Chloroplast FtsY, Chloroplast Signal Recognition Particle, and GTP Are Required to Reconstitute the Soluble Phase of Light-harvesting Chlorophyll Protein Transport into Thylakoid Membranes*

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The integration of light-harvesting chlorophyll proteins (LHCPs) into the thylakoid membrane proceeds in two steps. First, LHCP interacts with a chloroplast signal recognition particle (cpSRP) to form a soluble targeting intermediate called the transit complex. Second, LHCP integrates into the thylakoid membrane in the presence of GTP, at least one other soluble factor, and undefined membrane components. We previously determined that cpSRP is composed of 43- and 54-kDa polypeptides. We have examined the subunit stoichiometry of cpSRP and find that it is trimeric and composed of two subunits of cpSRP43/subunit of cpSRP54. A chloroplast homologue of FtsY, an Escherichia coli protein that is critical for the function of E. coli SRP, was found largely in the stroma unassociated with cpSRP. When chloroplast FtsY was combined with cpSRP and GTP, the three factors promoted efficient LHCP integration into thylakoid membranes in the absence of stroma, demonstrating that they are all required for reconstituting the soluble phase of LHCP transport.

SRP mediates the cotranslational targeting of endomembrane and secretory proteins to the endoplasmic reticulum in eukaryotes and of polytopic membrane proteins to the cytoplasmic membrane in prokaryotes (1–3). Cytosolic forms of SRP are ubiquitous in eukaryotic and prokaryotic organisms. All contain, at a minimum, a 54-kDa GTPase subunit and an RNA (1, 2). Membrane targeting is facilitated by an interaction between SRP and an SRP receptor (4–6). In eukaryotes, the receptor consists of two GTPases, a peripheral protein (the SRP receptor α-subunit), and an integral membrane polypeptide (the SRP receptor β-subunit) (7, 8). The localization of the SRP receptor to the membrane may facilitate, but is not essential for, targeting (9). A key feature of the SRP/SRP receptor interaction is the ability of the SRP receptor α-subunit and SRP54 to reciprocally stimulate their GTP hydrolysis activities upon mutual binding in the presence of SRP RNA and thereby to regulate the GTP hydrolysis cycle associated with SRP-dependent protein targeting (10, 11).

Recently, a specialized SRP was found in the chloroplast (12, 13). cpSRP contains a homologue of SRP54 (14), but differs from cytoplasmic forms, as it lacks an RNA, contains a novel 43-kDa subunit, and interacts with substrates post-translationally (12, 15, 16). Both genetic and biochemical evidence indicates that the 43-kDa subunit is essential for this post-translational interaction (12, 15, 17). The known substrates of cpSRP are the LHCPs, hydrophobic proteins that are synthesized in the cytoplasm and are post-translationally transported to the internal membranes of the chloroplast via the soluble phase (18, 19). The solubility of LHCP is maintained in the stroma by its binding to cpSRP to form the targeting intermediate termed the transit complex (12, 16, 20).

The transit complex can be reconstituted in vitro from purified cpSRP and LHCP, suggesting that it is composed of cpSRP54, cpSRP43, and LHCP. However, one unresolved issue is the subunit stoichiometry of cpSRP and the transit complex. The molecular weight estimate of the transit complex from nondenaturing gel analysis is 120,000 (20), whereas the molecular weight of cpSRP from gel filtration is 200,000 (12). Also unresolved is the fact that the soluble form of LHCP is incapable of inserting into the thylakoid membrane unless additional stroma is added (12, 20). The requirement for additional stroma has fueled the speculation that two stromal factors are involved in LHCP integration: one factor, cpSRP, binds LHCP to form the intermediate, and the second facilitates membrane insertion (12, 20). This idea is directly supported by the observation that LHCP integration does not occur when the stroma is immunodepleted of cpSRP, but does occur when the immunodepleted stroma is supplemented with cpSRP (12).

