Functional Similarity between the Chloroplast Translocon Component, Tic40, and the Human Co-chaperone, Hsp70-interacting Protein (Hip)∗S

Jocelyn Bédard, Sybille Kubis, Sarat Bimanadham, and Paul Jarvis†

From the Department of Biology, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom

Tic40 is a component of the protein import apparatus of the inner envelope of chloroplasts, but its role in the import mechanism has not been clearly defined. The C terminus of Tic40 shares weak similarity with the C-terminal Sti1 domains of the mammalian Hsp70-interacting protein (Hip) and Hsp70/Hsp90-organizing protein (Hop) co-chaperones. Additionally, Tic40 may possess a tetratricopeptide repeat (TPR) protein-protein interaction domain, another characteristic feature of Hip/Hop co-chaperones. To investigate the functional importance of different parts of the Tic40 protein and to determine whether the homology between Tic40 and co-chaperones is functionally significant, different Tic40 deletion and Tic40:Hip fusion constructs were generated and assessed for complementation activity in the Arabidopsis Tic40 knock-out mutant, tic40. Interestingly, all Tic40 deletion constructs failed to complement tic40, indicating that each part removed is essential for Tic40 function; these included a construct lacking the Sti1-like domain (ΔSti1), a second lacking a central region, including the putative TPR domain (ΔTPR), and a third lacking the predicted transmembrane anchor region. Moreover, the ΔSti1 and ΔTPR constructs caused strong dominant-negative, albino phenotypes in tic40 transformants, indicating that the truncated Tic40 proteins interfere with the residual chloroplastic protein import that occurs in tic40 plants. Remarkably, the Tic40:Hip fusion constructs showed that the Sti1 domain of human Hip is functionally equivalent to the Sti1-like region of Tic40, strongly suggesting a co-chaperone role for the Tic40 protein. Supporting this notion, yeast two-hybrid and bimolecular fluorescence complementation assays demonstrated the in vivo interaction of Tic40 with Tic110, a protein believed to recruit stromal chaperones to protein import sites.

Most chloroplast proteins are nucleus-encoded, synthesized in precursor form (each one with a cleavable transit peptide), and post-translationally imported into the organelle (1–4).

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‡ To whom correspondence should be addressed. Tel.: 44-116-223-1296; Fax: 44-116-252-3330; E-mail: rpj3@le.ac.uk.

Import is mediated by translocon complexes in the outer and inner envelope membranes, termed TOC2 and TIC, respectively. Once translocation through the TOC is initiated, this complex associates with the TIC allowing preprotein transport across both membranes simultaneously. Several components of the TIC complex have been identified, including Tic40 and Tic110, but the functions of many of these proteins remain unclear (1–4). The TIC complex also recruits stromal chaperones, which are thought to drive protein import and mediate the folding of newly imported proteins (5–8).

Tic40 was identified by its association with preproteins undergoing import (9, 10) and is located in the inner envelope membrane in close association with Tic110 (11). Like Tic110, the bulk of Tic40 protrudes into the stroma where it may interact with stromal chaperones (12, 13). Interestingly, Tic40 shares homology with eukaryotic Hip and Hop co-chaperones (11, 12). Weak sequence similarity between Tic40 and Hip/Hop is restricted to ~60 C-terminal residues, which in the co-chaperones constitutes a conserved region termed the Sti1 domain (Sti1 is the yeast homolog of Hop). However, in silico and immunological studies have revealed that the C-proximal region of Tic40 immediately upstream of the putative Sti1 domain likely contains a TPR domain, which is another characteristic feature of Hip/Hop co-chaperones (12).

Hip interacts with Hsp70 and regulates its ATPase cycle by stabilizing the ADP-bound, high substrate affinity form of the chaperone (14). In addition, Hip possesses intrinsic chaperone activity, binding specifically to unfolded proteins and preventing their aggregation (14, 15). Hop interacts with both Hsp70 and Hsp90 to mediate their association (16, 17) but, unlike Hip, does not seem to function as a chaperone itself (18, 19). Together, Hip and Hop facilitate the transfer of some Hsp70-bound protein substrates (e.g. steroid hormone receptors) to Hsp90, in order for them to undergo further folding and reach their final conformation (20). In addition to Tic40, the Arabidopsis genome encodes two Hip homologs, AtHip-1 and AtHip-2 (21). The former is structurally similar to mammalian Hip along its entire length, and so it is likely to function as a canonical Hip co-chaperone. The latter possesses a truncated
Hip-like region and a thioredoxin domain, suggesting an involvement in redox regulation. Neither are closely related to Tic40.

The homology between Tic40 and Hip/Hop co-chaperones suggests that Tic40 may play similar (co)chaperone roles during chloroplast import. In mitochondrial protein import (which is functionally similar to chloroplast import), matrix Hsp70 (mtHsp70) is the key component of the presequence translocation-associated motor, the complex that drives preprotein translocation (22, 23). In addition to mtHsp70, the presequence translocation-associated motor contains Tim44, which recruits mtHsp70 to the import site, and a range of (co)chaperones that regulate mtHsp70 activity (22).

