Evidence That the Plastid Translocon Tic40 Components Possess Modulating Capabilities*

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The transport of proteins into the plastid is a process that faces changing cellular needs such as the situation found in different plant organs or developing tissues. The plastid translocon must therefore be responsive to the changing cell environment to deliver efficiently different arrays of structurally diverse proteins. Although the Tic40-related envelope proteins appear to be translocon components designed to address the varying needs of protein translocation, details of their involvement remain elusive. This study was thus designed to combine plant-based experiments and yeast mitochondrion-based approaches for unveiling clues related to how the Tic40 components may behave during the protein translocation process. The main findings related to how Tic40 proteins may work are: 1) natural fluctuations are apparent in developing tissues, in different organs of the same plant, and in different species; 2) transgenic Arabidopsis seedlings can tolerate functionally a wide range of variations in Tic40 levels, from partial suppression to excessive production; 3) the Tic40 proteins themselves exhibit configurational changes in their association with yeast mitochondria in response to different carbon sources; 4) the presence of Tic40 proteins in yeast mitochondria influences regulatory aspects of the mitochondrial translocon; and 5) the Tic40 proteins associate with mitochondrial translocon components involved in regulatory-like events. The combined data provide evidence that Tic40 proteins possess modulating capabilities.

Plastids are diverse in structure and function and occupy key roles in a variety of biosynthetic activities that take place in the ever changing environment of a plant cell. The role of plastids requires frequent adjustments to accommodate varying cellular needs such as those occurring in different organs, during development, during adaptation to external stimuli, or even a combination of needs. The adjustments required can be relatively subtle or distinct. The ability of the plastid to address such needs is dependent on its compositional status, e.g. chloroplasts versus leucoplasts. Although it is well established that changes to plastidial structure and function are governed predominantly by mechanisms that control gene expression (nuclear and organellar), there is growing evidence that the protein transport process contributes additionally to certain facets of these regulatory mechanisms. The involvement of protein transport in the various mechanisms of plastidial change is not unfounded because most of the plastid proteins involved are made as larger precursors before incorporation into organelles. The protein transport machinery of the plastid envelope (the plastid translocon) must therefore be accommodative in its ability to handle in an efficient manner a diverse, ever changing array of proteins. The proteins being handled range from different proteins to structural variations of the same protein. The plastid translocon is also afforded a position where additional “modulating” mechanisms can be introduced and exercised. The translocation of proteins across the envelope is a multistep process involving factors and signals that mediate recognition, specificity, and movement (1). There are thus many opportunities for the different translocon components to exercise their modulating capabilities (2–5).

Although there are potential scenarios in which modulation can be detected, these activities remain the most difficult aspect of the plastid protein transport process to study. One of the least understood aspects is how a seemingly “general” translocon attends to an array of structurally diverse precursor proteins during the various developmental and adaptive phases of a plant cell. Studies to date have provided insight into a number of potential “mechanistic provisions” for handling certain aspects of plastid protein transport. The mechanisms unveiled thus far appear to center predominantly on the GTPase-based receptors Toc159 (plus homologs Toc132/120/90)1 and Toc33/34 (two variants) and their preferences for different precursor types (6–14). The removal of Toc159 (via mutagenization) selectively affects the accumulation of photosynthetic rather than nonphotosynthetic proteins (10) because of Toc159 recognition of specific transit peptides (6). Like Toc159, Toc33 appears to be more involved in the transport of photosynthetic proteins than Toc34 (8, 9, 15–18). These receptors also exhibit profiles that change with tissue type and developmental stage, suggesting that mechanisms exist for the “regulation” or “modulation” of the plastid translocon at the site of the GTPase-based receptors to accommodate the transport needs of the tissue or cell. Indications of such mechanisms in action were observed, e.g. during

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1 The abbreviations used are: Toc, translocon component of the plastid outer envelope; Tic, translocon component of the plastid inner envelope; Tim, translocon component of the mitochondrial inner membrane; Tom, translocon component of the mitochondrial outer membrane.
the preferential in vitro import of proteins into chloroplasts versus leucoplasts (19), for “younger” versus “older” plastids (20), or plastids of nitrogen-fixing versus nonfixing nodules (21). Toc75-encoding mRNA levels, the outer envelope channel protein, also change in a tissue-specific manner (leaf versus roots) (16). Toc75 mRNA reductions parallel the observed changes in Toc75 protein levels between leaves and roots (22).