Whereas LHCP integration requires GTP hydrolysis (21), the formation of the transit complex is not GTP-dependent (12, 20). Therefore, it is likely that the second chloroplast protein participates in the regulation of the GTPase activity of cpSRP54; and hence, a likely candidate would be a homologue of the SRP receptor. An essential Escherichia coli protein, FtsY (22), is homologous to the soluble α-subunit of the SRP receptor (23). Recently, a putative ftsY gene was detected on chromosome II of Arabidopsis (Bacterial Artificial Clone number F4118.25 and GenBank™ accession number ATAC00465). In the present work, we demonstrate that the FtsY homologue is a chloroplast protein and, together with cpSRP and GTP, is required for reconstituting the soluble phase of LHCP transport. Furthermore, using these functionally active proteins, we have measured the subunit stoichiometry of cpSRP.

EXPERIMENTAL PROCEDURES

DNA constructs

GST43 Translation Vector (pSPUTKGSTchaos)—The chaos cDNA encoding cpSRP43 was subcloned into the E. coli expression vector pGTK+ (generously provided by John Walker) by PCR amplification of the plasmid pBSSK′sschaos with the primers GGAATTCCGCCTGCGGCCGCGC.
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TACAAAGAAAC, which introduces an EcoRI site just 5' to the processing site, and GTAATCACTACTATAGCCC (T7 primer), which results in the introduction of a 3'-XhoI site from the polylinker. The resulting PCR product was digested with EcoRI and XhoI and subcloned into the same sites of pGKT to form plasmid pGTK. This plasmid encodes a GST43 fusion protein. To make the translation construct of GST43, the insert from pGTK was subcloned in two steps. First, pGTK was PCR-amplified with ACTATCCATGGCCCTTACTAGGG, which introduces an NcoI site at the initiation codon of GST, and CGGGGATCCTCATTCTGTTGTTG, a reverse primer that hybridizes to the 3'-end of the cDNA. The PCR product was digested with NcoI and BamHI, and the 760-bp pair fragment encoding GST was subcloned into the NcoI and BamHI sites of pSPUTK to form pTU3. The remaining portion of the insert from pGTK was subcloned as a BamHI-ClaI fragment into similarly digested pTU3 to form pSPUTKGSTchaos.

GST43 Expression Vector (pGTK 54his) pTU3 was digested with HindIII and partially digested with EcoRI. The 1500-base pair fragment was subcloned into the same sites in pGTK to form pGTK 54his. GST43 Translation Vector (pSPUTK54his) pEU1 was digested with BamHI and HindIII and cloned into the 3.6-kilobase vector fragment from pSPUTKGSTchaos digested with BamHI and partially digested with HindIII to form pSPUTK 54his. This vector (RNeasy kit, Qiagen Inc.) from Arabidopsis leaf tissue and used to amplify the ftsY DNA by reverse transcription-PCR using a kit from Life Technologies, Inc. To clone the FtsY precursor into a translation vector, the forward and reverse primers CCTTGACACAGCCTGCGAGCATCTCT and GGTCTTAAGCTTAAGAGATATGACATTGAC were used, respectively. To introduce NcoI and HindIII sites at the initiation methionine and stop codons of the open reading frame, and the resulting PCR product was cloned into the same sites of pSSS.5NcoI (14) to form pTU1. For overexpressing FtsY in E. coli, the forward primer GGCGATCGCCGGCACGGGGCATGTCGTT, which introduces a BamHI site at the predicted processing site, and the above reverse primer were used to PCR amplify cDNA that was cloned into the BamHI and HindIII sites of pQE30 to form pTU2. The T4 DNA expression vector (pGEX4Tchaos(m)), the cpSRP54 translation vector (pGTK), and the LhcB1 translation vector (pAB80) have been previously described (12, 12, 13).

Antibodies and Immunoblot Analysis

Recombinant FtsY was expressed from pTU2 in the E. coli strain XL-Blue. Cells were grown in LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin to an absorbance of 0.6–1.0, and expression was induced by the addition of 0.1 mM isopropyl-p-b-thiogalactoside for 3 h. Cells were harvested and frozen at −80 °C until use. Overexpressed protein was purified on Ni2+-NTA-agarose (Qiagen Inc.) as suggested by the manufacturer. Recombinant protein was further purified by SDS-PAGE, eluted from gel slices, and injected into rabbits to raise antibodies (Cocalico Biologicals, Inc., Reamstown, PA). The IgG fraction was prepared from crude serum and affinity-purified on antigen cross-linked to Affi-Gel 10 (25). Immunoblot analysis was done as described (24). Antibodies against LHC-P (12), cpSRP43 (15), and cpSRP45 (17) were previously described.

