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Interactions of ribosome nascent chain complexes of the chloroplast-encoded D1 thylakoid membrane protein with cpSRP54

Robert Nilsson[1], Josef Brunner[2], Neil E.Hoffman[3] and Klaas J.van Wijk[1,4]

[1]Department of Biochemistry, Stockholm University, S-10691 Stockholm, Sweden, [2]Department of Biochemistry, Swiss Federal Institute of Technlogy (ETH), CH-8092 Zurich, Switzerland and [3]Department of Plant Biology, Carnegie Institution of Washington, 260 Panama Street, Stanford, CA 94305, USA

[4]Corresponding author
e-mail: klaas@biokemi.su.se

The mechanisms of targeting, insertion and assembly of the chloroplast-encoded thylakoid membrane proteins are unknown. In this study, we investigated these mechanisms for the chloroplast-encoded polytopic D1 thylakoid membrane protein, using a homologous translation system isolated from tobacco chloroplasts. Truncated forms of the *psbA* gene were translated and stable ribosome nascent chain complexes were purified. To probe the interactions with the soluble components of the targeting machinery, we used UV-activatable cross-linkers incorporated at specific positions in the nascent chains, as well as conventional sulfhydryl cross-linkers. With both cross-linking approaches, the D1 ribosome nascent chain was photocross-linked to cpSRP54. cpSRP54 was shown to interact only when the D1 nascent chain was still attached to the ribosome. The interaction was strongly dependent on the length of the nascent chain that emerged from the ribosome, as well as the cross-link position. No interactions with soluble SecA or cpSRP43 were found. These results imply a role for cpSRP54 in D1 biogenesis.

Keywords: amber suppression/cpSRP54/cross-linking/D1 protein/homologous chloroplast translation system

Introduction.

Chloroplasts in higher plants possess circular DNA containing ~105 identified genes. About 30 genes encode essential proteins located in the thylakoid membranes within the chloroplasts and are constituents of large membrane complexes involved in photosynthesis. The mechanisms of targeting, insertion and assembly of the chloroplast-encoded thylakoid membrane proteins are unknown. In this study, we address the targeting and insertion of the chloroplast-encoded D1 protein. This protein is a polytopic thylakoid membrane protein with five transmembrane domains (TMs) which is centrally located in photosystem II, a 700 kDa dimeric complex of the thylakoid membrane system.

Based on a number of observations in chloroplasts, such as run-off translations of thylakoids with bound ribosomes ('rough thylakoids') and detection of translation intermediates in the membrane, it can be postulated that insertion of the polytopic chloroplast-encoded membrane proteins occurs co-translationally (Margulies and Michael, 1975; Herrin and Michaels, 1985; Klein *et al*., 1988; van Wijk *et al*., 1996). Translation initiation inhibitors decreased the amount of bound mRNA, while translation elongation chain inhibitors (such as lincomycin and chloramphenicol) prevented most of the loss of bound mRNA (reviewed in Boschetti *et al*., 1990; Jagendorf and Michaels, 1990). To reconstitute the targeting and insertion process, a homologous chloroplast *in vitro* initiation/translation system is required in which plasmid-derived transcripts can be translated faithfully. The recent development of a translation system isolated from tobacco chloroplasts has opened up novel possibilities to address these important processes at a molecular level (Hirose and Sugiura, 1996).

Initiation of translation in the chloroplast takes place on 70S ribosomes, creating so-called ribosome nascent chain complexes (RNCs). After initiation, elongation proceeds and the nascent chain emerges out of the ribosome tunnel. In *Escherichia coli* and the endoplasmic reticulum of eukaryotes, the RNCs subsequently are targeted via soluble components interacting with the nascent chains emerging from the ribosome tunnel. Alternatively, translation continues in the soluble phase and the proteins are then post-translationally targeted to the membrane. For a number of mitochondrial inner membrane proteins, it has been shown that synthesis and assembly are strongly dependent on the interaction of proteins with 5′-untranslated leaders of their respective mRNAs (Sanchirico *et al*., 1998). For several chloroplast-encoded proteins, such as the polytopic D1 reaction center protein of photosystem II, translation initiation also involves several nuclear-encoded RNA-binding proteins (Rb proteins) (reviewed in Rochaix, 1996; Cohen and Mayfield, 1997). It has been postulated that these Rb proteins could be involved in targeting within the chloroplast (Rochaix, 1996; Zerges and Rochaix, 1998).

In *E.coli*, two soluble components, the signal recognition particle (SRP) and SecB, have been found to be involved in targeting of RNCs. A third component, trigger factor, has been shown to interact with nascent chains, but its role currently is unclear (reviewed in Hesterkamp and Bukau, 1996; Rapoport *et al*., 1996; de Gier *et al*., 1997; Duong *et al*., 1997). SRP in *E.coli* is a ribonucleoprotein complex consisting of a protein (referred to as P48 or Ffh) and a 4.5 S RNA, and binds tightly to N-terminal signal sequences or hydrophobic domains of nascent proteins when still associated with the ribsome. Targeting of SRP-RNC to the membrane is achieved by interaction with an SRP receptor (FtsY) on the inner membrane (Miller *et al*., 1994; Valent *et al*., 1998). At the cytoplasmic membrane translocon, the ATPase SecA can help to insert the protein (reviewed in Duong *et al*., 1997; Matlack *et al*., 1998).

As chloroplasts are of prokaryotic origin, it is not surprising that several of the chloroplast targeting and translocon components share strong homology with those in *E.coli.* A chloroplast-localized homolog of Ffh, designated cpSRP54, was identified (Franklin and Hoffman, 1993). Unexpectedly, cpSRP54 was determined to be involved in post-translational targeting of nuclear-encoded light-harvesting complex protein (LHCP; Li *et al*., 1995) when assembled into a 170 kDa complex together with a 43 kDa protein, named cpSRP43 (Scheunemann *et al*., 1998). *Arabidopsis* mutants lacking cpSRP54 had a strong pleiotropic disturbance in chloroplast biogenesis (Pilgrim *et al*., 1998), suggesting a broad role in protein targeting. In chloroplasts, cpSecA was shown to exist mostly in a soluble form, and purified cpSecA supported post-translational translocation of a number of nuclear-encoded lumenal proteins (Nakai *et al*., 1994; Yuan *et al*., 1994). A deletion mutant of cpSecA in maize (*tha1*) showed a loss of post-translational targeting of a specific class of nuclear-encoded proteins and, interestingly, also an effect on at least one chloroplast-encoded protein, cytochrome *f* (Voelker *et al*., 1997).