In chloroplasts, Hsp70 is not associated functionally with the TIC (6–8). Instead, the Hsp100 homolog, Hsp93, associates with the translocon in an ATP-dependent fashion and is thought to be functionally analogous to mtHsp70 (6–8). Tic110 has been proposed to recruit Hsp93 to the stromal face of the TIC (cf. Tim44 in mitochondria) and to provide an initial binding site for preproteins as they emerge into the stroma (6–8, 24). More recently, Tic40 was reported to mediate the regulation of Hsp93 activity and the transferal of translocating preproteins between other components of the TIC complex (25). The former role is supported by the fact that yeast Hop (Sti1) is able to interact with Hsp104, another Hsp100 chaperone (26). Interestingly, Tic110, Hsp93, and Tic40 were found to associate with a translocating preprotein at a similar late stage of import (12). Furthermore, the Arabidopsis knock-out mutations, tic110, hsp93, and tic40, interact genetically, supporting the notion that the proteins cooperate functionally in vivo (13).

To further investigate the hypothesis that Arabidopsis Tic40 (atTic40) plays (co)chaperone roles during import, we assessed its functional similarity with human Hip; domain-swap constructs were generated and tested for their ability to complement the tic40 knock-out mutation in Arabidopsis plants. Additionally, to determine the importance of different domains of Tic40, a series of atTIC40 deletion constructs were used in similar tic40 complementation studies.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth—All Arabidopsis thaliana plants were of the Columbia-0 ecotype. The tic40-4 mutant used has been described previously (13). Plants were grown on Murashige-Skoog medium using published procedures (13, 27). Constructs were stably introduced into tic40-4 plants using the floral dip method (28).

Complementation Construct Generation—cDNA fragments for construct assembly were amplified using Proof Start polymerase (Qiagen, Crawley, UK). The atTic40 template for amplification was cDNA 144K24T7 (accession T76608), whereas the hsHsp-Tac sequence included the full coding sequence between the Ncol and Kpnl sites of pSPUTK (Stratagene, La Jolla, CA). Constructs were assembled from PCR fragments and cDNA restriction fragments downstream of the atTIC40 promoter in pSP72 (Promega, Madison, WI). Promoter-construct fusions were transferred to a pBluescript II KS (Stratagene) derivative containing the cauliflower mosaic virus (CaMV) 35S terminator. These cassettes were transferred to the pBin19 T-DNA vector for Agrobacterium and plant transformation. Because the atTIC40 promoter used in these constructs failed to drive robust expression, it was replaced by the CaMV 35S promoter. A detailed account of these cloning procedures is provided as Supplemental Material.

Characterization of Transgenic Lines—Chlorophyll quantification, immunoblotting, reverse transcription-PCR, electron microscopy, chloroplast isolation, and protein import assays were all conducted using standard procedures, as described previously (13, 27).

Yeast Two-hybrid Analysis—Assays were done using the Matchmaker GAL4 Two-hybrid System 3 (Clontech). GAL4 DNA-binding domain (GBD) fusions were made in the pGBK7 vector, and GAL4 activation domain (GAD) fusions were made in the pGADT7 vector.

Sequence encoding the soluble domain of atTic40 (Tic40ΔN; residues 129–447 of the precursor) was cloned from cDNA 144K24T7 as a Dral-BamHI fragment into Smal-BamHI-cut pGBK7 or pGADT7; note that the pGBK7-Tic40 clone was unsuitable for interaction analysis, because it caused strong auto-activation of the HIS3 histidine reporter.

Sequence encoding the soluble domain of atTic110 (Tic110ΔN; residues 144–1016 of the precursor) was amplified from cDNA RAFL09-95-B13 (accession AY099850) using the following primers: Tic110 Smal forward, 5′-acc ggt tgc agg tag ctc-3′; Tic110 Xhol reverse, 5′-act cga gga ttt aaa cga aat tgc c-3′. After sequencing, the Smal-Xhol-cut Tic110 fragment was cloned into Smal-Xhol-cut pGADT7 and into Smal-Sall-cut pGBK7. The constructs were co-transformed into yeast strain AH109 for the plate growth assays. The control clones, pGBK7-p53 (pGBK7-T53) and pGADT7-SV40T (pGADT7-T), were supplied by the manufacturer (Clontech).

Quantitative growth assays were conducted using yeast strain HF7c, essentially as described previously (29) except that cultures were grown to an A600 of 0.5 prior to spotting. The difference in growth on synthetic dropout (SD) medium lacking histidine, tryptophan, and leucine (SD-HTL) versus SD medium lacking tryptophan and leucine (SD-TL) was measured after 1 day only.