Cross-linking

20 ng of recombinant cpSRP43 or GST-cpSRP43 were incubated with 1 mM disuccinimidyl tartarate in 20 mM HEPES-KOH, pH 8.0, and 150 mM NaCl for 2 h on ice in a final volume of 20 µl. The cross-linking reaction was quenched by the addition of 1.5 µl of 10 mM Tris-HCl, pH 8.0, and incubation for 15 min at room temperature. Samples were separated on 8% SDS-polyacrylamide gels and detected by immunoblotting as described (24).

Subunit Stoichiometry

cpSRP was assembled by incubating 3.4 µCi of cpSRP54his and 2.7 µCi of GST43 translation products with incubation buffer (20 mM HEPES-KOH, pH 8.0, 50 mM KCl, and 10 mM MgCl2) for 15 min at 25 °C. The reaction was mixed end-over-end with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. The beads were washed three times with 1.5 ml of washing buffer (20 mM HEPES-KOH, pH 8.0, 0.3 M KCl, 10 mM MgCl2, and 1% Tween 20). The beads were transferred to Wizard minicolumns (Promega), and the protein was eluted in 50 µl of 10 mM glutathione in incubation buffer. The eluted sample was diluted to 120 µl with incubation buffer and incubated with Ni2+-NTA-agarose beads for 1 h at 4 °C. The beads were transferred to a second Wizard column, washed three times with 1.5 ml of washing buffer, and eluted in 45 µl of 200 mM imidazole in 20 mM HEPES-KOH, pH 8.0. The sample was analyzed by SDS-PAGE on 13% acrylamide gels, and radioactivity was quantitated by radioimaging on a PhosphorImager (Molecular Dynamics, Inc.). Normalized pixel values were calculated by dividing each band by the number of methionines within the protein, and the ratio of the normalized values was used to calculate the molar ratio of the two subunits. To investigate cpSRP43-mediated dimerization of cpSRP54, 0.5 µg of GST54 was incubated with 3.4 µCi of cpSRP54his, 0.5 µg of cpSRP43, or both under standard conditions; purified on glutathione-Sepharose; and analyzed by SDS-PAGE as described above.

Isolation of Stroma, Salt Washing of Thylakoids, and Gel Filtration

The stroma was collected from chloroplasts lysed in 20 mM HEPES-KOH, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) at 2 mg of chlorophyll/ml and centrifuged for 10 min in a microcentrifuge. The thylakoid pellet was resuspended in lysis buffer containing the indicated salt solution. Samples were rotated end-over-end for 10 min at 4 °C and centrifuged, and the pellet was washed a second time as described before and resuspended in lysis buffer at a concentration of 0.5 mg of chlorophyll/ml. Arabidopsis stroma, pea stroma, or purified recombinant protein was fractionated on a Superose 6HR gel filtration column (Amersham Pharmacia Biotech) in 20 mM HEPES-KOH, pH 7.0, 0.5 mM MgCl2, and 180 mM NaCl at 0.5 ml/min and analyzed as described (12).

Reconstitution

Pea thylakoids were washed one time in 20 mM HEPES-KOH, pH 8.0, and 2 mM KOAc and twice in 50 mM HEPES-KOH, pH 8.0, and 330 mM sorbitol. Thylakoids containing 25 µg of chlorophyll were re-suspended in a total of 100 µl of the indicated reaction mixture. Each reaction mixture contained 10 mM MgCl2, 10 mM methionine, 0.15 mM GTP, 82 mM sorbitol, and 13 mM HEPES-KOH, pH 8.0. cpSRP54, FtsY, and LHC-P were translated either in a wheat germ translation extract or a commercial rabbit reticulocyte lysate. The amounts added for wheat germ and rabbit reticulocyte lysate translations were as follows: 300 µg of cpSRP54, 20 and 20 µl, respectively; cpFtsY, 40 and 30 µl, respectively; and LHC-P, 5 and 40 µl, respectively. Recombinant cpSRP43 (150 ng), pea stroma (equivalent to 90 µg of chlorophyll), and aprylase (1 unit) were added as noted. Reactions were incubated for 30 min at 25 °C, followed by trypsin treatment and analysis by SDS-PAGE and fluorography.