In this study, we have set out to evaluate the interaction of the soluble stromal components cpSRP54, cpSRP43 and cpSecA with the chloroplast-encoded D1 thylakoid membrane protein using an optimized homologous chloroplast translation system isolated from tobacco chloroplasts (Hirose and Sugiura, 1996). To trap the elongation process at different stages, truncated forms of D1 protein of different length were generated from truncated transcripts, and purified D1 RNCs were used to study the interaction with soluble stromal components. Using both conventional sulfhydryl cross-linkers as well as UV-activatable cross-linkers incorporated into the nascent chains at specific positions (see Brunner, 1996), we show that cpSRP54 interacts tightly with D1 RNCs dependent on the length of the D1 nascent chain.

Results

Optimizations and selection of nascent chains

The study of Hirose and Sugiura (1996) was the first to present a homologous chloroplast translation system in which exogeneously added *in vitro* transcribed mRNA of chloroplast-encoded proteins could be translated faithfully. To increase the activity of the chloroplast translation extract, we shortened the isolation procedure of the chloroplasts and added additional protease inhibitors to decrease proteolysis of nascent chains during translations (see Materials and methods).

We first wished to examine the interaction of soluble stromal proteins with the D1 nascent chain emerging out of the ribosome tunnel. To trap the elongation process at different stages, truncated forms of the D1 protein of different lengths were generated by translation of truncated *psbA* mRNA, without a stop codon at the 3′ end (Figure 1). Upon translation, the ribosome halts at the end of the transcript and a stable RNC should be generated.

We decided to produce four D1 nascent chains with lengths of 36, 87, 107 and 189 amino acids, respectively, denoted D1–36, D1–87, D1–107 and D1–189 (Figure 1). It is generally assumed that ~30–40 amino acids are buried in the ribosome tunnel or groove when the nascent chain is still attached to the ribosome. Thus, in the case of the shortest nascent chain D1–36, the hydrophilic N-terminus is either still shielded by the ribosome or is just emerging. The D1–86 nascent chain has one TM, which is expected to have at least partially emerged from the ribosome. The D1–107 nascent chain has the complete first TM out of the ribosome, whereas D1–189 has two complete TMs and part of the third TM freely exposed to the stroma (Figure 1).

Translation kinetics and isolation of stable ribosome-associated D1 nascent chains

Translation of all four truncated transcripts yielded stable proteins of the expected size (Figure 2A). Translation rates of all four transcripts were linear for at least 35 min, without significant degradation, as is shown for the D1–189 (Figure 2B). To diagnose whether nascent chains aggregated during the translations, translation mixtures were subjected to a 10 min centrifugation at 15 000 *g*. The pellet contained $<5\%$ of nascent chains, indicating that aggregation was not significant. To purify stable D1 RNCs, the translation inhibitor chloramphenicol was added at the end of the translation and the translation mix was spun on sucrose cushions. Depending on translation temperature and duration, 50–90% of the nascent chains were found in the pellet (Figure 2C). To verify that the nascent chains in the pellets were indeed associated with ribosomes, we attempted to destabilize the RNCs by addition of puromycin and high salt (500 mM KOAc). The translation reactions were incubated for 5 min with puromycin (a tRNA analog), followed by addition of 500 mM KOAc. Centrifugation on sucrose cushions of those treated translation reactions led to a loss of nascent chains from the pellets, indicating that the pellets indeed represent D1 RNCs (Figure 2C). The higher molecular weight bands between 25 and 35 kDa which were observed in the pellets also disappeared upon addition of puromycin (Figure 2C) or incubation at high pH (not shown), and were most significant after short translation times at lowered translation temperatures (26°C). These bands probably represent nascent chains to which the last tRNA remained acylated, similarly to that described for eukaryotic translation systems (e.g. Borel and Simon, 1996). A weak background translation originating from the very abundant, chloroplast-encoded large subunit of RUBISCO was sometimes found in the supernatants of the sucrose cushions (Figure 2C). Fractionation of D1 translation reactions on sucrose gradients demonstrated that nascent chains sedimented with 70S ribosomes (not shown), confirming that stable D1 RNCs were produced by translation of truncated transcripts.

Incorporation of UV-activatable cross-linkers at site-specific positions in the D1 nascent chain

To identify soluble components interacting with the D1 RNCs, we wanted to cross-link components interacting with the nascent chains and subsequently identify these interacting proteins by immunoprecipitation. We decided to use amber suppression technology to incorporate highly reactive photo-activatable cross-linkers at specific, predetermined positions in the D1 nascent chains. This would allow highly specific cross-linking and determination of the interacting domains of the D1 protein, as well as the