Fluorescence Microscopy—Full-length coding sequences for atTic40 and atTic110, as well as the atTic40 ΔTM deletion mutant (all three lacking stop codons), were amplified by PCR using cDNA templates described above and the ΔTM binary construct with the following primers: Tic40 Xhol forward, 5′-act cga gat atg gag aac ctt acc cta g-3′; Tic40 Kpn1 reverse, 5′-agg tcc tcc cta gta gaa gat g-3′; Tic110 Xhol forward, 5′-tgt ctc gat gaa cct ctc act c-3′; and Tic110 Kpn1 reverse, 5′-gac gcc cag gac cta ctc aa cgg aag tgg tgt ctc tcc gtc c-3′. To generate full-length YFP fusions, all three sequences were cloned as XhoI-Kpn1 fragments into pwen18 (30). To generate half-YFP fusions for the bimolecular fluorescence complementation (BiFC) assays, the two full-length sequences were cloned into the vectors pwen18 and pwen18-1 (30).

Protoplasts were prepared from 14-day-old, wild-type Arabidopsis seedlings essentially as described previously (31). Seedlings were chipped with a razor blade in solution containing 2% cellulose and 0.08% macerozyme and incubated for 3.5 h. For transfection, 5 μg of plasmid DNA (per clone) was used with
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100 μl of protoplast suspension (2 × 10⁶ per ml) and 110 μl of 40% PEG4000 solution. Fluorescence analysis was performed using a Nikon Eclipse TE-2000E inverted fluorescence microscope equipped with filters for analyzing YFP (exciter HQ500/20x, emitter HQ535/30m) and chlorophyll autofluorescence (exciter D480/30x, emitter D660/50m) (Chroma Technologies, Rockingham, VT).

For BiFC analysis, samples were analyzed 16–24 h after (co)transfection. The pWEN-NY-Tic110 with pWEN-CY-Tic40 combination was repeated three times and with relevant controls; the empty vectors (pWEN-NY and pWEN-CY) and the pWEN-NY-Tic110 with pWEN-CY-Tic40 clones did not produce detectable fluorescence when transformed alone (data not shown; supplemental Fig. S5).

RESULTS

atTic40 Contains Several Conserved Domains—To identify regions of Tic40 that may be important for function, Tic40 sequences from different plant species were aligned (supplemental Fig. S1). The degree of conservation varied considerably along the length of the alignment. Distinct, highly conserved regions corresponding to the transit peptide cleavage site and the predicted transmembrane domain (11) were detected. In addition, three conserved motifs were found in the central part, upstream of a highly conserved C-terminal region. Very high conservation in the C-terminal ~150 residues suggested that this region may contain one or more important functional domains.

Interestingly, the C terminus of atTic40 was found to display weak similarity to the C termini of eukaryotic Hip and Hop co-chaperones, as reported previously (11, 12). However, only an ~60 residue C-terminal region of atTic40 shows clear homology to the Hip/Hop proteins (Fig. 1A); previous comparisons of Hip and Hop led to the detection of this conserved domain, termed the Sti1 domain (14). The human Hip (hsHip) Sti1 domain shares low but significant similarity with the human Hop (hsHop) Sti1 domain, equivalent to ~25% identity across 60 residues. As shown in Fig. 1B, the atTic40 C terminus is more similar to the Sti1 domains of mammalian Hip proteins (35% identity) than to those of mammalian Hop proteins (19% identity). This homology with the Hip/Hop Sti1 domains suggests that this region of atTic40 may constitute a conserved Sti1 domain. Moreover, the fact that it resides at the extreme C terminus suggests that it may perform a similar function.

Deletion Mutants of the atTic40 Protein—To investigate the functional importance of different parts of the Tic40 protein, three deletion constructs were made from a full-length atTIC40 cDNA (Fig. 1C): ΔTM lacked codons 95–136, including the predicted transmembrane domain; ΔSti1 lacked codons 391–447 and contained a premature in-frame stop codon (introduction of these mutations also generated an I390L missense mutation); and ΔTPR lacked the entire region between the predicted transmembrane and Sti1 domains (codons 139–389), including the C-proximal region proposed to constitute a TPR domain (an in-frame fusion between residues 138 and 390 was generated, as well as an A137V missense mutation).

The deletion constructs (and the full-length atTIC40 cDNA, termed WT40) were inserted into a T-DNA vector carrying the CaMV 35S promoter and terminator sequences and used to transform tic40 knock-out plants. Multiple (≥19) transformants (T₁ plants) were identified for each of the four constructs. Single insertion lines (segregating three resistant T₂ plants for every sensitive T₁ plant) were further propagated to identify homozygous lines. These were subjected to PCR genotyping, to confirm homozygosity of the tic40 mutation and the presence of the appropriate atTic40 transgene (supplemental Fig. S2), before further analysis.