General

Previously described conditions were used for import of FtsY into chloroplasts (26), in vitro transcription/translation (26), and reconstitution and purification of GST fusion proteins (12). Mature cpSRP43 was generated by thrombin cleavage of GST43 using biotinylated thrombin and purified from GST and thrombin using streptavidin-agarose (Novagen) as suggested by the manufacturer.

RESULTS
cpSRP43 Is a Dimer—We previously determined that cpSRP43 is a monomer based on gel filtration analysis of cpSRP45 translation products (Fig. 1A) (22). To determine the oligomeric state of cpSRP43, we examined the gel filtration characteristics of cpSRP43 from the stroma of ftc1-2, an Arabidopsis mutant that lacks cpSRP54 (17). Stromal cpSRP43 eluted as a 70-kDa protein (Fig. 1B), which is identical to the elution profile of purified recombinant cpSRP43 (data not shown). The same molecular mass estimate was obtained by nondenaturing gel electrophoresis (data not shown) (27). Whereas Arabidopsis cpSRP43 migrates as a 42-kDa polypeptide during SDS-PAGE, its molecular mass determined from cDNA sequence is 35 kDa (15). Hence the 70-kDa species identified by gel filtration probably represents a homodimer. This notion is further supported by cross-linking experiments. Following treatment of dilute protein solutions with a cross-linking reagent, part of the Arabidopsis cpSRP43 and cpSRP43 fused to glutathione S-transferase (GST43, 66 kDa) migrate as a 90- and 126-kDa species, respectively, on SDS-polyacrylamid-
amide gels (Fig. 1B). Furthermore, based on the cDNA sequences, cpSRP43 has chromodomains that could mediate dimerization (15).

**Stoichiometry of cpSRP**—To determine the subunit stoichiometry of cpSRP, the complex was assembled *in vitro* from cpSRP54his and GST43 translation products. cpSRP was purified by successive passes of the sample over glutathione-Sepharose and Ni²⁺-NTA-agarose, and the final eluate was analyzed by SDS-PAGE (Fig. 2a). GST43 and cpSRP54his alone did not bind to Ni²⁺-NTA-agarose and glutathione-Sepharose, respectively. However, these proteins did copurify on the resins after assembly. The eluate from successive passes of the assembled complex over the two resins was quantitated by radioimaging, and the GST43:54his ratio was determined to be 2:1 in two independent experiments. This ratio suggested that cpSRP is a trimer composed of one cpSRP43 dimer and one cpSRP54 monomer. An alternative possibility inconsistent with the binding data, but more consistent with the previously determined molecular mass estimation by gel filtration (200 kDa) (12) is that each cpSRP is a tetramer consisting of two cpSRP54/cpSRP43 heterodimers. A qualitative test to distinguish these two possibilities was conducted using GST54 and cpSRP54his, which are discernible by size and binding affinity for glutathione-Sepharose. Recombinant GST54 was incubated with the cpSRP54 translation product in the presence and absence of cpSRP43. If cpSRP43 causes cpSRP54 to dimerize, then radiolabeled cpSRP54his should bind GST54 in the presence of cpSRP43. However, no additional cpSRP54his bound to GST54 in the presence of cpSRP43 (Fig. 2b). Immunoblotting of the fractions revealed that cpSRP43 bound to GST54, and this interaction was reduced by the presence of cpSRP54his, indicating that both forms of cpSRP54 bound cpSRP43. That cpSRP54his bound to cpSRP43 is also evident from the retention of cpSRP54his on glutathione-Sepharose in the presence of GST43 (Fig. 2b). Together, these data indicate that cpSRP is a trimer.

**Arabidopsis Contains a Chloroplast Homologue of Bacterial FtsY**—An alignment of the Arabidopsis FtsY homologue with related sequences is shown in Fig. 3. High similarity among all sequences is observed in the C terminus containing the GTP-binding and hydrolysis domains. An acidic N terminus present in *E. coli* and *Synechocystis* FtsY is absent in the *Arabidopsis* protein. The N terminus of the Arabidopsis protein is predicted to contain a chloroplast transit peptide cleaved after Arg-40 based on analysis by the ChloroP program (28).