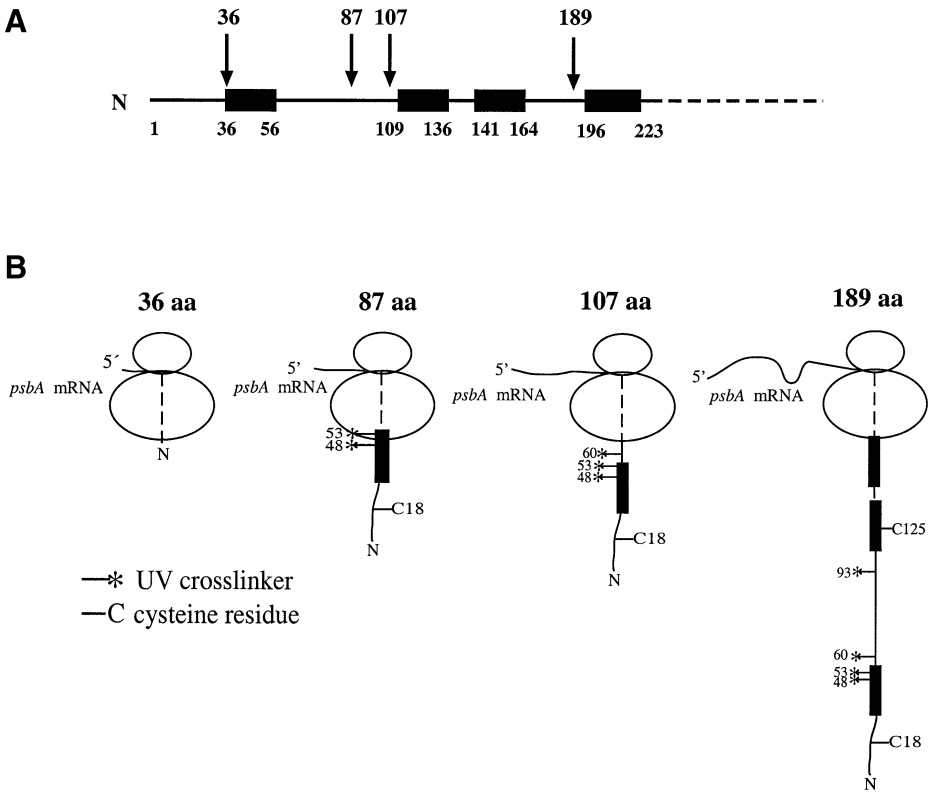

Fig. 1. Production of D1 ribosome nascent chain complexes, cross-linking sites and topology. (**A**) Schematic representation of the N-terminal part of the D1 protein. Arrows indicate the C-termini of the truncated nascent chains. Filled boxes indicate the transmembrane domains. The amino acid numbering is indicated. (**B**) Schematic presentation of RNCs of truncated *psbA* mRNAs encoding 36, 87, 107 and 189 amino acids, and positions of the cross-linking sites. C = cysteine residue, * = photo-activatable amino acid (Tmd)Phe incorporated by amber suppression. The assumed length of the nascent chain buried in the ribosome tunnel is 35 amino acids.

optimal nascent chain length (Noren *et al*., 1989; Brunner, 1996; Martoglio and Dobberstein, 1996). Stop codons were introduced in the first TM at positions F48 and F53 and in the first loop at positions I60 and F93 (Figure 1).

Amber suppression was first tested using increasing concentrations of L-4′-(3-trifluoromethyl)-3H-diazirin-3-yl)phenylalanine [(Tmd)Phe]-tRNA^{sup}, as is shown for D1–107 with a stop codon at position 48 (Figure 3A). Suppression efficiency correlated directly with the (Tmd)Phe-tRNA^{sup} concentration, and up to 50% suppression was achieved. In the absence of added (Tmd)Phe-tRNA^{sup}, no read-through was observed, indicating that a single stop codon was sufficient to block translation (Figure 3A and B). As is demonstrated for D1–189, the amber suppression efficiency was approximately equal for all four amber positions.

To demonstrate that we could obtain specific UV-induced cross-linking, D1–189 with a stop codon at either position 53 or 60 was translated in the presence of (Tmd)Phe-tRNA^{sup}. After translation, D1 RNCs were purified on sucrose cushions and the pellets were incubated in stromal extract for 10 min at 26°C. Immediately after incubation in the stroma, samples were transferred to ice and kept in room light or irradiated for 90 s by long wavelength UV (Figure 3C). In parallel, the tops of the sucrose cushions, containing ‘free’ nascent chains, were also irradiated by UV (not shown). After illumination, samples were precipitated and analyzed on SDS–PAGE. As is shown in Figure 3C, cross-linked bands could be observed with RNCs for both constructs only when samples had been illuminated by UV. No photocross-linked products were observed with the nascent chains which were released from the ribosome and were accumulating on top of the sucrose cushions, indicating that the free nascent chains did not interact with soluble components (not shown). Similarly, a UV dependence of cross-linking was obtained for D1–87 and D1–107, independent of the position of the stop codon (not shown).

We thus conclude that the amber suppression functioned well in the chloroplast translation system, with no read-through in the absence of added (Tmd)Phe-tRNA^{sup} and with good suppression efficiency. Cross-linking was entirely dependent on irradiation with UV, and no background cross-linking was induced by ambient room light, indicating that the moment of cross-linking could be controlled very well.

Probing the interaction of the D1 nascent chain with cpSRP54, cpSRP43 and cpSecA

We verified by Western blotting that the chloroplast translation extract contained the potentially interacting components cpSRP54, cpSRP43 and cpSecA using antisera directed against these chloroplast proteins (not shown). All three components could be immunoprecipitated under denaturing conditions (not shown).

To evaluate the affinity of cpSRP54 and other soluble stromal products for D1, D1–87, D1–107 and D1–189 containing a stop codon in the first TM at position 53 were synthesized. After 30 min translation, the samples were illuminated with UV, and RNCs were purified on