As expected, the positive control construct, WT40, complemented tic40 fully, restoring wild-type-like appearance and growth in most transformants (Fig. 2A). By contrast, none of the atTic40 deletion constructs gave clear tic40 complementation (Fig. 2A). A total of 24, 34, and 38 transformants were identified for ΔTM, ΔSti1, and ΔTPR, respectively. All ΔTM transformants displayed a tic40-like phenotype (Fig. 2A). Interestingly, identification of ΔTPR transformants (and, to a lesser extent, ΔSti1 transformants) was more difficult, because many plants displayed a more severe phenotype than the tic40 mutant; i.e. seedlings with very small, albino cotyledons. Most ΔTPR transformants (23 of 38) produced T₂ progenies containing seedlings with albino cotyledons, whereas only three of the ΔSti1 transformants expressed the albino phenotype (Fig. 2A). The effects of the various deletion constructs were quantified by measuring chlorophyll concentrations in several representative lines (Fig. 2B). As expected, chlorophyll levels in WT40 plants were comparable with those in the wild type, and those in tic40-like ΔTM and ΔSti1 transformants were comparable with those in tic40. By contrast, albino ΔTPR seedlings contained drastically reduced levels of chlorophyll (~11% of the amount in tic40).

atTic40 Deletion Mutants Are Expressed and Targeted to Chloroplasts—Failure of the deletion constructs to complement tic40 suggested that the deleted proteins were nonfunctional or that they were not expressed and/or targeted to chloroplasts properly. To eliminate the latter possibilities, we assessed accumulation of the proteins by immunoblotting, using an antibody raised against the pea Tic40 (psTic40) C terminus (residues 128–436) (11). As expected, WT40 control plants overexpressed a Tic40 protein of the same size (~44 kDa) as that in wild type (Fig. 3A); that the apparent size of mature atTic40 is larger than predicted (41 kDa) is consistent with previous observations and may be due to its high proline content (12). This protein could also be detected in isolated chloroplasts, where it was resistant to exogenously applied thermolysin protease, demonstrating proper targeting to the organelle (Fig. 3B).

Expression products were also detected in ΔTM, ΔSti1, and ΔTPR plants (Fig. 3A). In the ΔTM lines, two faint bands were detected: one at ~43 kDa and another more diffuse band at ~36 kDa (Fig. 3A). It was recently reported that the atTic40 precur- sor is subjected to two distinct proteolytic processing events during its targeting to the inner envelope membrane (32). The first processing event is carried out by the stromal processing peptidase, which cleaves after residue 42 to yield an intermediate form of the protein in the stroma; the second event occurs concomitantly with inner membrane insertion and is mediated by a thus far unidentified, envelope-associated peptidase that...
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The stroma. We therefore suggest that the additional, diffuse band at ~36 kDa includes stromal proteolytic fragments of the ΔTM protein (Fig. 3A). Consistent with this interpretation, this band was resolved into at least two distinct fragments in Fig. 3B. As expected, all detected ΔTM forms (~43- and ~36-kDa fragments) were completely protected from protease in isolated chloroplasts, indicating that all bands contained fully imported protein (Fig. 3B).

In the ΔSti1 lines, a single and abundant protein of ~35 kDa was detected. This is most likely the mature, processed ΔSti1 protein (calculated size, 34 kDa), because it was also detected in isolated, protease-treated chloroplasts (Fig. 3B). Finally, a protein of the expected size (13 kDa) was detected in ΔTPR seedlings (Fig. 3A). It was not possible to isolate chloroplasts from these albino seedlings, but the strong effect of the ΔTPR protein on chloroplast biogenesis (Fig. 2, A and B) strongly suggests that it too is properly targeted to the organelle.

The fact that the ΔTM, ΔSti1, and ΔTPR proteins are expressed (and, in the case of ΔTM and ΔSti1, properly targeted to chloroplasts) means that their failure to complement tic40 can be attributed to effects of the deletions on protein functionality. Thus, we conclude that the transmembrane, Sti1, and central regions of atTic40 (the latter incorporating the putative TPR domain) are essential for the activity of the protein.

Dominant-negative Effects of the atTic40 Deletion Mutants—Overexpression of the ΔTPR protein (and, to a lesser extent, the ΔSti1 protein) gave rise to an albino phenotype, much more severe than the tic40 knock-out phenotype, in a large number of seedlings (Fig. 2, A and B). Interestingly, albino ΔTPR and ΔSti1 seedlings reverted to tic40-like growth after 12–14 days (presumably because of the onset of transcriptional silencing of the transgene) but then re-initiated albino growth after transplantation to soil in many cases, producing plants with variegated or fully albino rosette leaves (Fig. 4, A and B). In addition, some of the ΔSti1 transformants that did not display the albino phenotype in seedlings nevertheless developed the phenotype in more mature plants. The fact that a significant proportion ΔSti1 and ΔTPR transformants displayed albino growth at some point

cleaves after residue 76 (supplemental Fig. S1). Because the transmembrane domain of atTic40 is essential for membrane insertion (32), our ΔTM protein is predicted to accumulate as an intermediate in the stroma. Thus, the ~43-kDa band in Fig. 3A most likely corresponds to the intermediate form of the ΔTM protein, which has a calculated size of 40 kDa but is expected to have a larger apparent size (12). Consistent with this notion, a ΔTM-YFP fusion protein yielded distinctly stromal fluorescence in transfected Arabidopsis protoplasts, which was in marked contrast with the envelope-associated fluorescence provided by a full-length atTic40:YFP fusion (supplemental Fig. S3).