The number of mechanisms so far uncovered is clearly not sufficient to fulfill all possible dynamic activities operating during the plastid protein transport process. Because other modulating mechanisms are likely to be in play, there is a need to uncover clues as to how other translocon components attend to structurally diverse proteins, especially during other stages of the process. It was stated prominently in two recent reviews (2, 23) that regulatory mechanisms associated with translocon function represent a new, exciting investigative direction. The 44-kDa envelope proteins (multiple forms or variants termed collectively as Tic40-related translocon components) appear to be one particular candidate that warrants such an analysis. The Tic40 components seem to be designed to meet the various protein translocation needs in a manner different from the receptor-based mechanisms or during other stages of the process. Tic40 proteins are often observed to be heterogeneous with respect to abundance, gel mobility, and different apparent configurations in the envelope (24–27). The overall endogenous levels often display fluctuations that seem to parallel the import activity of the tissue or developmental stage (25). Tic40 components can also be found in close association with translocating proteins early in the process at the outer envelope, as well as at more advanced stages at the inner envelope (24). Two select forms of the Tic40-related components (Toc36 and Tic40) exhibit preferential interactions with different precursor (or structural) forms of the thylakoid lumen protein Oee1 (28). Partial suppression of Tic40 in transgenic Arabidopsis appears to correlate with lower steady-state Oee1 levels (28). The same components were recently defined as co-chaperones by Chou and co-workers (29) and were shown to affect the overall efficiency of protein import when mutated in Arabidopsis (null mutations of Tic40). Despite these observations, the aspect of the protein translocation process in which Tic40-related translocon components work remains largely unknown. There is clearly a need to advance our understanding of Tic40 function and how Tic40 associates functionally with the rest of the translocon. Therefore, we need to develop parallel strategies aimed at fundamental aspects to track how Tic40 conducts its role. This study thus combines approaches for unveiling clues related to where Tic40 proteins work during the protein translocation process, e.g., in regulation or as structural requirements. The first approach was to profile trends of how Tic40-related components behave in plant tissues, using several semiquantitative assessment models where there is a reasonable expectation of change in the demand on the protein transport process. The natural way in which the Tic40 proteins continuously change, in terms of abundance and forms, provides basic clues on function. We then developed a “cross-organelle” strategy using the analogous system, the yeast mitochondrion, to map out where Tic40 components are likely to associate and exert their role. Because the yeast mitochondrial system is well established relative to the plastid translocon, we utilized known functional aspects of the mitochondrial translocon to search for clues related to the role of Tic40 proteins by determining how the mitochondrial translocon is affected by the presence of Tic40. The combined data provide evidence for the aspect of the protein transport process in which Tic40 components work and that these polypeptides possess modulating capabilities related to their role(s) in protein transport.

EXPERIMENTAL PROCEDURES

Procedures for Plant-based Experiments—The plants used were propagated in growth chambers set at 21 °C with a 16:8 h light:dark photoperiod (fluorescent-incandescent lighting). Total cellular protein extracts were prepared for the various indicated tissues by grinding fresh samples in chilled buffer as described previously (30).

Standard DNA cloning techniques were employed in the construction of the transgene-containing binary plasmids (pEND4K-based) (31). The scheme for producing the various transgenic Arabidopsis thaliana plants is described in Ref. 28. Composition of the transgenes used in this study is as follows: 35S-labeled CaMV promoter, bnToc36B or rcTic40 (both without any internal manipulations), nopalin synthase termination signal. Tic40 proteins were expressed using cDNAs derived from Brassica napus cv. Topas (bnToc36B, a version of Toc36 (24, 25)), or Ricianus communis (rcTic40, a version of Tic40). Plastids were prepared from seedlings grown on media plates as described (32, 33). Chlorophyll determinations were performed as described previously (34). Standard immunoblotting techniques were used to assess all protein extracts, which were quantitated and normalized before assessment. Immunoreactive bands were scanned and quantitated relative to appropriate internal references.

Construction of Yeast Expression Plasmids—All Tic40-related constructs were inserted into the yeast Escherichia coli shuttle vector YEpLac185 (35). Expression was achieved using the alcohol dehydrogenase promoter. The rcTic40 gene was inserted without any internal manipulations. A form equivalent to BnToc36B (a version of Toc36) was generated from rctic40 (designated Rctoc36 or Rctoc36) using a unique 5′-BstEII site in the rctic40 cDNA (28). Histidine tags were added to the carboxyl termini of both Rctoc40 and Rctoc36 using a 3′-BstNI site. The fusions occurred at the 3rd residue from the carboxyl end. This deletion had no observable impact on the transport or functionality of the Tic40 proteins (28). Plasmids were introduced into the yeast strain INVSc1 (Invitrogen) using standard transformation techniques.

Analysis of Yeast Mitochondria—The isolation and subfractionation of mitochondria were conducted as described previously (36). Treatment of mitochondria with the proteases thermolysin and trypsin was carried out according to Refs. 37 and 38, respectively. Pull-down assays were conducted according to Ref. 39 using nickel-nitrilotriacetic acid-agarose (Qiagen). All protein samples were quantitated and normalized before assessment. Associating proteins were detected immunologically. The anti-Tic40 IgGs used were equally immunoreactive for all Tic40 forms, independent of derivation source (yeast or plant species) (25).

