The major chloroplast envelope polypeptide is the phosphate translocator and not the protein import receptor

Ulf-Ingo Flügge*, Andreas Weber*, Karsten Fischer*, Fritz Lottspeich†, Christoph Eckerskorn†, Karin Waegemann‡ & Jürgen Soll§

* Julius-von-Sachs-Institut für Biowissenschaften mit Botanischem Garten der Universität Würzburg, Mittlerer Dallenbergweg 64, 8700 Würzburg, Germany
† Max-Planck-Institut für Biochemie, Am Klopferspitz 18, 8033 Martinsried, Germany
‡ Botanisches Institut der Universität München, Menzinger Strasse 67, 8000 München 19, Germany
§ Botanisches Institut, Universität des Saarlandes, 6600 Saarbrücken, Germany

DURING photosynthetic CO_2 fixation, fixed carbon is exported from the chloroplasts in the form of triose phosphate by the chloroplast phosphate translocator, which is the principal polypeptide (E29) from spinach chloroplast envelopes[1]. We have sequenced this nuclear-coded envelope membrane protein from both spinach and pea chloroplasts[2,3]. An envelope membrane protein, E30, has been identified as a possible receptor for protein import into pea chloroplasts using an anti-idiotypic antibody approach[4–6]; antibodies raised against purified E30 inhibited binding and import of proteins into chloroplasts[7]. The amino-acid sequence of E30 deduced from its complementary DNA[7] turned out to be highly homologous to that of E29, assigned by us as the spinach phosphate translocator[2], and was identical to the corresponding polypeptide from pea chloroplasts[3]. Differences in the binding properties to hydroxylapatite of E30 and the phosphate translocator suggested that E30 was not responsible for the chloroplast phosphate-transport activity but was the chloroplast import receptor[7]. Here we present evidence that argues against this and which identifies E30 as the chloroplast phosphate translocator.

Polypeptide profiles of envelope membranes from spinach and pea chloroplasts are shown in Fig. 1*a*. The main polypeptides of these membranes have different apparent relative molecular masses (M_r) of 29,000 (29 K; E29 (spinach); lane 1), and 30K (E30 (pea); lane 2). When the precursor proteins are synthesized from their cDNAs[3] and processed *in vitro*, the mature forms have slightly different electrophoretic mobilities (Fig. 1*a*, lanes 6 and 7), which is consistent with the pea protein being larger by six amino-acid residues[3]. When intact chloroplasts from spinach or pea leaves are incubated with micromolar amounts of tritiated 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid ([^{3}H]DIDS), which strongly inhibits phosphate transport activity[8,9], the tritium label in the envelope membrane is associated with E29 and E30, respectively (Fig. 1*a*, lanes 4 and 5). Other higher-molecular weight envelope polypeptides besides E29 are labelled when higher concentrations of [^{3}H]DIDS are used and the resulting fluorogram is overexposed (Fig. 1*a*, lane 3).

To assess whether E29 is involved in phosphate transport we purified E29 and measured its activity in a reconstituted system[10]. We have used hydroxylapatite to purify solubilized phosphate-transport activity and found that it does not bind hydroxylapatite and so can be enriched by this procedure[10]. Subsequent chromatography of the unbound fraction containing this phosphate-transport activity on heparin–Sepharose CL-6B or chelating Sepharose 6B purified E29 to apparent homogeneity (Fig. 1*b*). In parallel, the specific reconstituted phosphate-transport activity increased (Table 1). Table 1 also shows that [^{3}H]DIDS-labelled E29 can be purified 9-fold from envelope membranes, as determined from the [^{3}H]DIDS/protein ratio. About 12% of the chloroplast envelope membrane protein therefore consists of E29. The reconstituted transport activity was enriched by only 5-fold, probably because of its functional instability in detergent[10]. Taken together these results demonstrate that E29 and [^{3}H]DIDS-labelled E29, respectively, represent the phosphate translocator protein.