The low abundance of the ΔTM intermediate (Fig. 3A) probably reflects the instability of the protein when mis-localized to the stroma. We therefore suggest that the additional, diffuse band at ~36 kDa includes stromal proteolytic fragments of the ΔTM protein (Fig. 3A). Consistent with this interpretation, this band was resolved into at least two distinct fragments in Fig. 3B. As expected, all detected ΔTM forms (~43- and ~36-kDa fragments) were completely protected from protease in isolated chloroplasts, indicating that all bands contained fully imported protein (Fig. 3B).

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![Image](https://example.com/image.png)
Thus, the accumulation of all three deleted proteins seems to induce a dominant-negative effect under certain conditions. This variegated phenotype was reminiscent of, but not identical to, the pale or albino sectors during later development (data not shown). This variegated phenotype was associated with profound chloroplast ultrastructural defects; organelles in albino ΔTPR seedlings were underdeveloped in comparison with tic40 chloroplasts (Fig. 2C, panel i) and many were completely devoid of a thylakoid network (Fig. 2C, panel ii). Furthermore, many of the cells in ΔTPR albinos seemed to completely lack recognizable chloroplasts (Fig. 2D). Consistent with these observations, immunoblot analyses revealed that the albino seedlings were strongly deficient in a range of photosynthetic (SSU, OE33, and FNR) and nonphotosynthetic (CPO, Cpn60, and Hsp93) chloroplast proteins (Fig. 2E). Interestingly, however, Tic110 did not appear to be significantly reduced in the ΔTPR albinos.

Functional Similarity between ΔTPR and Human Hip—The failure of the ΔSti1 and ΔTPR constructs to complement tic40 indicated that the Sti1 domain and the central region of
atTic40, including the putative TPR domain, are important for function. Because similar domains are present in the Hip co-chaperone, we generated domain-swap constructs to assess functional similarity between the proteins (Fig. 1C). Domains of human Hip (hsHip) were used, rather than those of Hop, primarily because the Hip Sti1 domain shows greater similarity to atTic40 (Fig. 1B). First, to determine whether the putative Sti1 domain of atTic40 is functionally equivalent to that of Hip, the 40:HipC domain-swap construct was generated. Second, to determine whether Tic40 performs (co)chaperone functions equivalent to those performed by Hip (14), the 40TP/TM:Hip construct was made. This comprised the transit peptide and transmembrane regions of atTic40 fused to nearly full-length hsHip; the small N-terminal region of hsHip that was removed carries an oligomerization domain (33, 34).

Transgenic tic40 lines were identified for both constructs and characterized in the same manner as the deletion construct transformants (supplemental Fig. S2). Remarkably, all 42 transformants carrying the 40:HipC construct were at least partially complemented, and most were indistinguishable from wild type (Fig. 5A). In contrast, none of the 24 40TP/TM:Hip transformants displayed clear signs of complementation (Fig. 5A). Complementation efficiency in representative lines was quantified by measuring chlorophyll concentrations. Although chlorophyll levels in 40TP/TM:
Hip transformants were similar to those in tic40, those in 40:HipC plants were comparable with wild-type levels (Fig. 5B). Chloroplasts in true leaves of representative 40:HipC plants were indistinguishable from wild-type organelles (Fig. 5C), and isolated 40:HipC chloroplasts imported preproteins at least as efficiently as wild-type chloroplasts (Fig. 5D). These data demonstrate that the C terminus of atTic40 is a bona fide Sti1 domain functionally equivalent to the hsHip.

**FIGURE 5. Functional similarity between atTic40 and human Hip.** A, typical 10-day-old in vitro-grown seedlings from representative tic40 lines carrying the indicated constructs, alongside wild-type (WT) and untransformed tic40 controls. B, chlorophyll levels in whole 10-day-old in vitro-grown tic40 seedlings carrying the indicated constructs. Values are means derived from four samples, each one containing 5 or 15 seedlings, taken from each of three individual, representative transgenic lines. Error bars show S.D. C, first-leaf chloroplast ultrastructure in 10-day-old 40:HipC seedlings. A representative organelle is shown alongside typical wild-type and tic40 control chloroplasts. Scale bar, 1 μm. D, chloroplast protein import efficiency in 10-day-old 40:HipC seedlings. Radiolabeled ribulose-bisphosphate carboxylase/oxygenase small subunit (ats1A) precursor was incubated with equal numbers of 40:HipC and wild-type chloroplasts for 2.5–10.0 min, as indicated, at 25 °C. Samples were then analyzed by SDS-PAGE and fluorography. Precursor (p) and mature (m) forms of ats1A are indicated; IVT denotes the ats1A in vitro translation product. An image of the SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue, is shown to give an impression of sample loading. The large and small subunits of ribulose-bisphosphate carboxylase/oxygenase (LSU and SSU) and light-harvesting chlorophyll-binding protein (LHCP) are indicated, as are the positions of molecular weight standards. Data shown are representative of three independent experiments. The chart shows the mean amount of imported, mature ats1A at each time point, normalized relative to endogenous SSU. Error bars show S.E.
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Sti1 domain, which is somewhat surprising given that the two domains share only limited homology (35% identity; Fig. 1B). Given that Sti1 domains are a unique and characteristic feature of co-chaperones, these data are strongly supportive of a co-chaperone role for Tic40.