RESULTS

Tic40 Components Exhibit Continuous Fluctuation in Tissues Undergoing Plastid Biogenesis and/or Conversion and between Different Plant Varieties—The manner in which a plastid translocon component behaves in tissues undergoing plastid biogenesis and/or conversion is likely to provide valuable information on how the component conducts its role in the protein transport process. We therefore utilized a number of plant-based assessment models to develop semiquantitative profiles for Tic40-related components in tissues where there is a reasonable expectation of change in the demand on the process of plastid protein transport. The tissues selected represent developmental programs involving plastid biogenesis or conversion. Profiles were compiled for whole dark grown seedlings exposed to light for a 24-h period (“greening” model for developing plastids and conversion), developing seeds (whole seed), developing seeds (postpollination to maturity) and from different organs of the same mature plant. The “whole tissue” samples represent collections of different cell types rather than a specific cell type. The resulting protein samples thus reflect all Tic40-related activities present in the tissues sampled and are likely to possess a compilation of different programs related to
A mature seed). Tic110 was again used as a semiquantitative reference point for tissues collected from pea and Brassica. Profiles for pea and Brassica are presented. Whole seedlings or developing seeds were used to prepare total cell protein extracts. The experiments were designed to measure changes in a continuous fashion, hourly for greening seedlings (after germination in the dark) and in 2–3-day increments for developing seeds (indicated accordingly). A mature leaf sample was included in the Brassica greening seedlings experiment (lane marked ML). Tic110 was analyzed for the same samples and is presented as a semiquantitative reference point. The arrows indicate the positions of smaller Tic40-related proteins (bands migrating around 30 kDa as opposed to the 40 kDa range) that appear during certain stages. The overall trends observed in each of the assessment models clearly indicate that Tic40 components display fluctuation-related activities (relative to Tic110). The Tic40 proteins in the various tissues or stages of development not only fluctuate in total amounts but also as different band patterns. The patterns of fluctuation were not strictly progressive from an early point to the last stage sampled, i.e. the changes were not in a strict incremental manner from one stage to the next. Other forms of fluctuation within the stages examined were observed, and these may represent other programs taking place in the different cell types within the whole seedlings or whole seeds being assessed. This phenomenon was clearly not the result of errors in the preparation and handling of protein samples because it was observed in all replicates and for different plant species (Pisum sativum, B. napus, A. thaliana, Phaseolus vulgaris, Glycine max, as well as other bean varieties (the latter data are not shown)). The trends appear to be part of the collective developmental program of the tissue rather than a product of protein degradation events (occurring either in intact cells or during extraction). Furthermore, Tic110 levels, unlike the Tic40 components, remain relatively stable throughout the same sampling period (in the greening seedling and developing seed experiments). The presence of other forms of fluctuation provides another indication that Tic40 components fluctuate in a natural setting and between different cell types. Fluctuations between different cell types were also evident in the different organs assessed. Samples prepared from different organs of the same mature plant exhibit a fair amount of fluctuation (Fig. 1C). Even more striking is the degree of variation observed in the endogenous levels of Tic40 proteins between different species (Fig. 1, A and B). The fluctuation-related activities exhibited by Tic40 components are likely reflective of the way in which these proteins work. There appears to be a high degree of flexibility in the way the Tic40 proteins operate, such as that observed between different developmental stages and especially between different species. Tic40 functionality thus appears to be linked to aspects involving changes in stoichiometry and the level of different configurations present. These characteristics together point to the possibility that the Tic40 role(s) involves some form of modulating activity.

Arabidopsis Seedlings Can Tolerate Manipulations in the Level of Tic40—Relative to the core plastid translocon components, Tic40-related components clearly exhibit a fair degree of
natural fluctuation between tissues of different organs and developmental stages (nontransgenic plants). The trends of fluctuation observed appear to reflect in a logical manner the level and/or mode of importation-based activities that is likely to exist in the different organs (during plastid conversion) or developmental phases (developing plastids). Moreover, the degree of variation observed for Tic40 proteins in different species (nontransgenic) does not appear to influence the plant or its tissues in a manner that can be readily detected or defined as a positive or negative impact. This particular fluctuation phenomenon indicates that there is considerable flexibility in the system, allowing different levels of Tic40 components to work. Because the fluctuations appear to be related to some aspect of function with respect to the protein transport process, we further examined the effects of manipulating “artificially” the level of Tic40 components within a plant species, in our case, Arabidopsis seedlings (Fig. 2). Whole transgenic seedlings (excluding roots) were used for the preparation of plastid samples. The samples thus represent a collection of plastids from different tissues of the harvested seedlings. The analysis was repeated three times, and representative profiles are shown (Fig. 2). All profiles were designed for semiquantitative analysis only. The overall data indicate that the seedlings (green tissues) can tolerate a high degree of fluctuation generated by the transgenes. Partial suppression of steady-state Tic40-related proteins correlated with a general decrease in steady-state levels of the three internal plastid proteins assessed (Fig. 2, see transgenic lines M1–M3 (medium overproduction) and H1–H3 (high overproduction)). There may be subtle phenotypic changes that occur in specific tissues (e.g., seeds), in different developmental stages (e.g., early development), and under different environmental parameters (e.g., stress), but these aspects have yet to be examined. More interestingly, the other core translocon components assessed (Toc159, 75, 33/34, Tic110, Com70) appear to remain generally stable in terms of their relative levels. There does not appear to be any obvious detriment related to either the mild suppression or the excessive production of Tic40 proteins in seedlings propagated under normal growth conditions. This type of behavior leads to the interpretation that the Tic40 role(s) associates with an aspect of the protein transport process where there is a considerable amount of operational flexibility, an environment appropriate for modulating activities.