After our study was complete, but prior to the publication of our results, Kogata *et al.* (29) reported that the *Arabidopsis* FtsY homologue was a chloroplast protein localized to the thylakoid membrane. We tested whether the putative *ftsY* clone encodes a chloroplast protein by incubating the corresponding translation product with isolated pea chloroplasts as described (26). The precursor (44 kDa) was processed to a 37-kDa protease-protected mature form, indicating that the protein was imported into the chloroplast (Fig. 4A, lanes 1–3). Upon subsequent fractionation, the imported protein was found to associate with both the thylakoid membrane and the stroma. Antibodies raised in rabbits and affinity-purified against the recombinant protein readily detected 5 ng of antigen (Fig. 4A, lane 4). The same antibodies cross-reacted with a single Arabidopsis chloroplast protein that comigrated with the mature form of the imported protein, whereas the corresponding pea protein migrated slightly faster on SDS-PAGE (Fig. 4A, lanes 5 and 6). In contrast to the results reported by Kogata *et al.* (29), the majority of the cross-reacting protein was localized in the stroma, although a significant fraction was found associated with the thylakoid membrane (Fig. 4A, lanes 2, 3, 6, and 7). Putative *ftsY* was effectively removed from the thylakoid membrane after two washes in 0.5 M KOAc. Together, these data indicate that the *ftsY* clone encodes a soluble chloroplast...
protein that has an affinity for the thylakoid membrane.

cpFtsY Is a Monomer—To determine if other factors associate with putative cpFtsY, we fractionated the stroma by fast protein liquid chromatography and used antibodies to quantify the protein in the different fractions. cpFtsY eluted with an estimated molecular mass of 25 kDa (Fig. 4B), implying that the stromal form is a monomer. The same blot probed with antibodies against cpSRP54 and cpSRP43 revealed that, in contrast and as previously observed, cpSRP54 and cpSRP43 coeluted in the 200-kDa fraction (12). These data indicate that soluble cpFtsY does not form a stable complex with cpSRP. As such, it would remain in the stroma after immunodepletion of cpSRP, a characteristic of the second factor required for LHCP integration.

**DISCUSSION**

This work establishes four new and important points. First, we show that cpSRP43 is a dimer. Second, we demonstrate that cpSRP is a trimer consisting of two cpSRP43 subunits and one cpSRP54 subunit (Fig. 6). Third, we show that cpFtsY is a soluble chloroplast protein that has a weak affinity for cpSRP. Fourth, we conclusively demonstrate that cpFtsY is the second soluble factor that is required to reconstitute the soluble phase of LHCP transport.

cpSRP is distinctive in its ability to interact with members of the LHCP protein family post-translationally. It is likely that this specialized role is mediated directly or indirectly through cpSRP43. From an analysis of Arabidopsis mutants that lack cpSRP43, it appears that only members of the LHCP protein family are adversely affected (15, 17). Thus, it appears that cpSRP43 functions primarily, if not exclusively, in LHCP biogenesis. Mutant plants lacking cpSRP54 show wider effects; chloroplast encoded proteins whose targeting is cotranslational are affected in addition to LHCP (17, 24). A substantial pool of cpSRP54 is dissociated from cpSRP43 and associated with 70 S ribosomes (14). Furthermore, cpSRP54 has been shown to directly interact with the nascent chain of a chloroplast protein synthesized in a chloroplast translation extract (30). Therefore, we think it likely that cpSRP54 free of cpSRP43 mediates cotranslational targeting, whereas the presence of cpSRP43 enables the post-translational interaction between cpSRP and LHCP. Cross-linking data clearly demonstrate that cpSRP54 mediates the cpSRP-dependent integration of LHCP into the thylakoid membranes.
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Fig. 4. A, import of the FtsY precursor into pea chloroplasts and localization of the processed protein. The FtsY precursor was synthesized in vitro (26), and the radiolabeled product was incubated with intact pea chloroplasts and subsequently treated with 0.1 mg/ml thermolysin (42). Chloroplasts were osmotically lysed by resuspending in 10 mM HEPES-KOH, pH 8.0, and 10 mM EDTA; stromal (Str) and thylakoid (Tk) fractions were separated by centrifugation; and the fractions were analyzed by SDS-PAGE and fluorography. Samples prepared from intact purified chloroplasts containing the indicated amounts of chlorophyll (Chl) or 5 ng of FtsY antigen were immunoblotted against cpFtsY antisera. B, FtsY does not co-chromatograph with cpSRP. The stroma was prepared from intact pea chloroplasts and fractionated by fast protein liquid chromatography on a Superose 6HR column. Each fraction was immunoblotted against cpSRP54, cpSRP43, and cpFtsY antisera and quantitated as described (12). Arab, Arabidopsis; pFtsY, precursor FtsY; mFtsY, mature FtsY; Trans, translation product; Clps, chloroplast.