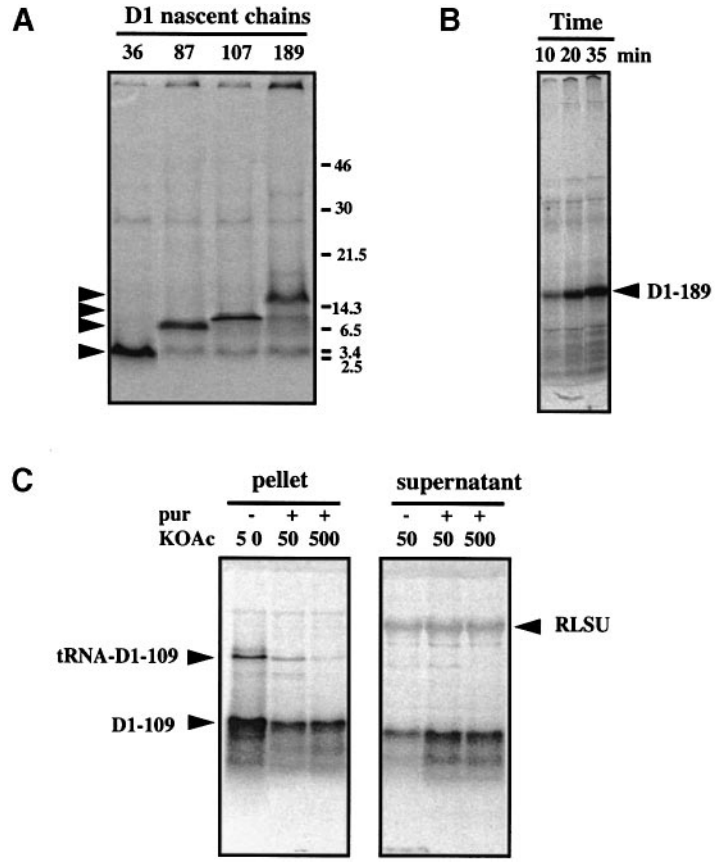

Fig. 2. Synthesis of truncated D1 nascent chains, translation kinetics, isolation of stable D1 RNCs and effect of puromycin. (**A**) Autoradiogram of D1 nascent chains of different lengths produced in the chloroplast translation system using truncated mRNA templates (the number of amino acids is indicated). (**B**) Kinetics of translation of D1–189 using truncated *psbA* mRNA. Translation in the tobacco translation system was carried out for 10, 20 or 35 min at 30°C, after which proteins were precipitated by TCA, solubilized and separated on SDS–PAGE. (**C**) Purification of stable RNCs on sucrose cushions and effect of incubation with puromycin. After 30 min translation of truncated *psbA* mRNA coding for the first 107 amino acid residues, the translation mix was spun on a 450 µl 0.5 M sucrose cushion (with 50 or 500 mM KOAc) containing solution A and 250 µg/ml chloramphenicol for 50 min at 70 000 r.p.m. in a TLA100.3 rotor. Where indicated, translation reactions were incubated in 2 mM puromycin for 5 min at 30°C after the translation reations. Non-pelleted proteins in the sucrose cushion were precipitated by addition of TCA. The arrows indicate the presence of the large subunit of Rubisco (RLSU), D1–107 and tRNA-D1–107.

sucrose cushions. A total of 10% of the pellets were solubilized directly in SDS buffer. The remainder of the pellets and the supernatants containing released D1 nascent chains were denatured in SDS and immunoprecipitated with antisera against cpSRP54, cpSRP43 and cpSecA (Figure 4A). A large amount of cpSRP54 was found to interact with D1–107 RNCs, forming a complex of apparent mol. wt 60–65 kDa. A significant but smaller amount of cross-linked cpSRP54 was immunoprecipitated with D1–87, but no precipitation was observed with D1–189. Neither cpSecA (Figure 4A) nor cpSRP43 (not shown, but see next section and Figure 5B) interactions were observed. No immunoprecipitation of nascent chains in the supernatants was detected, indicating that cpSRP54, cpSRP43 and cpSecA did not interact with D1 nascent chains released from the ribosome (not shown).

Photocross-linking with the constructs containing stop codons at other positions (at position 48 for D1–87 and at positions 48, 60 and 93 for D1–189) did reveal a weak interaction between cpSRP54 and D1–87 but no or very weak interactions with the longer nascent chain (Figure 4B and C). The weak interaction of D1–87 might be related to the possibility that the residues at positions 48 and 53 are buried in the ribosome tunnel.

Comparing the cross-linking efficiences for the four amber positions in D1–107 indicates that cpSRP54 interaction is strongest with the cross-linker at position 53, weaker at position 48 and absent with the cross-linkers incorporated in the lumenal loop (at positions 60 and 93) (Figure 3D). Naturally, the absence of cross-linking in the case of D1–107 amber 93 is logical as the cross-linker is buried in the ribosome. The absence of cross-linking at position 60 probably indicates that interaction of SRP occurs with the first TM at the N-terminus. Incubation of purified RNCs in stroma only slightly increased the amount of cross-linked cpSRP54 (Figure 3D).

Apart from the cross-linked product that contained cpSRP54, a number of other cross-linked bands can be observed (Figures 3C and D, and 4A–D). Some of the lower molecular weight products became more abundant when the RNCs were incubated in stromal extract prior to cross-linking (Figure 4D). It is likely that they represent cross-links to as yet unidentified stromal components.

We extensively experimentally investigated the question of whether the cpSRP54 interaction with the D1 protein is really dependent on the association with the ribosome. Cross-linking experiments with the amber suppression system were carried out using two different set-ups. On

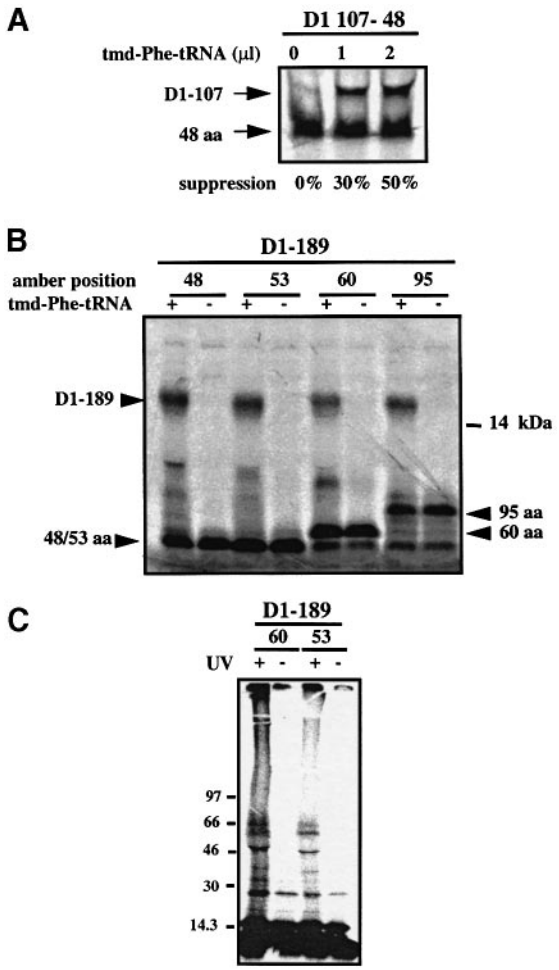

Fig. 3. Testing amber suppression, incorporation of (Tmd)Phe into the nascent chain and UV-activatable cross-linking in the chloroplast translation system. (**A**) Truncated *psbA* mRNA with a stop codon at position 48 was translated for 30 min at 30°C in the chloroplast translation system in the absence or presence of different amounts of (Tmd)Phe-tRNA^{sup}. The percentage of amber suppression is indicated. Proteins were separated on 14% Tricine gels. (**B**) Comparison of amber suppression efficiencies and read-through for four amber codons (at positions 48, 53, 60 and 93) in the D1–189 construct. The four different amber constructs were translated for 30 min in the absence or presence of (Tmd)Phe-tRNA^{sup}. The arrows show the different D1 nascent chains with the number of amino acid residues indicated. Proteins were separated in a 7–17% gradient SDS–PAGE. (**C**) mRNAs coding for D1–189 with stop codons at positions 54 and 60 were translated in the presence of suppressor (Tmd)Phe-tRNA^{sup} for 30 min at 30°C. After translation, 50% of each sample was irradiated by long wavelength UV for 90 s, after which RNCs from all samples were purified on sucrose cushions and solubilized (– stroma samples). Alternatively, RNCs were purified on sucrose cushions directly after translation, incubated for 10 min at 26°C in stromal extract, irradiated by long wavelength UV for 90 s on ice, precipitated in ice-cold TCA and solubilized directly (+ stroma samples). Proteins were separated in a 7–17% gradient SDS–PAGE.