To confirm the identity of the [^{3}H]DIDS-labelled E29 with the mature cDNA clone[3], we sequenced a tryptic fragment of [^{3}H]DIDS-labelled E29 from the amino terminus. The N-terminal amino-acid residues of a 26.4K fragment were ThrGlyPheLeuGluLysTyrProAla, corresponding to the sequence of the mature cDNA clone[3] (amino-acid residues 11–19) and confirming the identity between them.

As shown in Fig. 1*c* and *d*, lanes 1–3, most of the functional translocator activity was not bound to hydroxylapatite at 4 °C and neither were E29 or [^{3}H]DIDS-labelled E29, but the other solubilized envelope membrane proteins were largely absorbed and could only be eluted with high concentrations of phosphate. If the solubilized membrane proteins were exposed to hydroxylapatite for 30 min at room temperature as in the conditions used by Schnell *et al.*[7], however, then most of the membrane proteins, including E29, remained bound to the column and could only be eluted with high concentrations of phosphate (Fig. 1*c*, lanes 4 and 5). The same holds for [^{3}H]DIDS-labelled E29 (Fig. 1*d*, lanes 4 and 5). The absence of any immunoreactive E29 in this unbound fraction led to the conclusion[7] that E29 was not the chloroplast phosphate translocator (expected in the unbound fraction) and could not therefore be responsible for chloroplast phosphate transport. But why no immunoreactive E29 was detected in the unbound fraction was because there was no E29 present in this fraction. The prolonged incubation at higher temperature probably favours denaturation of E29, causing it to be adsorbed by hydroxylapatite. Indeed, hydroxylapatite has been used to separate denatured and native membrane proteins[11,12]. Presumably, the E29 (Fig. 1*c*), [^{3}H]DIDS-labelled E29 (Fig. 1*d*), and E29 imported *in vitro* (Fig. 1*e*) which elute from hydroxylapatite only in high phosphate are partially denatured. This fraction also has a greatly reduced phosphate-transport activity.

TABLE 1 Isolation of phosphate-translocator activity and [^{3}H]DIDS-labelled E29 from spinach chloroplast envelope membranes

Preparation	Protein (mg)	Reconstituted transport activity (nmol min^{-1})	Specific transport activity (nmol mg^{-1} min^{-1})	[^{3}H]DIDS-labelled E29 (d.p.m. 10^{-3} per mg)
Triton X-100-solubilized chloroplast envelope membranes	6.06	32.7	5.4	4.6
Chromatography on hydroxylapatite	0.50	8.4	16.8	30.1
Chromatography on heparin–Sepharose CL-6B or chelating Sepharose 6B	0.10	2.2	22.0	42.0

For experimental details see legend to Fig. 1*b*.

