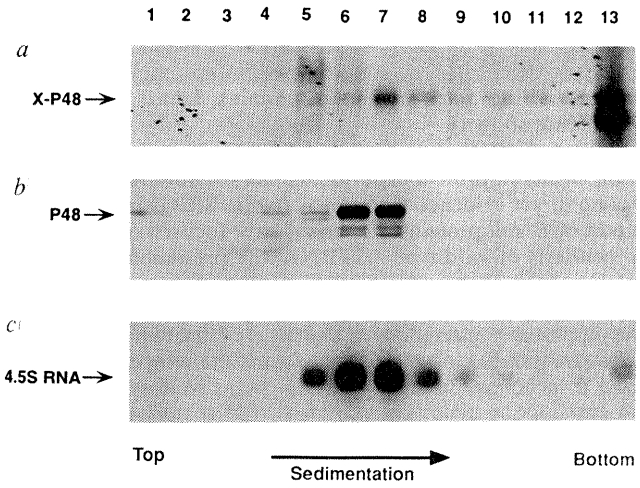

FIG. 3 *E. coli* P48 interacts with nascent preprolactin as part of a ribonucleoprotein particle. Crosslinked complexes were released from the ribosome by addition of 2 mM puromycin and 500 mM potassium acetate followed by incubation for 5 min at 25 °C. The samples were centrifuged for 30 h in a Beckman SW40 rotor at 39,000 r.p.m. and 4 °C through a 5–20% sucrose gradient containing 500 mM KCl, 2 mM $MgCl_2$, 50 mM tetraethylammonium-HCl, pH 7.5, 0.1% β-mercaptoethanol and 0.1 mM PMSF. Fractions of 1 ml were collected from the top, and portions were analysed by SDS-PAGE and fluorography (*a*), by western blotting using an antiserum directed against P48 (*b*) and by northern blotting using a 4.5S RNA-specific probe (*c*).
METHODS. Crosslinked complexes were prepared as described in Fig. 1 except that emetine was added to 2 mM instead of cycloheximide, to inhibit further chain elongation. Western blotting to detect P48 and northern blotting to detect 4.5S RNA were done essentially as described[9]. Bound antibodies were visualized on the western blot by enhanced chemiluminescence (Amersham).

P48 *in vivo* affects the membrane translocation of several exported proteins (G. J. Phillips and T. Silhavy, manuscript in preparation).

We propose that the role of the *E. coli* SRP is that of a chaperone, that is specific for the signal sequence of nascent preproteins and maintains them in a translocation-competent conformation. Dissociation of the SRP from the signal sequence may be induced by binding of other chaperones either to the nascent chain or to the released polypeptide. Alternatively, the release of the preprotein from the ribosome may force the dissociation of the SRP. Subsequently, chaperones like SecB and GroEL/ES may maintain the preprotein in an intermediate folding state that is still translocation-competent[14–16]. Release of the *E. coli* SRP from nascent chains may be mediated by direct interaction with a cognate receptor, similar to the interaction between mammalian SRP and its receptor, the docking protein[4]. Our crosslinking assay should allow the identification of such a receptor. □

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