**Tic40 Components Incorporated into Yeast Mitochondria Act in a Manner Resembling the Plastid Counterparts**—To gain a further insight into the possibility of a modulating role for the Tic40-related proteins, we developed additional strategies to unveil clues related to how these components may function, especially in a biologically relevant live cell context. As one way to address this need, we developed a cross-organelle strategy to study where in the mitochondrial protein transport process the Tic40 proteins are likely to display some form of association. This approach is likely to be feasible to an extent because mitochondria and plastids face similar challenges in the transport of proteins. Both organelles utilize analogous multisubunit translocons to import proteins and may thus facilitate the process in a similar fashion. There are likely to be, if not for the entire process, commonalities for at least certain aspects of the process. Hence, there is a reasonable chance that the site of the mitochondrial translocon with which Tic40 proteins “associate” may provide an indication of the Tic40 mode of operation. The next series of experiments was thus designed to assess the impact of incorporating Tic40 proteins into yeast mitochondria. Impact was assessed several ways: basic expression characteristics, importation and localization properties, configurational patterns, and response to changes in carbon source. The results indicate that Tic40 proteins are utilized by mitochondria in a biologically relevant manner, possibly reflective of their role in plastids. The Tic40 proteins (two select forms, RcTic40 and

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**Fig. 2. Transgenic Arabidopsis seedlings can tolerate Tic40 fluctuations.** Steady-state levels of plastid proteins from various transgenic lines with manipulations to Tic40 levels were assessed. Plastids were prepared from whole seedlings (excluding roots) grown on solid media. A semiquantitative immunoblot analysis of the plastid translocon (Tic40-related proteins, Toc159, Toc33/34, Toc75, Tic110, and Com70) and three internal plastid proteins (Oee1, Rbcs, and Lhcb1*1) are shown (panels titled Core Translocon Components and Plastid Proteins, respectively). Individual lines assessed are grouped as high (H1–H3), medium (M1–M3), low (L1–L3), and wild type (WT). The lines designated low display partial suppression of Tic40 levels (0.15–0.22), whereas the medium and high lines exhibit higher Tic40 levels relative to the wild type (set at 1).
RcToc36) were expressed and stable (Fig. 3). The transgenic yeast cells did not display any observable growth detriment in glucose-supplemented media. Immunoblots of mitochondrial fractions and the accompanying aqueous lysates (postmitochondrial fractions) indicate that the Tic40 proteins were successfully and efficiently targeted to mitochondria (Fig. 3 A).

Although the mechanism for Tic40 targeting remains to be elucidated, it does not appear to be dominated by "mistargeting" events. Hexokinase was found predominantly in the cytosol-containing fraction, whereas Tic40 proteins were detected mainly in the same mitochondrial fraction as Arg8, a matrix protein (Fig. 3 A).

Yeast mitochondria appear to assemble the Tic40 proteins in a manner similar to plastids. The multiple immunologically related Tic40 protein bands in mitochondria mirror the patterns observed previously in other systems (plants and bacteria) (25–26, 29, 40, 41). Several Tic40 proteins from different plant species were tested, and they all behave in the same manner in yeast (data not shown). Most of the RcTic40 proteins were present in the inner membrane. There were also less abundant forms in the intermembrane space and associated with the outer membrane (Fig. 3 B). RcToc36 was found in the intermembrane space and inner membrane (Fig. 3 B). The identity of the mitochondrial subfractions and the degree of subfractionation were assessed using IgGs specific for the various subcompartments; Tim44, an inner membrane protein; porin, an outer membrane protein; Arg8, a matrix protein; and CCPO, an intermembrane space protein.