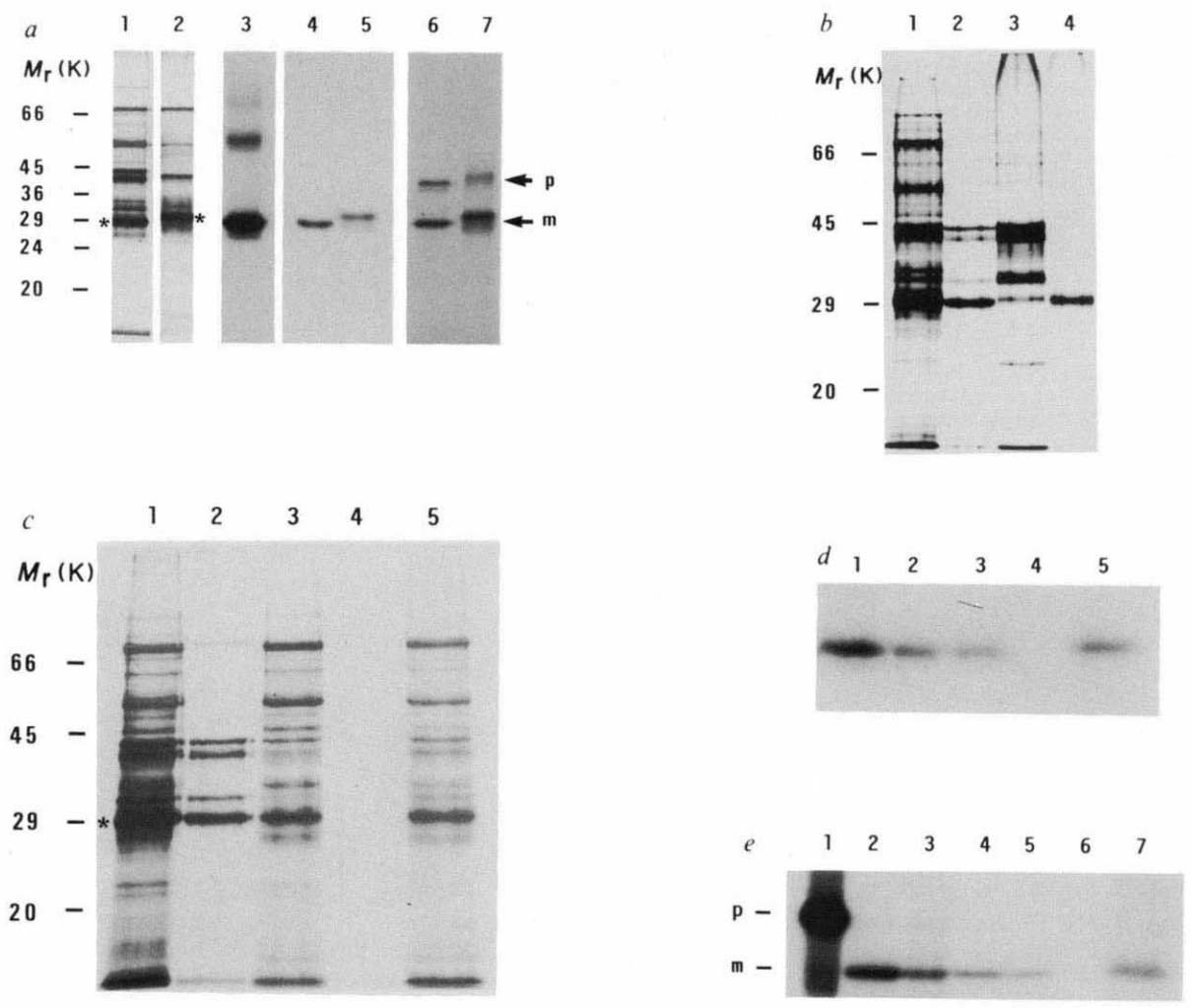

FIG. 1 The envelope membrane polypeptide E29 can be purified to homogeneity and does not bind to hydroxylapatite. *a*, SDS–PAGE of envelope membrane proteins from spinach (lanes 1, 3, 4) and pea (lanes 2, 5) chloroplasts, respectively. Lanes 1 and 2, silver-stained envelope membrane proteins E29 and E30, respectively, are marked by an asterisk; lanes 3–5, fluorographic analysis of [^{3}H]DIDS-labelled envelope proteins: chloroplasts were incubated with 10 μM [^{3}H]DIDS (lane 3) or 2 μM [^{3}H]DIDS (lanes 4 and 5). Lanes 6 and 7, cDNA clones encoding E29 (spinach, lane 6) and E30 (pea, lane 7) were transcribed *in vitro*[3] and translated in the presence of [^{35}S]methionine, processed *in vitro*[3] and analysed by SDS–PAGE and fluorography; p and m represent the precursor and the mature forms of E29 and E30, respectively. *b*, Purification of E29 by chromatography on hydroxylapatite followed by chromatography on heparin-Sepharose CL-6B. Triton X-100-solubilized envelope membranes (lane 1) were fractionated on hydroxylapatite and the unbound fraction (lane 2) chromatographed on heparin–Sepharose CL-6B (lane 3, pass-through fraction; lane 4, fraction eluted with 0.6 M NaCl). *c* and *d*, Chromatography of solubilized envelope membranes (*c*, SDS–PAGE and silver staining; the position of E29 is marked by an asterisk) and solubilized [^{3}H]DIDS-labelled envelope membranes (*d*, SDS–PAGE and fluorography) on hydroxylapatite at 4 °C (lanes 2 and 3) and 22 °C (lanes 4 and 5). Lanes 2 and 4 represent unbound fractions, lanes 3 and 5 fractions eluted from hydroxylapatite with 0.8 M sodium phosphate/0.1% SDS. *e*, Chromatography on hydroxylapatite of solubilized envelope membranes containing ^{35}S-labelled E29 imported *in vitro* which had been synthesized from the corresponding cDNA clone[2]. Lane 1, precursor form of E29; lane 2, total envelope membrane proteins; lanes 3–5, fractions unbound to hydroxylapaptite; lane 6, wash; lane 7, fraction eluted from hydroxylapatite with 0.8 M sodium phosphate/0.1% SDS. Fractions were analysed by SDS–PAGE and fluorography. In *a–c*, relative molecular mass calibration is shown on the left (in thousands).