one hand, UV cross-linking was done directly in the translation mix, and subsequently the D1–RNCs and released nascent chains were separated on the sucrose cushions, followed by immunoprecipitation under denaturing conditions (as described for Figure 4). On the other hand, we also used an alternative set-up: after translation, D1–RNCs and released nascent chains were first separated on sucrose cushions and subsequently both fractions were illuminated by UV. In neither set-up did we detect significant radioactive cross-linked bands in the nascent chain population released from the ribosome, even though significant amounts of nascent chains were found in the supernatants. This indicates that when D1 nascent chains are released from the ribosome they do not remain associated with cpSRP54. This also means that released D1 nascent chains do not interact with cpSRP54, at least not with the N-terminal domain which we probed with UV cross-linkers. Finally, to evaluate the interaction of cpSRP54 with free nascent chains, we did not need to stimulate nascent chain release from the ribosome by puromycin incubations. The reason was that translations for amber cross-linking were carried out at 30°C for 30 min to maximize the amount of translation products. Under those conditions, ~30–50% of the nascent chains were released, as mentioned earlier.

Cross-linking of interacting proteins with conventional cross-linkers

The D1 protein contains two cysteines (C18 and C125) but no lysines (Figure 1B). C18 is positioned close to the N-terminus prior to the first TM, whereas C125 is located in the middle of the second TM. In addition to the amber cross-linking, we used these cysteines to investigate interacting stromal components further. After translation of the truncated proteins (87, 107 and 189 amino acids), stable RNCs were purified and interacting proteins were cross-linked by addition of the homo-bifunctional sulfhydryl cross-linker bismaleimidohexane (BMH) and the hetero-bifunctional cross-linker *m*-maleimidobenzoyl-*N*-hydroxysulfo-succinimide ester (S-MBS) (sulfhydryls/amines). After quenching of the cross-linker, the samples were precipitated, solubilized in SDS and immunoprecipitated with antisera directed against cpSecA, cpSRP54 and cpSRP43 (Figure 5).

With all three constructs and with both cross-linkers, a clear and distinct cross-linking pattern was observed, whereas without addition of cross-linkers, no cross-linked bands were observed (Figure 5A). The cross-linking pattern achieved with the two cross-linkers was quite different, indicative of specific cross-linking. No immunoprecipitation of cross-linked D1 nascent chains with antisera directed against cpSecA (not shown) or cpSRP43 (Figure 5B) was found. However, immunoprecipitation with cpSRP54 antiserum immunoprecipitated a product of 50–60 kDa with D1–87 after cross-linking with BMH and with D1–107 after cross-linking with S-MBS (Figure 5B). Thus, clearly, both nascent chains interacted with cpSRP54, but apparently not with cpSecA or with cpSRP43. It should be noted that cpSRP54 was co-purified with the RNCs on the sucrose gradients and, prior to cross-linking, no additional stroma or SRP was added, indicating a strong affinity of cpSRP54 for the nascent chains.

In an attempt to increase the amount and number of cross-linked products, D1 RNCs were purified and incubated with stromal extracts in the absence or presence of ATP and GTP. Subsequent cross-linking revealed a number of strong interactions with different proteins smaller than 40 kDa (not shown), and experiments are in progress to identify these interacting proteins.