Thermolysin and trypsin treatments of yeast mitochondria were conducted to compare the pattern of Tic40 association with the subcompartments and the plastidial Tic40 profiles reported previously by various laboratories (Fig. 3 C). Similarities in the pattern of subcompartment association between mitochondria and plastid would be another indication that the Tic40 proteins are expressing a certain level of functionality in yeast. The protease treatment results demonstrate that most of the incorporated proteins (RcTic40 and RcToc36) were inaccessible to proteases and were probably protected by the inner membrane. This behavior was also reported previously for plastids (25). Like plastids, there were also subtle protease-dependent events indicating that incorporated RcTic40 exists as different configurations. Both thermolysin and trypsin were
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accessible to the largest RcTic40 form, indicating that this form was accessible from the cytosolic side of the outer membrane. Thermolysin treatment gave rise to at least one additional minor band with a lower relative molecular mass (Fig. 3C, left panel, marked by an asterisk). Trypsin treatment gave rise to a band similar in size to the one generated by thermolysin plus a low amount of a smaller 35 kDa band (Fig. 3C, left panel, marked by an asterisk). The protease-generated Tic40 patterns for yeast mitochondria (for both thermolysin and trypsin) are similar to the profiles generated previously for chloroplasts (25). The matrix protein Arg8 remained protected in all treatments (see Fig. 3C, left panel, bottom row marked Arg8). The combined protease accessibility results indicate that the Tic40 proteins associate with mitochondrial membranes in a manner similar to plastids.

We further compared the protease treatment patterns discussed above (obtained using cells grown in glucose-containing media) with profiles generated for mitochondria isolated from cells grown in glycerol-supplemented media, as a way to determine whether changes to mitochondrial respiration affected the Tic40 configurations. Configurational changes, even subtle ones, would likely represent another indicator of functionality. The results indicate that there were subtle changes to at least one of the Tic40 configurations in glycerol-grown cells. Although the overall Tic40 levels (RcTic40 and RcToc36) were much lower compared with the glucose-grown cells, most of the incorporated Tic40 proteins were inaccessible to added proteases and were protected by the inner membrane. A low abundance RcTic40 form became protease-accessible (for thermolysin or trypsin) in glycerol-grown cells versus its inaccessible configuration found in glucose-grown cells (Fig. 3C, left versus right panel). The position of the RcTic40 band that became accessible is marked with an asterisk in the corresponding lanes (Fig. 3C, right panel under Glycerol-grown cells). There were no comparable changes in protease accessibility observed for RcToc36 in glycerol-grown cells. Although much lower in quantity as a result of growth in glycerol-containing media, the matrix protein Arg8 was largely protected from the added proteases (see Fig. 3C, right panel, bottom row marked Arg8). At the general level, the subtle changes observed for some of the Tic40 configurations signify events related to function and respiratory status. In other words, specific configurations of the incorporated Tic40 proteins exhibit an ability to respond, a sign of functionality. The significance of this mechanism/response is the subject of another study and will not be addressed here.

Yeast Cells Expressing Tic40 Display Changes in Growth Characteristics Related to Mitochondrial Status—From a physical perspective, the protein-based data compiled above indicate that the incorporated Tic40-related proteins are displaying functionality. Tic40 incorporation into yeast mitochondria appears to proceed in a manner that may bear significance in terms of affecting the functional behavior of the compartment. The transgenic yeast strains were therefore assessed to determine whether there was any measurable impact (directly or indirectly) on aspects attributable to mitochondrial function. Key indicators of mitochondrial function were thus used to assess the impact of Tic40. Growth studies were conducted in media containing fermentable (glucose) versus nonfermentable carbon sources (glycerol) as well as in combination with temperature stress (25–30 °C versus 37 °C). The growth studies provide evidence that the presence of RcTic40 allowed the cells to sustain growth in glycerol-containing media in a manner more similar to growth in glucose-containing media (Fig. 4). This behavior was observed in all independent lines tested. In contrast to the RcTic40-containing yeast strain, both control and RcToc36-containing strains grew weakly (or normally) in glycerol-containing media. The RcToc36 strain appeared to grow less vigorously (a small observable difference) than the control strain in glycerol-containing media, despite the presence of RcToc36 in the mitochondria. There were no obvious differences among the three strains when grown in glucose-containing media, for both solid and liquid media at 25–30 °C.

Interestingly, differences in growth were observed at 37 °C. Temperature stress was used primarily to exacerbate growth in glycerol-containing media. All three strains (control, RcTic40, and RcToc36) grew in a similar manner at 37 °C when propagated in glucose-containing media. Noticeable differences in growth were observed only when the cells were propagated at 37 °C in glycerol-containing media. The RcTic40-containing yeast strain was noticeably more “tolerant” during 37 °C growth in glycerol-containing media, whereas the control and RcToc36-containing strains were less tolerant. At the general level, the observed changes in growth characteristics signify that the Tic40 proteins express functionality in mitochondria. These physiological or phenotypic manifestations at the whole cell level may be partly related to the mitochondrial translocon events observed in the following experiments.