Slightly higher protein import rates were observed in the 40:HipC assays than in wild-type assays (Fig. 5D), although these differences were not statistically significant (Student’s two-sample t test, p > 0.3). This subtle difference may be explained as follows. Even though 40:HipC chloroplasts in true leaves were indistinguishable from equivalent wild-type chloroplasts (Fig. 5C), a proportion of those in 40:HipC cotyledons were larger than the wild-type organelles (by ~50% in terms of cross-sectional area) but nevertheless were fully developed internally (data not shown). This presumably reflects the fact that the 35S promoter is not active during early embryogenesis (such that the increased chloroplast size aspect of the tic40 phenotype (35) is not fully complemented), because a similar size phenotype was observed in the cotyledons of WT40 lines (data not shown). The import assays contained equal numbers of chloroplasts, and so experiments with larger organelles might have contained a slightly greater number of protein import sites.

Like the deleted proteins, the fusion proteins were assessed for proper expression and targeting by immunoblotting (Fig. 3, A and B). The abundant ~41-kDa protein in 40:HipC seedlings is almost certainly the mature protein, because it co-migrates with mature atTic40 as expected (Fig. 3A; the proteins have a similar calculated size and proline content). The additional protein of ~48 kDa likely corresponds to the intermediate form (32) of 40:HipC, because it is of the expected size. The hsHip domain of 40:HipC may reduce its targeting efficiency (due perhaps to its association with stromal or envelope chaperones), such that complete cleavage to the mature form does not occur. The minor bands likely correspond to proteolytic fragments.

Unfortunately, the 40TP/TM:Hip protein could not be detected using either the psTic40 antibody (because all Tic40 epitopes used to generate the antibody are absent from the fusion protein) or a polyclonal rat Hip antibody (probably because this antibody cross-reacts only weakly with hsHip). However, reverse transcription-PCR clearly demonstrated that the 40TP/TM:Hip transgene is transcribed in several independent lines (supplemental Fig. S4). Furthermore, 40TP/TM:Hip lines were found to behave in a similar fashion to the ΔTM lines, in that some plants developed a variegated (probable dominant-negative) phenotype during later development (data not shown), strongly suggesting that the protein is expressed and targeted to chloroplasts. Thus, although the Sti1 domains of atTic40 and hsHip are functionally equivalent, it would appear that other parts of the proteins are not.

Direct Interaction between atTic40 and atTic110—The partial functional equivalence shared by atTic40 and hsHip strongly suggests that Tic40 plays a co-chaperone role during chloroplast import. Previous observations led to the hypothesis that stromal chaperones (in particular, Hsp93) are recruited to the TIC complex by Tic110 (6–8, 24). Thus, if Tic40 does indeed serve to regulate the activity of a stromal chaperone, one might expect it to be closely associated with Tic110. In fact, cross-linking studies have shown that the two proteins are in close proximity (11), and genetic analyses have suggested that they may co-operate functionally (13). However, at the time of this study, no evidence for a direct interaction between the proteins had been presented (although see Ref. 25). To address this possibility, we subjected atTic110 and atTic40 to yeast two-hybrid interaction analysis. As shown in Fig. 6 (A and B), the results provided clear evidence of a significant, if rather weak, interaction between the proteins. Visible differences in growth between control and interaction test strains (Fig. 6A) were corroborated by quantitative analysis using established procedures (29) (Fig. 6B). The differences shown in Fig. 6B are highly statistically significant, as was revealed by a Student’s two-sample t test (p < 0.05).

Attempts were made to conduct the reciprocal yeast two-hybrid assay (using atTic40 as the bait and atTic110 as the prey), but meaningful experiments were precluded by the fact that the atTic40 bait caused strong levels of constitutive auto-activation of the auxotrophic reporter (data not shown). This problem was observed consistently in repeated experiments using both Gal4- and LexA-based yeast two-hybrid systems, even under conditions of elevated stringency. Such problems were not reported following a similar study using a psTic40 bait (25), and so these observations may reflect inherent differences between the Arabidopsis and pea proteins.

To ensure that our yeast two-hybrid data (and those reported previously (25)) were truly reflective of the in planta situation, we went on to assess the Tic110-Tic40 interaction using bimolecular fluorescence complementation (BiFC) in transiently transformed protoplasts; BiFC involves the reconstitution of YFP fluorescence upon association of nonfluorescent, N-terminal (NY) and C-terminal (CY) fragments of YFP, brought together as a consequence of their fusion to interacting proteins (29, 36). As controls for these experiments, we also analyzed the in vivo distribution of atTic110 and atTic40 fusions to full-length YFP in singly transformed protoplasts.