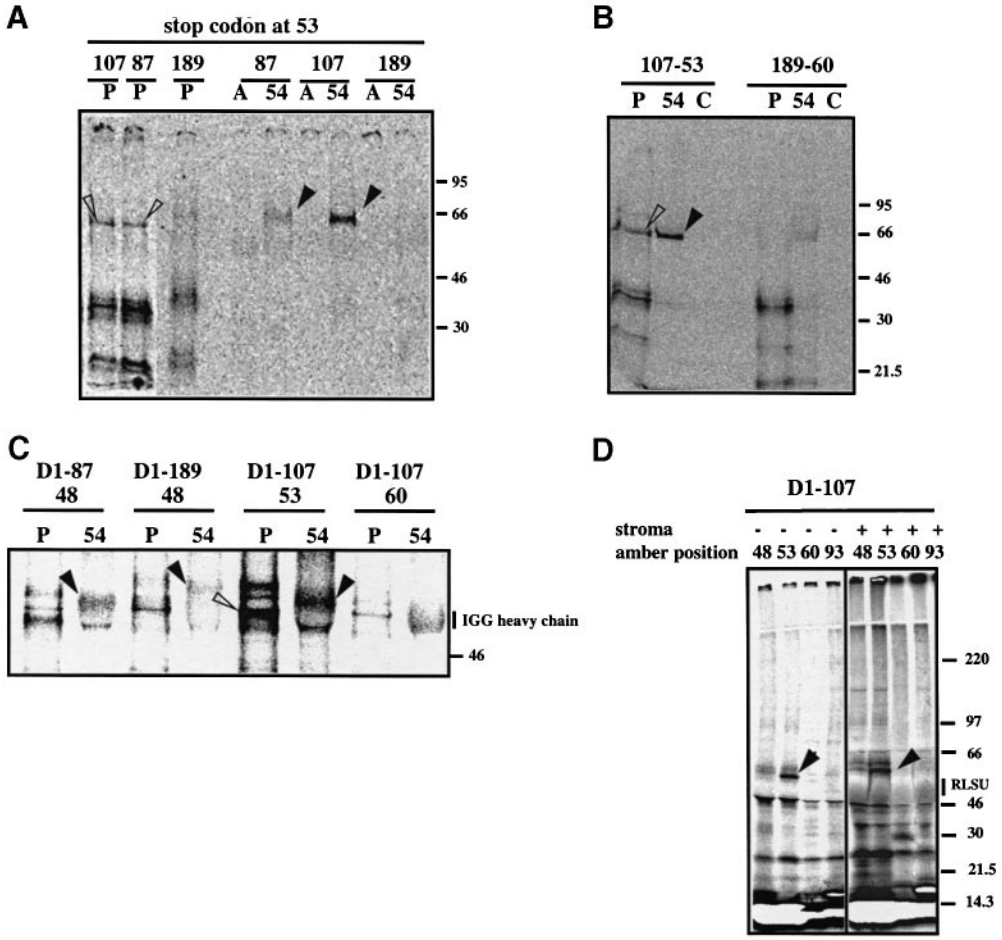

Fig. 4. Probing the interaction between cpSRP or SecA and D1 RNCs by photo-activatable cross-linkers and immunoprecipitation. Truncated *psbA* mRNAs containing stop codons at different positions were translated in the chloroplast translation system in the presence of (Tmd)Phe-tRNA^{sup}. After translation, samples were illuminated for 90 s on ice and RNCs were purified by centrifugation on sucrose cushions. Ribosomal pellets were solubilized and immunoprecipitated with antisera against cpSRP54, cpSRP43 or cpSecA, as indicated. Ten percent of the RNC pellets were loaded directly on the gels (indicated by 'P'). Precipitated proteins were released by SDS solubilization buffer, separated on 14% Tricine gels, dried and exposed to phosphoimager screens. Filled arrows indicate the D1 nascent chains cross-linked to cpSRP54 as detected by immunoprecipitation. Open arrows in the lanes with the pellets prior to immunoprecipitation indicate the most likely candidate cpSRP54 cross-linked product. (**A**) UV cross-linking and immunoprecipitation of D1–87, D1–107 and D1–189 containing a stop codon at position 53. Immunoprecipitation was carried out on the cross-linked ribosomal pellets, using antisera directed against SecA (indicated by 'A') or cpSRP54 (indicated by '54'). The two lanes on the left hand side (87 P and 107 P) were run on a separate gel. (**B**) UV cross-linking and immunoprecipitation of D1–107 and D1–189 containing an amber codon at position 53 or 60, respectively. Immunoprecipitation was carried out on the cross-linked ribosomal pellets, using antisera against cpSRP54 (indicated by '54') or a control serum (indicated by 'C'). (**C**) UV cross-linking and immunoprecipitation of D1–87, D1–189 with a stop codon at position 48 and D1–107 containing a stop codon at position 53 or 60. Immunoprecipitation was carried out on the cross-linked ribosomal pellets, using antisera directed against cpSRP54 (indicated by '54'). (**D**) mRNAs encoding D1–107 containing amber codons at position 48, 53, 60 or 93 were translated in the chloroplast translation system in the presence of (Tmd)Phe-tRNA^{sup}. After translation, RNCs were purified by centrifugation on sucrose cushions. Ribosomal pellets subsequently were resuspended in a buffer, supplemented with or without stromal extract. After incubation for 10 min at 26°C, samples were irradiated for 90 s by long wavelength UV, TCA-precipitated, solubilized directly and run on a 7–17% gradient SDS–PAGE.

Discussion

In this study, we have begun to address experimentally the mechanisms of targeting and insertion of the chloroplast-encoded thylakoid membrane proteins, using a homologous chloroplast translation system. We have used the D1 protein as our first thylakoid membrane protein for several reasons. The D1 protein has the highest turnover rate of the known thylakoid membrane proteins and *in vivo* it is constantly synthesized and inserted into the mature thylakoid membrane system (reviewed in Aro *et al*., 1993). We have shown that during run-off translation on rough thylakoids, the D1 protein is inserted into the thylakoid membrane and assembled into the photosystem II complex (van Wijk *et al*., 1996, 1997). Furthermore, transcriptional and translational regulation of the D1 protein is under study by several laboratories, and information about 5′- and 3′-untranslated regions is available. These untranslated regions are important for mRNA stability and regulation of translation initiation (reviewed in Gillham *et al*., 1994; Rochaix, 1996; Cohen and Mayfield, 1997) and are possibly also involved in the targeting process (Rochaix, 1996; Zerges and Rochaix, 1998).

Here we show that the homologous chloroplast translation system can produce stable D1 RNCs when different truncated *psbA* mRNAs are translated. No premature termination of translation occurred, as was found in an *E.coli* homologous translation system (Valent *et al*., 1997). We show that the amber suppression system, as was first described by Schultz and co-workers (Noren *et al*., 1989),

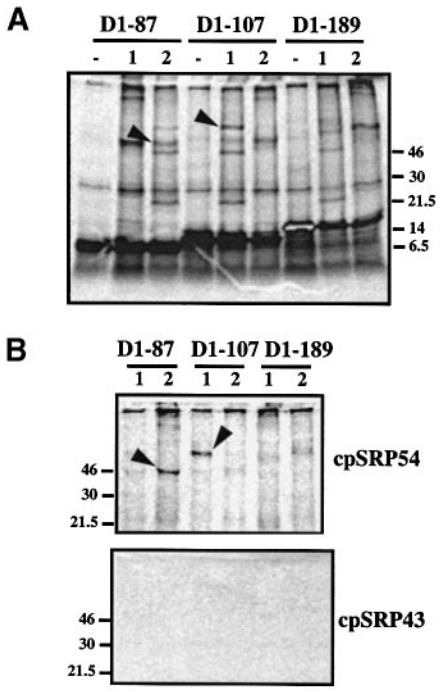

Fig. 5. Interaction between D1 RNCs and cpSRP54 after cross-linking to cysteine residues. Truncated *psbA* mRNAs encoding D1–87, D1–107 and D1–189 were translated in the chloroplast translation system. After translation, RNCs were purified by centrifugation on sucrose cushions. Ribosomal pellets subsequently were resuspended in a buffer and incubated with a homo- or heterobifunctional cross-linker (BMH or S-MBS, respectively). After incubation for 10 min at 26°C, the cross-linkers were quenched for 30 min on ice, denatured in SDS and immunoprecipitated with antisera directed against cpSRP54 or cpSRP43. Ten percent of the pellets before cross-linking (–) or after cross-linking with BMH (1) or S-MBS (2) were loaded directed on an SDS–PAGE gel (**A**). The remainder of each pellet was denatured with SDS and immunoprecipitated with antiserum against cpSRP54 (**B**) or cpSRP43 (**C**). The arrows indicate cross-links between cpSRP54 and D1 nascent chains. Proteins were separated on Tricine gels.

is compatible with the chloroplast translation system. No read-through occurred without addition of suppressor tRNA, and good suppression efficiencies were obtained. This allowed us to incorporate UV cross-linkers at predetermined, specific positions into the nascent D1 chains. The UV cross-linking technique is favorable over more conventional cross-linking techniques since UV cross-linking is not dependent on suitable amino acids in the neighboring proteins but instead can cross-react with any part of the protein in proximity. The UV cross-linker employed in this study was activatable by long wavelength UV, and the carbene cross-linker has a short lifetime. Therefore, cross-linking is likely to reveal relevant interactions. An additional advantage is that free amino acids present in the translation system do not interfere with the UV cross-linking, thus allowing cross-linking to be carried out at any point in time during the translation, without the need for purification of the nascent chains (Brunner, 1996; Martoglio and Dobberstein, 1996).