The Presence of Tic40 in Mitochondria Influences the Regulatory Aspect of the Mitochondrial Translocon—The results from the yeast-based experiments so far indicate that Tic40-related proteins possess a certain level of functionality in yeast mitochondria and will likely manifest as events at the protein transport level. To determine whether there were such events occurring, we examined further the influence of Tic40 by profiling compositional and/or stoichiometrical responses of the yeast mitochondrial translocon. Profiles were generated separately for mitochondria prepared from glucose- and glycerol-grown cells. The particular aspect of the translocon affected should provide additional clues concerning how Tic40 proteins work in the mitochondrial translocon transport process. Key mitochondrial translocon components were quantitated with respect to levels, normalized, and expressed as arbitrary units relative to Tim44 (mitochondria from glucose-grown cells) or Tom40 levels (mitochondria from glycerol-grown cells). Tim44 and Tom40 were selected as the internal translocon reference points because their levels appear to be steady for all of the yeast strains examined. The data generated were grouped into functional collections, the Tom complex and the two Tim complexes (Tim22 and Tim23), to help delineate aspects of the translocon affected. The overall data indicate that the influence of Tic40 (or Toc36) on the mitochondrial translocon is most discernible at the Tom complex (Fig. 5) when grown in glucose-supplemented media. Three Tom components display a response to the presence of Tic40 (RcTic40 or RcToc36). Tom22 levels increased by at least 20% in response to the presence of Tic40 proteins (RcTic40 and RcToc36) (Fig. 5). Tom40 and Tom70 levels were influenced by RcTic40 and RcToc36, respectively. Tim50 appears to be the only component in the Tim23 complex responding to RcTic40. From the Tim22 complex, Tim10 levels were affected by RcToc36 (Fig. 5). The observed changes indicated by asterisks were statistically significant (Tim 40 in response to RcToc36, F = 22.9, p = 0.0087; Tom70 in response to RcTic40, F = 155.43, p = 0.00024; Tom22 in response to RcTic40, F = 8.10, p = 0.047; Tom22 in response to RcToc36, F = 28.54, p = 0.0058; Tim50 in response to RcTic40, F = 84.64, p = 0.00075; Tim10 in response to RcToc36, F = 12.16, p = 0.025).

As a strategy to help validate the responses observed with mitochondrial translocons prepared from glucose-grown cells, we repeated the analysis with mitochondria from glycerol-grown cells. The switch in carbon source may give rise to an overlapping but different pattern of responses in the translo-
The ability to alter the translocon in response to mitochondrial status would be a reflection of functionality. Different responses affecting stoichiometrical or compositional aspects of the mitochondrial translocon were indeed observed with glycerol-grown cells (Fig. 5). Only components exhibiting a response are shown for the glycerol-based experiment. Like the glucose-grown cells, Tom22 appears to respond to RcTic40 or RcToc36, albeit differently. The level and direction of Tom22 responses were different from the glucose-grown cells. There were no significant changes observed for Tom40, Tom70, Tim10, or Tim50 levels, which is in contrast to the situation with the glucose-grown cells. Instead, two other changes were observed, and they appear to occur with the more internal Tim23 translocon components. The level of Tim23 appears to increase in the presence of RcTic40 or RcToc36. Again, the observed changes indicated by asterisks were statistically significant (Tom22 in response to RcTic40, $F = 61.94$, $p = 0.001409$; Tom22 in response to RcToc36, $F = 9.017$, $p = 0.0398$; Tim23 in response to RcTic40, $F = 9.31$, $p = 0.037$; Tim44 in response to RcTic40, $F = 11.109$, $p = 0.0398$; Tim44 in response to RcToc36, $F = 10.45624$, $p = 0.0318$). The profiles from the two series of assessments were useful in helping delineate aspects of the mitochondrial translocon where Tic40 proteins are likely to display an influence (directly or indirectly). More interestingly, the responses appear to change between cells grown in different carbon sources. The influence of Tic40 appears to move inward from the Tom complex to the Tim23 complex. The combined results provide evidence that the Tic40 influence appears to be directed at regulatory aspects of the yeast mitochondrial translocon.