METHODS. *a*, Intact chloroplasts from spinach and pea leaves were labelled by incubation with 2 μM or 10 μM [^{3}H]DIDS[9] and analysed by SDS–PAGE[26] with silver staining and fluorography[27]. The cDNA clones encoding E29 and E30 were transcribed and translated *in vitro* as described[3], processed *in vitro*[3] and then subjected to SDS-PAGE and fluorography. *b*, Hydroxylapatite chromatography of purified envelope membranes was essentially as described[10]: envelope membranes (5 mg ml^{-1}) in 10 mM Tricine buffer, pH 7.4 were solubilized in Triton X-100 (final concentration, 3% v/v) for 2 min at 4 °C and added to hydroxylapatite (1.2 g per ml solubilized protein) that had been equilibrated with 10 mM Tricine buffer, pH 7.4, containing 0.2% Triton X-100 (buffer A). After 2 min on ice, the mixture was centrifuged (10,000*g* for 1 min) and the unbound fraction applied to a heparin-Sepharose CL-6B column (1.5 ml bed volume per ml sample; equilibrated with buffer A) which was eluted with a 0–1.0 M NaCl gradient. Aliquots of each fraction were assayed for protein[28], reconstitution of phosphate-transport activity[10], and analysed by SDS–PAGE. *c* and *d*, Envelope membranes (*c*) or [^{3}H]DIDS-labelled envelope membranes (*d*) were solubilized in Triton X-100 and incubated with hydroxylapatite for 5 min at 4 °C or for 30 min at 22 °C, respectively. The unbound fraction was collected by centrifugation and the hydroxylapatite washed three times with buffer A. The hydroxylapatite-bound proteins were eluted by washing the gel material with 0.8 M sodium phosphate, pH 7.2, 0.1% SDS and were then analysed by SDS–PAGE and silver staining (*c*) or by SDS–PAGE/fluorography (*d*). *e*, The ^{35}S-labelled precursor of E29 was synthesized by *in vitro* transcription/translation and imported into intact chloroplasts[2]. The protein import buffer contained 0.05 M HEPES–KOH, pH 8.0, 0.3 M sorbitol, 10 mM methionine, 25 mM potassium gluconate, 0.2% bovine serum albumin, 50 mM lysine, 2 mM $MgSO_4$, 2 mM ATP. After 15 min at 22 °C, chloroplasts were treated with thermolysin (30 μg ml^{-1} for 15 min at 0 °C). Envelope membranes were isolated from the import assay[2], solubilized in Triton X-100 and chromatographed on hydroxylapatite.