As expected, YFP fluorescence in cells expressing either of the full-length YFP fusions (pWEN18-Tic110 and pWEN18-Tic40) was exclusively associated with the chloroplast envelope (Fig. 6C). These data nicely corroborate previous findings based on biochemical analysis (which showed that Tic110 and Tic40 are envelope proteins) and indicated that BiFC fluorescence associated with an atTic110-atTic40 interaction should localize to the envelope. Indeed, when we analyzed doubly transformed protoplasts co-expressing fusions of atTic110 and atTic40 to NY and CY, respectively, clear envelope-associated fluorescence was detected (Fig. 6C). Although the Tic110-Tic40 BiFC fluorescence was not as strong as that associated with the full-length YFP fusions, it was substantially stronger than the BiFC signal produced by a previously reported protein–protein interaction (that associated with AtFtsZ2–1 homodimerization; see supplemental Fig. S5) (29). When considered in conjunction with the yeast two-hybrid data, these results strongly support the proposal that atTic110 and atTic40 interact directly.

The BiFC data shown in Fig. 6C demonstrate the interaction between these components in planta for the first time, corroborating the conclusions of previous cross-linking, genetic, and yeast two-hybrid experiments, and indicating that Tic40 is ide-
ally situated to regulate the activity of any chaperones bound to Tic110, such as Hsp93. Attempts were made to assess for a direct interaction between Tic40 and Hsp93 in similar yeast two-hybrid experiments, but no evidence of an interaction was detected. It is likely that the putative Tic40-Hsp93 interaction is either very weak or transient, because attempts to demonstrate an interaction in vitro using recombinant proteins also failed (data not shown). Nevertheless, Hsp93 remains the most likely chaperone partner of Tic40, because cross-linking data have shown that it acts at the same stage in the import mechanism as Tic110 and Tic40 (12), and genetic data have indicated that it likely cooperates functionally with Tic110 and Tic40 (13). Recently published data are certainly supportive of this conclusion (25), as discussed below.

DISCUSSION

The fact that all three atTic40 deletion constructs (∆TM, ∆Sti1, and ∆TPR) failed to complement tic40 (Fig. 2), despite efficient expression and chloroplast targeting of the proteins (Fig. 3), indicates that each of the deleted regions (the transmembrane domain, the Sti1 domain, and the central region incorporating the TPR domain) is essential for function. In the case of ∆TM, this is consistent with the idea that the deleted sequence is essential for proper localization (11, 12, 32). This hydrophobic domain is predicted to form a transmembrane α-helix by various programs, overlaps with a well conserved region, and is followed by two Lys residues that may dictate topology (supplemental Fig. S1) (37). Recently published data on the targeting of Tic40 (32), together with the localization data presented in supplemental Fig. S3, indicated that the ∆TM protein accumulates in the stroma. It is possible that a proportion of this stromal protein associates peripherally with the TIC complex in order to cause the sporadic dominant-negative effects we observed. Similar deletion of the N-terminal transmembrane anchor of atTic110, a topologically similar protein, led to its accumulation in the stroma in vivo (38).

For the two deletion constructs that caused strong albino phenotypes, ∆TPR and ∆Sti1, abnormal growth correlated with overexpression of the truncated proteins (Fig. 4). Recently, transgenic expression of truncated forms of Tic110 (lacking
sections of its large, stromal, C-terminal domain), in wild-type plants, was found to cause similar dominant-negative effects (38). The resultant albino or pale phenotypes were associated with defective chloroplast protein accumulation, caused by a specific effect on inner membrane translocation. Furthermore, the translocation defect was correlated with disturbances in the functioning and assembly of import complexes, because of the incorporation of the truncated Tic110 proteins (38). Given the phenotypic similarities, it is possible that the Tic40 dominant-negative effects are due to similar processes. However, it is important to bear in mind that although the Tic110 dominant-negative phenotypes were substantially less severe than the tic110 null phenotype (embryo lethality), the Tic40 dominant-negative phenotypes actually exceed the tic40 knock-out in terms of severity. This suggests that the normal role of Tic40 is to increase the efficiency of an aspect of the import mechanism (an aspect that functions at reduced but nevertheless significant levels in the absence of Tic40), and that the deleted Tic40 proteins titrate an important factor (or factors) involved in this process (e.g. a molecular chaperone that is able to function with reduced efficiency in the absence of Tic40), or otherwise interfere with the functioning of the TIC complex.

The ΔSti1 mutant demonstrated that the atTic40 Sti1-like domain is essential for functionality (Fig. 2). Therefore, the fact that the 40TP/TM:Hip construct fully complemented tic40 indicates that the atTic40 C terminus is functionally equivalent to the hsHip Sti1 domain (Fig. 5). Because the Sti1 domain is a characteristic feature of Hip/Hop co-chaperones, it seems likely that it performs co-chaperone-specific functions and, by extension, that Tic40 functions as a co-chaperone. However, the precise role played by the Sti1 domain in Hip and Hop remains to be elucidated. Using an in vitro progesterone receptor maturation assay, a process in which Hip, Hop, Hsp70, and Hsp90 all participate, it has been shown that the Sti1 domains of the two co-chaperones are not equivalent (39). Despite this apparent specificity, the homology between the domains suggests that they play analogous roles. This notion is supported by the fact that the Sti1 regions provide both Hip and Hop with a degree of resistance to proteolysis, implying that Sti1 domains form a tightly folded, protease-resistant structure (39).