**Tic40 Proteins Appear to Associate with Regulatory Components of the Yeast Mitochondrial Translocon**—The compositional or stoichiometrical changes observed above suggest that there may be physical associations (direct or indirect) between some of the mitochondrial translocon components and the Tic40-related proteins. Such associations would imply that the Tic40 proteins are functional in the yeast mitochondrial translocon, providing additional clues related to how the Tic40 components work. Potential associations between Tic40 proteins and mitochondrial translocon components were retrieved using immobilized metal affinity chromatography and histidine-tagged RcTic40 and RcToc36 (Fig. 6). The tagged Tic40 proteins were confirmed to be functionally identical to nontagged proteins (data not shown). Potential Tic40-mitochondrial translocon component associations were detected immunologically using the antibodies utilized above. Experiments were conducted for mitochondria from glucose- and glycerol-grown cells. Again, comparisons between glucose- and glycerol-grown cells were employed primarily to help validate the protein-protein interaction results. The ability to alter Tic40-translocon interactions in response to mitochondrial status would be a reflection of
The results indicate that there are interactions between the Tic40 proteins and a specific set of mitochondrial translocon components. Tom22 appeared to be the most distinct component displaying an association with the Tic40 proteins (both RcTic40 and RcToc36 for glucose-grown cells and RcToc36 for glycerol-grown cells) (Fig. 6). There was also an additional association observed between Tim44 and RcToc36 for glycerol-grown cells (Fig. 6, right panel). Control Tic40 proteins or mitochondrial extracts (RcTic40 or RcToc36 without histidine tags or control extracts prepared from cells containing vector only) did not give rise to any associations with Tom22 or Tim44. Likewise, other major mitochondrial translocon components such as the larger Tom70 did not show any detectable association with RcTic40 or RcToc36 (Fig. 6, left panel). The
histidine-tagged proteins were confirmed to be affinity-purified and intact using anti-Tic40 IgGs (Fig. 6, right panel). These results help delineate more narrowly where in the mitochondrial translocon the Tic40 proteins form physical associations. The associations observed appear to align with the responses documented in the above section. Tom22 is believed to represent one of the Tom receptors and a key “regulatory” component, and Tim44 is one of the three membrane-bound co-chaperones that interact with pre-proteins and mHsp70 (23). The associations between Tic40 proteins and Tom22 or Tim44 likely represent aspects of the protein transport process in which these proteins work. The mechanistic aspects are currently under investigation and will be reported separately.

**DISCUSSION**

Plastids are required to face the challenge of addressing different cellular needs and respond. The responses are generally protein-based, requiring importation by the plastid translocon that in turn would need to be functionally adaptive to deliver simultaneously an extensive array of structurally diverse proteins across changing microenvironments. The translocon must not only interact with translocating proteins, but associate in a functionally meaningful manner to facilitate efficient importation. Despite the need to be adaptive and productive, the plastid translocon appears to be relatively “simple” in composition; hence, there must be other creative ways to be adaptive. The mechanistic provisions for handling certain “adaptive” aspects of plastid protein transport unveiled thus far appear to center on the more external Toc components (Toc159/132/120/90/75/33/34) and their preferences for different precursor types (6–14). Although excellent progress has been made toward our understanding of how the Toc components listed above work, it is clear that there are likely to be additional mechanisms in play during other stages of the process involving more internal components. These additional mechanisms likely operate in partnership with the Toc components. The Tic40-related translocon components appear to be one such candidate designed to address the various needs of protein translocation in a manner different from the receptor-based mechanisms and during other stages of the process. Features documented to date for the Tic40 proteins that may bear meaning in terms of the components’ role are: 1) their heterogeneous nature with respect to abundance, gel mobility, and different apparent configurations in the envelope (24–27); 2) their association with pre-proteins at different stages of translocation (24); 3) their preferences when interacting with different precursor forms of the thylakoid lumen protein Oee1 (28); and 4) their co-chaperone properties (29). Despite these observations, the aspect of the protein translocation process in which Tic40 components work remains unknown. This study was thus designed to combine approaches for unveiling clues related to where the Tic40 proteins work. The first strategy was to profile trends related to how Tic40 components behave in plant tissue models where there are likely to be changes in the demand on the protein transport process (different plant organs, greening seedlings, developing seeds, different plant species, transgenic plants). This strategy provided important and much needed data concerning how the Tic40 proteins behaved on an hourly or daily basis in tissues undergoing organelle biogenesis, plastid development, or conversion. Although the trends compiled represent activities from a collection of cells rather than individual specific cell types, there is clearly fluctuation. The abundance of Tic40 proteins and the pattern of the different configurations change as seedlings go through light-induced greening (converting from dark-germinated tissues to green expanding plantlets) or as developing seed tissues (from postpollination green seeds to yellow mature seeds). This behavior indicates that a key feature of the Tic40 role involves some form of fluctuation or modulation, perhaps to accommodate the changing demands on the protein transport process. The changes in abundance and pattern are distinct and appear to follow a purposeful program, rather than being random by-products of protein degradation. Tic40 levels also exhibit variations between different organs of the same mature plant and between different species. All of the changes observed were assessed relative to the inner envelope translocon component Tic110. The trends together suggest that the plastid translocon works within a fairly wide range of naturally occurring Tic40 fluctuations, an indication that the Tic40 proteins probably operate in a modulating capacity. The ability of the plastid translocon to operate in a range of variations was again observed in the transgenic *Arabidopsis* seedlings with artificial manipulations to Tic40 levels. Although partial suppression of Tic40 reduced the steady-state levels of the imported proteins tested, the reductions were limited. At the other end of the spectrum, excessive production of Tic40 did not by itself increase accumulation of the imported proteins assessed. It should be noted that our transgenic *Arabidopsis* profiles are compilations of activities occurring across different tissues and cell types and do not reflect localized events or effects emanating from particular cells. There may be a dramatic impact on a specific cell type in a very localized manner, but these effects are not resolvable with our current approach. Nevertheless, our transgenic *Arabidopsis* findings are in agreement with the observations reported in Ref. 29, where disruption of the *Arabidopsis* Tic40 gene failed to reduce the accumulation of plastid proteins to an extent that would be detrimental to the full development of the plant, even though the import process was partially impaired. The behavior displayed by the Tic40 proteins also aligns well with the findings reported in a chloroplast proteome study (42). In contrast to the other plastid translocon components, Tic40 was observed to be either substoichiometric or not constitutive in nature. The authors also added the remark that a full description of the chloroplast proteome will likely require the analysis of all plastid types and all possible tissues. The fluctuating behavior displayed by Tic40 proteins points to a modulating role because the components do not appear to be essential to the core process of protein transport.