Using these innovations, we show that cpSRP54 interacts with the D1 nascent chain only when it is attached to the 70S ribosome. Moreover, variations of the nascent chain length (from 87 to 189 amino acid residues) showed that the affinity for cpSRP54 was lost when the nascent chain was 189 amino acid residues long. When the UV cross-linker was positioned in the first lumenal loop, no interaction between the nascent chain and cpSRP54 was observed. This probably indicates that cpSRP54 is not interacting directly with this D1 region. On the other hand, positioning of cross-linkers in the first TM revealed strong interaction with cpSRP54.

The use of conventional cross-linkers confirmed and supplemented the UV cross-linking results. The D1 protein does not contain any lysine residues, a prominent residue for cross-linking, but does contain a cysteine close to the N-terminus (at position 18) and a cysteine in the second TM (C125). We used the cross-linker BMH (cross-linking to cysteines) and S-MBS (cross-linking to cysteines and the ε amine of lysine as well as to primary amines at the N-terminus of proteins) to test whether interaction between D1 RNCs and cpSRP54 could be detected. With both cross-linkers and with all three nascent chain lengths, a clear and distinct cross-linking pattern was obtained. However, immunoprecipitation with cpSRP54 antiserum only revealed interactions of cpSRP54 with D1–87 and D1–107 and not with D1–189, despite the presence of a second cysteine (cross-linking site) in the longer nascent chain.

Thus, the UV cross-linking and conventional cross-linking data together demonstrate that cpSRP54 can interact with the D1 nascent chain when it is attached to the ribosome, however, only when elongation has not proceeded too far. In the endoplasmic reticulum (ER), it has been demonstrated that the affinity of the signal sequence for SRP decreases with chain length (Siegel and Walter, 1988) and it appears that there is only a certain time-window during which SRP can bind and target polypeptides to the ER membrane (see Rapoport *et al*., 1996).

Despite considerable effort to determine interactions with cpSRP43 or with cpSecA, no such interactions with the RNCs could be found in the absence of thylakoid membranes. The absence of cpSecA interaction, despite its presence in soluble form in the stroma, could indicate that cpSecA interacts when elongation of the D1 nascent chain has proceeded further than 189 amino acid residues. However, on the basis of a recent study in *E.coli*, it can be postulated that SecA only interacts with the nascent chains after the RNCs have associated with the membrane and subsequently transferred to the Sec translocon (Valent *et al*., 1998). Gray and co-workers showed that the nuclear-encoded protein OEC33 could be cross-linked to cpSecA at the thylakoid membrane surface during post-translational insertion (Haward *et al*., 1997). Thus, if cpSecA is involved in the insertion of D1 into the membrane, this interaction probably occurs at the thylakoid membrane surface. Preliminary cross-linking experiments have not yet revealed such interactions.

In a recent study, the interaction of cpSRP54 with different proteins was studied with RNCs generated in a wheat germ translation system (High *et al*., 1997). The wheat germ translation system is of a eukaryotic type and contains 80S ribosomes, whereas the chloroplast translational machinery is of a prokaryotic type with 70S ribosomes. The nature of the ribosome could prove to be important for the cpSRP54 affinity. Indeed, in studies on the SRP particle in *E.coli*, differences in cross-linking between a homologous translation system versus a hetero-

logous translation system were found. It was shown that in a homologous *E.coli* translation system (containing 70S ribosomes), the 54 homolog (named Ffh) did not compete with the soluble trigger factor for the interaction with nascent chains of PhoE, in contrast to results previously obtained in a heterologous system using wheat germ extract. This is possibly due to a difference in the association with the ribosome (Valent *et al*., 1997, 1998). It was also reported that *E.coli* SRP was unable to target nascent preproteins on wheat germ ribosomes to *E.coli* membrane vesicles (see Valent *et al*., 1997). Thus, clearly, the use of a homologous chloroplast translation system is crucial to study physiologically relevant interactions of soluble stromal proteins with nascent chains and also to reconstitute targeting and insertion to the thylakoid membrane.

In conclusion, we have developed an experimental system to address the targeting and insertion of the chloroplast-encoded thylakoid membrane proteins. Amber suppression can be used to incorporate UV-activatable cross-linkers into the nascent chains. We have shown that cpSRP54 interacts specifically with the D1 RNCs, implying that cpSRP is involved in biogenesis of the D1 protein. It is expected that these novel developments will enable us to identify more precisely the role of cpSRP54 and unravel mechanisms of targeting, insertion and assembly of thylakoid membrane proteins encoded by the chloroplast genome.

Materials and methods

DNA constructs

A plasmid containing the full-length 5′-untranslated leader (–85 to +1) and coding region of *psbA* (coding for the D1 protein) cloned behind the T7 promotor was a kind gift of T.Hirose and M.Sugiura (Hirose and Sugiura, 1996). For incorporation of UV-activatable cross-linkers using amber suppressers (Brunner, 1996; Martoglio and Dobberstein, 1996), stop codons were introduced into the N-terminus of the D1 protein at amino acid positions F48, F53, I60 and F93, by converting the codons into TAG by PCR, using the Stratagene 'quick-change' kit. To try to increase the suppression efficiency (Yarus and Curran, 1992), the downstream codon context of the amber codon at amino acid position 93 was changed from AAC to TAC, which changed the tyrosine C-terminal of F93 into an asparagine. All constructs were verified by nucleotide sequencing.

Preparation of mRNA templates

DNA templates for the transcription of truncated mRNA were prepared either by using appropriate restriction sites within the coding region or by a PCR approach. Plasmids were digested with *Sau*3A, *Ava*II or *Bst*NI for production of D1 nascent chains of respectively 36, 107 or 189 amino acid residues (denoted D1–36, D1–107 and D1–189). For production of an 87 amino acid nascent chain (D1–87), DNA templates were prepared by PCR according to Nilsson *et al*. (1994), using a forward primer annealing upstream of the T7 promoter and a reverse primer annealing within the coding region of *psbA*. Transcription with T7 polymerase was carried out according to the Promega Ribomax system. After transcription, DNA was digested by DNase I. Transcripts subsequently were purified by phenol/chloroform extraction and ethanol precipitation, according to standard procedures (Sambrook *et al*., 1989). The size and quality of the mRNA were verified routinely on 6% acrylamide/TBE/8 M urea gels or on agarose gels.