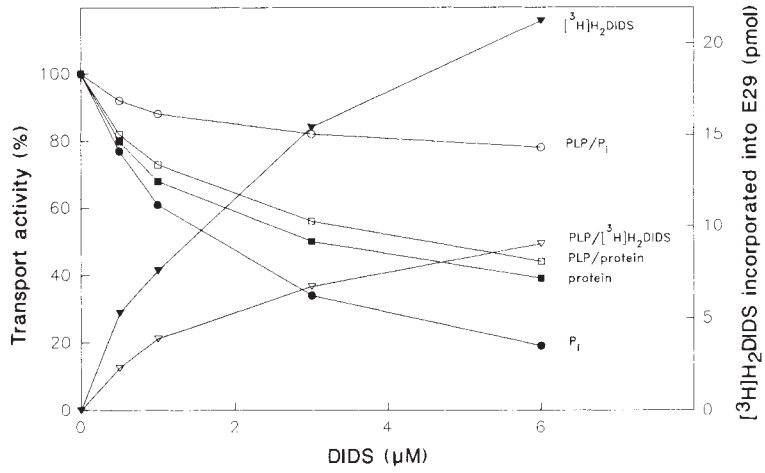

FIG. 2 [^{3}H]DIDS-labelling of E29 is linked to the inhibition of phosphate transport activity but not to protein import activity. *a*, Effect of pretreatment of the chloroplasts with pyridoxal-5'phosphate (PLP) on the inhibition of the transport of phosphate and on the inhibition of import of the ribulose-1,5-biphosphate carboxylase small subunit precursor (pSSU) by DIDS and, in addition, on the incorporation of [^{3}H]DIDS into E29. Phosphate transport (circles) and import of pSSU (squares) into chloroplasts that had been pretreated without (filled symbols) and with PLP (open symbols); triangles represent incorporation of [^{3}H]DIDS into E29 of chloroplast envelope membranes: ▼, control; ▽, effect of preincubation of the chloroplasts with PLP before the addition of [^{3}H]DIDS. METHODS. *a*, Intact chloroplasts (0.17 mg chlorophyll ml^{-1}) were incubated in 0.33 M sorbitol, 10 mM tricine buffer, pH 7.4, with or without 0.08 mM PLP for 30 min at 4 °C and then for a further 30 min with increasing concentrations of DIDS or [^{3}H]DIDS, respectively. They were then washed twice in 0.33 M sorbitol, 10 mM Tricine, pH 7.4, resuspended in protein import buffer (see legend to Fig. 1) and the phosphate transport activity[29], protein import activity and incorporation of [^{3}H]DIDS into E29 (ref. 9), respectively, were measured after 30 min. For quantification of [^{3}H]DIDS incorporated into E29 and of protein import activity, both E29 and the processed form of pSSU were cut out of and eluted from the unstained SDS-polyacrylamide gel by incubation with 5% SDS for 12 h at 37 °C (ref. 9). Aliquots of centrifugal supernatants were used for liquid scintillation counting.