The notion of a modulating role for Tic40 components was further supported by our cross-organelle work. The yeast mitochondrial system proved invaluable for mapping where Tic40 components are likely to associate and exert an influence. Because the Tic40 proteins appear to possess a role involving modulation, aspects of the yeast mitochondrial translocon that are likely to attract Tic40 proteins would be components involved in regulatory-like activities. The results arising from the yeast-based experiments did in fact unveil the association of Tic40 proteins with such components of the mitochondrial translocon. The presence of Tic40 proteins influences regulatory aspects of the translocon manifesting as changes to the levels of Tom22, Tom40, Tom70, Tim10, Tim23, and Tim50. The pull-down assays provided evidence for the existence of physical associations between Tic40 proteins and two of the translocon components, Tom22 and Tim44. The associations appear to favor regulatory or modulating aspects of the mitochondrial translocon or the protein transport process, matching the compositional observations. The associations do not appear to be fortuitous because there are signs of functionality. The Tic40 proteins appear capable of adjusting their influence and physical associations in response to a switch in carbon source and during temperature stress. The pattern of influence and association appears to migrate from the outer Tom complex to...
the inner Tim23 complex. This migration pattern may be related to the Tic40 configurational changes observed in the protease treatment experiments, where there was an overall change from more external configurations to more internal ones. There also appear to be differences in the associations formed by the two select Tic40 configurations (RcTic40 and RcToc36) and the way in which the two forms respond to the external changes imposed. The benefits of the new advantage provided by Tic40 proteins became distinct when the transgenic yeast cells were subjected to changes in growth conditions (different carbon sources) and temperature stress. It is important to note that the associations and influences observed exist for both outer and inner membrane translocons, further supporting the notion that Tic40 proteins are present as different configurations and in different locales. In plastids, Tic40 proteins, although found predominantly in the inner envelope and protected by their association with the membrane, are also present as protease-accessible configurations (25) and possess the capability of forming chemical cross-links with the outer Toc75 component (29). How the Tic40 proteins establish associations with different locations remains unknown. It is equally clear that the relationship(s) among the various configurations, associations, and influences needs detailed analysis. The combination of results, both previously reported and from this study, helps direct further studies into this particular aspect of the protein transport process.

Based on the observations to date, the term modulating appears to be the most appropriate to describe the Tic40 proteins role, as opposed to regulating. It appears that Tic40 proteins are not essential under normal growth conditions because plants can operate in a wide range of Tic40 fluctuations. The disruption of the gene has no discernible detrimental effect on the development of a plant. The Tic40 components instead appear to provide added benefits to the system, which may only manifest under conditions of need or stress, e.g. during certain stages of development or in the presence of external stress. The yeast cells, when grown in glycerol-containing media and temperature stress, appear to express this benefit. Bacterial cells containing Tic40 proteins also exhibit a benefit by sustaining protein transport under stress (azide, cold temperature, and high ampicillin concentrations), a characteristic that remains masked in optimal conditions (43). The need to accommodate different arrays of protein precursors was also the case for the existence of the Toc159 receptor family (Toc159/132/129/90) (7). Ivanova and co-workers (7) speculate that different transport pathways evolved to provide balanced importation of essential proteins role, as opposed to the notion that Tic40 proteins are present as different protease-accessible configurations (25) and possess the capability of forming chemical cross-links with the outer Toc75 component (29). How the Tic40 proteins establish associations with different locations remains unknown. It is equally clear that the relationship(s) among the various configurations, associations, and influences needs detailed analysis. The combination of results, both previously reported and from this study, helps direct further studies into this particular aspect of the protein transport process.
Evidence That the Plastid Translocon Tic40 Components Possess Modulating Capabilities

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