Isolation of the homologous chloroplast translation system and in vitro translations

The preparation of homologous chloroplast translation extracts from tobacco leaves was carried out essentially according to Hirose and Sugiura (1996). Tobacco was grown in the greenhouse at 26°C/21°C day/night temperatures under daylight with additional illumination and, after 30–40 days, leaves between 5 and 10 cm were harvested. Intact chloroplasts were isolated rapidly by a 4 min centrifugation at ~5500 *g* on a Percoll step gradient (30%/70% Percoll). Translation reactions were carried out essentially according to Hirose and Sugiura (1996). The protease inhibitors antipain and pepstatin were also added, increasing the amount of stable translation products on average by 30% (not shown). In summary, the optimized translation mixture contained 100 mM mannitol, 30 mM HEPES–KOH pH 7.7, 10 mM MgOAc, 60 mM KOAc, 8 mM creatine phosphate, 1% polyethylene glycol 6000, 1 mM ATP, 0.1 mM GTP, 2 mM dithiothreitol (DTT), 0.4 μg/μl creatine phosphokinase (type I), 0.4 μg/μl *E.coli* tRNA, 2.6 U/μl RNAsin (Promega), 0.45 μCi/μl [^{35}S]methionine, and 1 μg/ml leupeptin, antipain and pepstatin. Translations (10–50 μl volumes) were carried out routinely at 26–30°C for 20–30 min.

Isolation of RNCs

For isolation of RNCs, chloramphenicol was added to the translation reactions at a final concentration of 250 μg/ml and translations were overlaid on 0.5 M sucrose cushions containing 50 mM HEPES–KOH pH 7.7, 50 mM KOAc, 5 mM MgOAc, 2 mM DTT and a cocktail of protease inhibitors Pefablok (50 μg/ml), antipain and leupeptin (2 μg/ml), from here on denoted solution A. The elongation inhibitor chloramphenicol was added to a final concentrations of 250 μg/ml to stabilize the ribosomes further. Sucrose cushions were spun for 50 min at 70 000 r.p.m. in a TLA100.3 rotor (Beckman). To investigate further the presence of RNCs, translation reactions were spun at 40 000 r.p.m. in a SW41 rotor on a linear sucrose gradient (15–45% sucrose) containing solution A and chloramphenicol. After centrifugations, the gradients were fractionated into 20 equal fractions of 550 μl, trichloroacetic acid (TCA) precipitated and analyzed on SDS–PAGE.

UV cross-linking

To obtain site-specific cross-linking of the D1 nascent chain with interacting components, the photo-activatable amino acid Tmd(Phe) was incorporated at the introduced amber codons within the nascent chain using an amber suppressor tRNA (tRNA^{sup}) chemically aminoacylated with (Tmd)Phe. Translation beyond the amber codon only proceeds if the tRNA^{sup} has incorporated (Tmd)Phe successfully into the nascent polypeptide chain. Hence, each fully translated polypeptide contains the photoactivatable amino acid and can participate in a cross-linking reaction upon activation with UV light (Noren *et al*., 1989; Brunner, 1996; Martoglio and Dobberstein, 1996). tRNA^{sup} was produced by run-off transcription followed by purification by anion exchange chromatography (Graf *et al*., 1997). (Tmd)Phe-pdCpA was produced and stored with a Boc-protected group, which was removed prior to chemical aminoacylation (Graf *et al*., 1997). The different amber constructs were translated in the presence of the tRNA^{sup} aminoacylated with (Tmd)Phe. Cross-linking was performed by irradiation of the samples for 90 s on ice at 365 nm using a long wavelength UV lamp (UVP, 100 W).

Cross-linking with irreversible homo- and heterobifunctional crosslinkers

To identify components closely interacting with the nascent chains, RNCs were resuspended in 50 mM HEPES–KOH pH 7.2, 50 mM KOAc, 5 mM MgOAc and the cocktail of protease inhibitors and incubated in the irreversible homo- and heterobifunctional cross-linkers BMH (0.5–1.0 mM) or S-MBS (0.5–1 mM) (Pierce) for 10 min at 26°C or for 30–60 min on ice. BMH (16.1 Å spacer arm length) reacts specifically with sulfhydryl groups (at pH 6.5–7.5) whereas S-MBS (9.9 Å spacer arm length) reacts with sulfhydryl groups (cysteines), the ε-amine of lysines and α-amines on the N-termini of proteins. After incubation, the cross-linkers were quenched by addition of 100–200 mM β-mercaptoethanol (BMH) or 50–100 mM β-mercaptoethanol and 50–100 mM glycine (S-MBS) and kept for at least 30 min on ice.

Isolation of chloroplast stroma

Pea seedlings were grown for 7 days in a growth chamber with 12 h of artificial light per day. Intact chloroplasts were prepared from pea leaves essentially as described in Cline (1986). To liberate the soluble stroma, chloroplasts were lysed in solution A. In the case of cross-linking with BMH and S-MBS, DTT was omitted and the pH was set at 7.2.

Immunoprecipitations

For immunoprecipitations, the samples were denatured in 1% SDS in 50 mM Tris–HCl pH 8.0 in the presence of protease inhibitor cocktail and heated for 10 min at 37°C. Subsequently, samples were diluted 10- to 33-fold with 0.1–1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl

pH 7.5, 2 mM EDTA and the cocktail of protease inhibitors. Antibodies were added and incubated for 2–3 h at room temperature. Subsequently, protein A– or protein G–Sepharose [blocked in 5% bovine serum albumin (BSA)] was added and incubation was continued for an additional 1 h. Finally the beads were washed five times in 0.1–1% Triton X-100, 150 mM NaCl, 2 mM EDTA and 50 mM Tris–HCl pH 7.5, resuspended in Laemmli SDS solubilization buffer and heated for 10 min at 37°C or 5 min at 75°C.

Protein analysis

Samples were run on large gradient SDS–PAGE (7–17%) (Laemmli, 1970) or on 14% Tricine gels (Schägger and von Jagow, 1987). For autoradiography, gels were dried and exposed to film or phosphoimaging screens (Fuji). Western blotting was performed using standard techniques, and using chemiluminescence (horseradish peroxidase-conjugated goat anti-rabbit or anti-chicken IgG, Bio-Rad or Sigma) for detection.

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