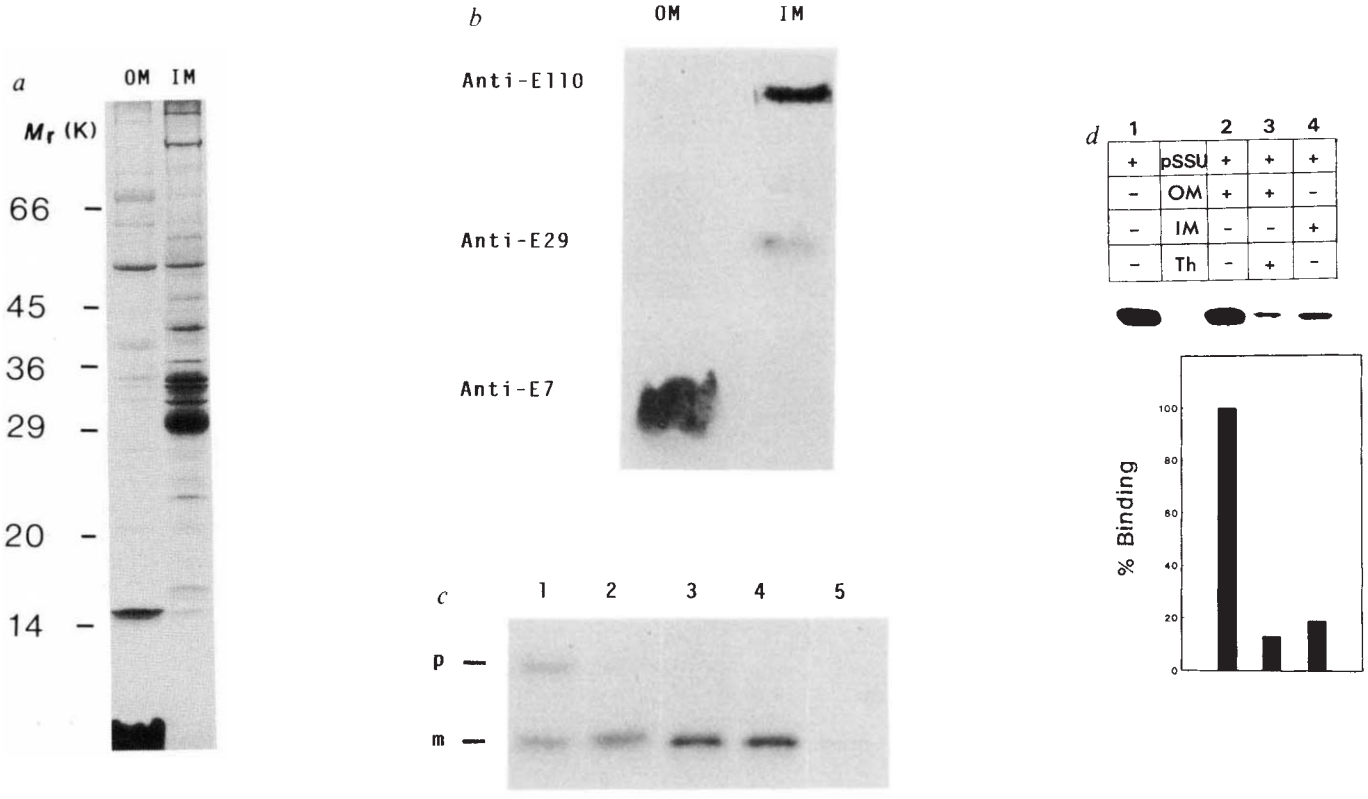

FIG. 3 E29 is a protease-resistant component of the inner envelope membrane but the protease-sensitive precursor binding activity is located in the outer envelope membrane. *a*, SDS-PAGE of purified outer (OM) and inner envelope membranes (IM) from pea chloroplasts. Relative molecular mass calibration shown on the left. Purified outer and inner envelope membranes were obtained by hypertonic lysis of chloroplasts followed by separation of the membrane vesicles on sucrose density gradients[19,30]. *b*, Western-blot analysis of purified inner and outer envelope membranes probed with antibodies against E110 (an inner envelope membrane protein[17]), E29, and E7 (an outer envelope membrane protein[18]). *c*, Effect of thermolysin-treatment of chloroplasts (30 μg ml^{-1} for 15 min at 4 °C) on *in vitro* imported ^{35}S-labelled E29 (lanes 1 and 2) and on [^{3}H]DIDS-labelled E29 (lanes 3 and 4). Envelope membranes were isolated from the assays and analysed by .SDS-PAGE and fluorography. Lane 5, before SDS–PAGE, envelope membranes containing imported E29 were treated with Triton X-100 (2%, v/v) in the presence of thermolysin (30 μg ml^{-1} for 15 min at 4 °C). Precursor and mature forms of E29 are indicated by p and m respectively. *d*, Histogram showing that outer envelope membranes specifically bind precursor proteins in a protease-sensitive way. Th, thermolysin pretreatment. Assay numbers are indicated at the top; + and −, presence or absence of components in the assay. Binding of pSSU synthesized *in vitro* to outer and inner envelope membranes was as described[19].

Figure 1*e* shows that E29 synthesized from its cDNA and imported *in vitro*, shares the properties of chloroplast envelope E29 [^{3}H]DIDS-labelled E29, and the phosphate-transport activity: for the most part it is not bound to hydroxylapatite at 4 °C after solubilization in Triton X-100. These findings indicate that the cDNA clone reported in refs 2, 3 and 7 encodes the chloroplast phosphate translocator.