directly interacts with LHCP (16). It remains to be shown whether cpSRP43 is also able to interact directly with LHCP or whether it simply modifies the conformation of cpSRP54 to facilitate the post-translational interaction. Previously, we entertained the possibility that cpSRP43 effectively dimerized cpSRP54 and thereby created a novel interaction between the SRP54 homologue and its substrate (12). The results from the present study clearly indicate that cpSRP, like cytoplasmic SRP, contains a single SRP54 subunit that presumably binds a single substrate molecule. Thus, the predicted molecular mass of cpSRP is 123 kDa. The large deviation of the mass estimate by gel filtration from the predicted value suggests that cpSRP is not a globular protein.

Previous work provided strong evidence that LHCP integration required multiple soluble factors (12, 20), a hypothesis that has now been validated. The present data also provide strong evidence that cpSRP, cpFtsY, and GTP are sufficient for reconstituting the soluble phase of LHCP transport. Purifying an active form of cpSRP54 is an obstacle that must be overcome to prove this point conclusively. For reconstitution experiments, recombinant and highly purified cpSRP43 was used, whereas cpSRP54, cpFtsY, and LHCP were synthesized by translation in either wheat germ extracts or rabbit reticulocyte lysates (data not shown). The fact that LHCP integration can be reconstituted using translation products synthesized in rabbit reticulocyte lysates indicates that no other chloroplast factors are required for the reaction.

In E. coli, SRP-dependent proteins are inserted into the membrane via the Sec translocon (31), which minimally consists of SecA/E/Y (32, 33). Homologues of all three proteins are found in the chloroplast and are required for the translocation of the luminal 33-kDa oxygen-evolving protein OE33 (34–37). One unresolved question is whether the Sec translocon is required for LHCP integration. The results presented here are inconsistent with the involvement of cpSecA in LHCP integration. cpSecA is a soluble protein that needs to be added as stroma or purified protein to reconstitute efficient translocation of OE33 across the thylakoid membrane (34, 38, 39). In the present work, efficient integration of LHCP occurred without specifically adding cpSecA. Together with the observations that azide (an inhibitor of cpSecA) does not inhibit LHCP integration (34), that OE33 is not a competitor of LHCP integration (40), and that maize SecA mutants have normal levels of LHCP (41), the data imply either that the cpSRP and cpSec pathways do not converge at the Sec translocon or, alternatively, that cpSec/YE is active in the absence of cpSecA. Either
case represents a fundamental departure from translocation events in E. coli.

We have now shown that cpFtsY is required for the activity of the specialized cpSRP. It remains to be determined whether cpFtsY also functions with cpSRP54 in the biogenesis of chloroplast encoded proteins. In either case, FtsY may regulate the cpFtsY also functions with cpSRP54 in the biogenesis of chloroplast encoded proteins. In either case, FtsY may regulate the cpSRP. It remains to be determined whether cpFtsY also functions with cpSRP54 in the biogenesis of chloroplast encoded proteins. In either case, FtsY may regulate the cpFtsY also functions with cpSRP54 in the biogenesis of chloroplast encoded proteins. In either case, FtsY may regulate the cpFtsY also functions with cpSRP54 in the biogenesis of chloroplast encoded proteins. In either case, FtsY may regulate the cpFtsY also functions with cpSRP54 in the biogenesis of chloroplast encoded proteins. In either case, FtsY may regulate

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