Our data imply that Tic40 functions in association with a molecular chaperone. In fact, it was recently reported that the Tic40 C terminus is able to stimulate the ATPase activity of Hsp93 (the proposed core of the putative chloroplast import motor), presumably via a direct interaction (25). This result is rather unexpected, because ATPase stimulation is not one of the proposed functions of Hip (or Hop), and our results demonstrate that the Sti1 domains of these proteins are functionally interchangeable (Fig. 5). However, the finding that tic40 and hsp93 knock-out mutations interact genetically (13) is consistent with the proposed functional link between these components, as is the fact that yeast Hop can bind to Hsp104, another member of the Hsp100 family (26). Because Hsp70 does not associate functionally with the TIC, it seems unlikely that Tic40 associates with stromal Hsp70. An alternative putative partner is Hsp90, because homologs of this chaperone have been identified in chloroplasts (40–42), and to our knowledge, their participation in chloroplast import has not been investigated.

Another possible role of the Tic40 Sti1 domain is suggested by the fact that Hip displays chaperone activity (14, 15); both the TPR domain and downstream C-terminal domains play a part in this activity (43). Because it is well established that preproteins are translocated through the TOC and TIC in an unfolded state, it is conceivable that Tic40 interacts with preproteins as they emerge into the stroma. This might serve to prevent aggregation of preproteins and/or assist their transfer to a nearby chaperone (e.g. Hsp93). Interestingly, it was recently shown that Tic40 can interact specifically with transit peptides in solution (44). Because transit peptides are largely unfolded in aqueous solution (45), it is possible that this affinity is actually a consequence of a less specific chaperone activity. However, another study suggested that Tic40 does not bind to preproteins directly (25).

The Hip Sti1 domain has been shown to interact specifically with a leucine-rich motif in mammalian chemokine receptors, in an agonist-dependent manner (46). Intriguingly, Tic110 is leucine-rich throughout its mature region (~11% in psTic110), and several lines of evidence now indicate that Tic40 is closely associated with Tic110 as follows: (i) yeast two-hybrid and pull-down assays indicated that the proteins interact directly (25) (Fig. 6, A and B); (ii) BiFC analysis demonstrated that the proteins are closely associated in planta (Fig. 6C); (iii) tic40 and tic110 knock-out mutations interact genetically (13); (iv) the two proteins were efficiently cross-linked using CuCl2, a chemical that promotes the formation of covalent bonds between juxtaposed cysteine residues (11). Interestingly, psTic40 and all other Tic40 homologs analyzed contain only one cysteine, and this lies within the Sti1 domain (supplemental Fig. S1). If the observed Tic40-Tic110 cross-links were direct, these observations would indicate that it is the Tic40 Sti1 domain that is in close association with Tic110.

However, more recent data suggest that it is actually the putative TPR domain of Tic40 that interacts with Tic110 (25). This is somewhat surprising, because the TPR domains of Hip and Hop mediate binding to their respective chaperone partners (26, 33, 34, 47). Nevertheless, these data and others led Chou et al. (25) to propose a model in which the Tic40-Tic110 interaction is favored when the transit peptide-binding site of Tic110 is occupied (24). Binding of Tic40 then leads to transit peptide release from Tic110, enabling the preprotein to associate with Hsp93. The Tic40 Sti1 domain subsequently stimulates the ATPase activity of Hsp93, enabling the chaperone to drive import in an ATP-dependent fashion. Thus, Tic40 was proposed to act as a timing device to coordinate sequential steps during the latter stages of chloroplast protein import.

Despite the functional equivalence of the Tic40 and Hip Sti1 domains, the 40TP/TM:Hip construct failed to complement tic40 (Fig. 5). There are two possible explanations for this. First, it is possible that the fusion protein is not properly expressed and/or targeted to chloroplasts. However, this seems unlikely because the transgene was transcribed efficiently (supplemental Fig. S4) and because the 40TP/TM:Hip construct was found to cause a dominant-negative phenotype in some lines (data not shown). The second and more likely possibility is that the central domains of the proteins are not equivalent. The fact that the proposed domain organization of Tic40 is more similar to that
Functional Similarity between Tic40 and Hip

of Hop than to that of Hip (the TPR and Sti1 domains are more closely juxtaposed in Tic40 and Hop) may be significant. In addition, it is possible that the central, conserved motifs of Tic40 (motifs 1–3; supplemental Fig. S1) are essential for function. Finally, it is quite likely that the Hip and Tic40 TPR domains are not functionally interchangeable, because such domains do show some level of recognition specificity (48). Although the Hip TPR domain is designed to bind Hsp70, it would appear that the putative Tic40 TPR domain interacts with Tic110 (25).

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