Figure 2 shows that DIDS inhibits the transport of both phosphate and protein, for example, the import of ribulose-1,5-biphosphate carboxylase small subunit precursor. Increasing amounts of [^{3}H]DIDS are incorporated into E29 as a function of [^{3}H]DIDS concentration. Chloroplasts were alternatively pretreated with 0.08 mM pyridoxal-5′phosphate (PLP), a potent inhibitor of phosphate transport[13] which affects protein import activity only at higher concentrations (not shown), and incubated with increasing amounts of DIDS or [^{3}H]DIDS. Subsequent treatment with lysine to hydrolyse the Schiff-base formed between PLP and the target protein, and to restore any PLP-inhibited activity, revealed that inhibition of protein import by DIDS was almost unchanged, whereas the inhibition of phosphate transport as well as the incorporation of [^{3}H]DIDS into E29 were both drastically reduced. These data indicate that the incorporation of [^{3}H]DIDS into E29 is linked to the inhibition of the transport of phosphate by DIDS and not to the protein import process. Thus, E29 is not involved in the translocation of proteins but rather of phosphate. One of the higher-molecular weight polypeptides that was labelled at higher [^{3}H]DIDS concentrations and was revealed by overexposing the fluorogram (Fig. 1*a*) might possibly be associated with protein import function.

We next addressed the localization of E29 in the envelope membranes. Metabolite translocator proteins like the phosphate translocator reside in the inner envelope membrane, whereas receptor proteins are thought to be in the outer envelope. This agrees with the observation that pretreatment of chloroplasts with the protease thermolysin abolishes binding of precursor proteins[14–16]. Schnell *et al.*[7], however, did not determine whether *in vitro*-synthesized E29 (their putative import receptor) was imported into the outer membrane or test whether it was protease-sensitive as would be expected for a receptor protein, nor did they establish whether antibody reactivity was restricted to the outer envelope. Figure 3*a* and *b* shows that E29 (and the 110K protein, an inner envelope membrane protein[17]) is exclusively localized to the inner envelope membrane. On the other hand, E7, an outer envelope membrane protein[17,18], could be detected only in the outer envelope fraction. In line with this is the observation that only the surface-bound precursor form of *in vitro*-imported E29 is protease-sensitive, whereas the mature form of *in vitro*-imported E29 and [^{3}H]DIDS-labelled E29 are both protease-resistant (Fig. 3*c*). In its solubilized form, however, E29 is completely digested by thermolysin (Fig. 3*c*). The localization of E29 to the inner envelope membrane was also demonstrated by western-blot analysis of envelope membranes from chloroplasts pretreated with thermolysin and from untreated controls (not shown).

Our localization of E29 could be attributed to the fractionation procedure of the inner and outer envelope membranes causing E29 to be pushed from putative contact sites between the inner and outer membranes into the inner envelope. We therefore investigated which membrane has the ability to bind precursor proteins. Figure 3*d* shows that in a functional envelope import system[19], only the outer envelope membrane (not containing E29) can bind precursor proteins in a protease-dependent way, which further indicates that E29 is not involved in protein receptor function. The observation that antibodies directed against E29 react with components associated with precursor binding activity[7] could be explained if the import receptor and the chloroplast phosphate translocator were to share common epitopes recognized by the antibody. This idea is supported by the observation that the mitochondrial 38K protein MOM38, which is part of the mitochondrial translocation apparatus, contains a sequence of ~70 amino acids with significant homology to an abundant 32K component of the inner mitochondrial membrane, the phosphate/OH^- carrier[20–22]. This protein has been assigned as the mitochondrial import receptor using an anti-idiotype approach[23,24]. It is feasible that in chloroplasts E29 as an integral constituent of the inner envelope membrane could also have regions homologous to components of the translocation apparatus such as the chloroplast import receptor. It is possible that some antibodies raised against abundant membrane proteins (for example, E29 or the mitochondrial phosphate/OH^- carrier) might also recognize less abundant import receptors and so affect protein translocation into organelles. It is unlikely that an import receptor would be a major constituent of the chloroplast envelope membrane like E29: assuming 3,000–5,000 precursor binding sites per chloroplast[25], the import receptor should correspond to about 0.02–0.04% of the envelope membrane protein, which is far less than the amount of E29 in the membrane (~12%). □

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