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Review

The TIC complex uncovered: The alternative view on the molecular mechanism of protein translocation across the inner envelope membrane of chloroplasts☆

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ABSTRACT

Chloroplasts must import thousands of nuclear-encoded preproteins synthesized in the cytosol through two successive protein translocons at the outer and inner envelope membranes, termed TOC and TIC, respectively, to fulfill their complex physiological roles. The molecular identity of the TIC translocon had long remained controversial; two proteins, namely Tic20 and Tic110, had been proposed to be central to protein translocation across the inner envelope membrane. Tic40 also had long been considered to be another central player in this process. However, recently, a novel 1-megadalton complex consisting of Tic20, Tic56, Tic100, and Tic214 was identified at the chloroplast inner membrane of *Arabidopsis* and was demonstrated to constitute a general TIC translocon which functions in concert with the well-characterized TOC translocon. On the other hand, direct interaction between this novel TIC transport system and Tic110 or Tic40 was hardly observed. Consequently, the molecular model for protein translocation across the inner envelope membrane of chloroplasts might need to be extensively revised. In this review article, I intend to propose such alternative view regarding the TIC transport system in contradistinction to the classical view. I also would emphasize importance of reevaluation of previous works in terms of with what methods these classical Tic proteins such as Tic110 or Tic40 were picked up as TIC constituents at the very beginning as well as what actual evidence there were to support their direct and specific involvement in chloroplast protein import. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. Introduction

Virtually all life forms on earth depend on organic materials produced by photosynthesis, either directly or indirectly. Plants and algae, the eukaryotes, and photosynthetic bacteria including cyanobacteria, the prokaryotes, perform this important reaction. In plants and algae, photosynthesis is carried out in a specialized organelle called chloroplast. Almost all chloroplasts in today's photosynthetic eukaryotes derive from one primary endosymbiotic event with a cyanobacterium-like ancestor thought to have occurred more than a billion years ago [1,2]. This was followed by massive transfer of genes from the endosymbiont to the host's nuclear genome, accompanied with the evolution of protein transport system that allows these nuclear-encoded proteins back into the endosymbiont. Extant chloroplasts can synthesize only ~100 proteins but must import more than 2000 different nuclear-encoded proteins synthesized outside the chloroplast, across the double envelope membranes surrounding this organelle, to fulfill their complex physiological roles [3].

In general, proteins cannot pass through biological membranes freely. Protein translocation across biological membranes requires supramolecular complexes, called translocons [4]. To date, only a limited number of translocons have been identified. Bacterial-type SEC and TAT translocons are widely distributed among eubacteria and archaebacteria and thus may be regarded as the most ancient types of translocons [5,6]. Homologous translocons are also found in eukaryotic ER and thylakoid membranes of chloroplasts [7,8]. Eukaryotic translocons located in distinct intracellular membranes appear to have arisen along with the evolution of various organelles [9]. They include mitochondrial TOM and TIM [10,11] and peroxisomal PEX complexes [12,13]. Chloroplasts require such translocons, namely TOC and TIC, in their double envelope membranes to import thousands of nucleus-encoded proteins synthesized in the cytosol [14–18]. These translocons differ entirely in their protein composition from each other, and therefore, elucidation of their detailed molecular architectures and underlying mechanisms is of fundamental importance in cell biology.

2. Overview of the protein translocation across the outer and inner envelope membranes of chloroplasts

Most nuclear-encoded chloroplast proteins are synthesized in the cytosol as a larger precursor protein called preprotein with an amino-

Abbreviations: TOC, protein translocon at the outer envelope membrane of chloroplasts; TIC, protein translocon at the inner envelope membrane of chloroplasts

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terminal so-called transit peptide which carries sufficient information on intracellular targeting to the chloroplast as well as intraorganellar sorting to their final destination (Fig. 1). In general, there are no consensus conserved sequences among various transit peptides but some common characteristics are present; in most cases, they are net positively charged with few acidic amino acid residues and are also rich in hydroxylated amino acid residues, especially serine [19]. Preproteins synthesized in cell-free translation systems can be imported into the isolated chloroplast post-translationally. Various cytosolic factors such as molecular chaperones may facilitate to keep import competency of the preproteins [20]. Then the preproteins are recognized by the TOC complex components at the outer envelope membrane of chloroplasts [21]. Toc159 family proteins and Toc34 family proteins are both GTP-binding proteins and function as receptors for preproteins [22–24]. Their GTP-binding domains protrude from the surface of the outer envelope to the cytosol. Toc75, another core component of the TOC complex, is a beta barrel protein of the well-known bacterial Omp85 family [25]. Toc159, Toc34 and Toc75 proteins form a rigid approximately 800–1000 kDa membrane protein complex at the outer envelope membranes [26,27]. The initial translocation of the amino-terminal portion of preproteins including a transit peptide across the outer envelope requires hydrolysis of low amount of ATP [28]. However, the exact reason for this ATP requirement remains unclear [29]. While GTP hydrolysis is likely essential for the import process as well [30], the exact functional roles of the two GTP-binding receptor proteins, namely Toc159 and Toc34, remain to be determined [31].

Amino-terminal portions of preproteins including a transit peptide emerged from the intermembrane space-side of the TOC complex should subsequently interact with the TIC translocon at the inner envelope membrane. There is only a limited amount of information about molecular details on preprotein transfer which must occur between TOC and TIC complexes. Tic22 protein is only known intermembrane space component which has been proposed to be involved in this process [32]. However, its exact physiological role remains unknown because double knock-out of genes for two Tic22 isoforms in *Arabidopsis* resulted in only slight phenotypic changes [33,34].

Complete translocation of preproteins across the inner envelope membrane through the TIC complex to the stroma occurs with much more expense of ATP where a TIC translocon-associated import motor has been believed to be involved in this process at the stromal side of the inner envelope [29,35]. With regard to the actual TIC constituents, many candidate proteins have so far been identified and characterized as so-called Tic proteins, which include Tic20, Tic21, Tic22, Tic32, Tic40, Tic55, Tic56, Tic62, Tic100, Tic110, and Tic214 [18]. Two seemingly mutually exclusive models have now been proposed [14–17], which is the main subject of this review article. While several stromal molecular chaperone proteins have been proposed to be responsible for the import motor function [36–40], because of the remaining uncertainty of the identity of the genuine TIC complex, the identity of actual TIC translocon-associated ATP-driven import motor also remains to be determined.

Finally, in the stroma, transit peptides of newly translocated preproteins are cleaved off by the stromal processing peptidase [41] and the remaining mature polypeptides are folded correctly and/or assembled with other protein subunits into oligomeric structures in the stroma with the aid of various stromal molecular chaperones [42,43] or are further transported to the thylakoid membranes *via* well-characterized bacterial type transport systems [44,45].

Two additional intriguing non-canonical protein transport pathways into the chloroplast have been proposed to exist. One is vesicle transport pathway through ER-Golgi endomembrane system to the chloroplast, which seems to be important for delivery of some glycosylated chloroplast proteins [46,47]. The other is a distinct transport pathway which seems to be responsible for some transit peptide-less chloroplast proteins especially destined for the inner envelope membrane [48].

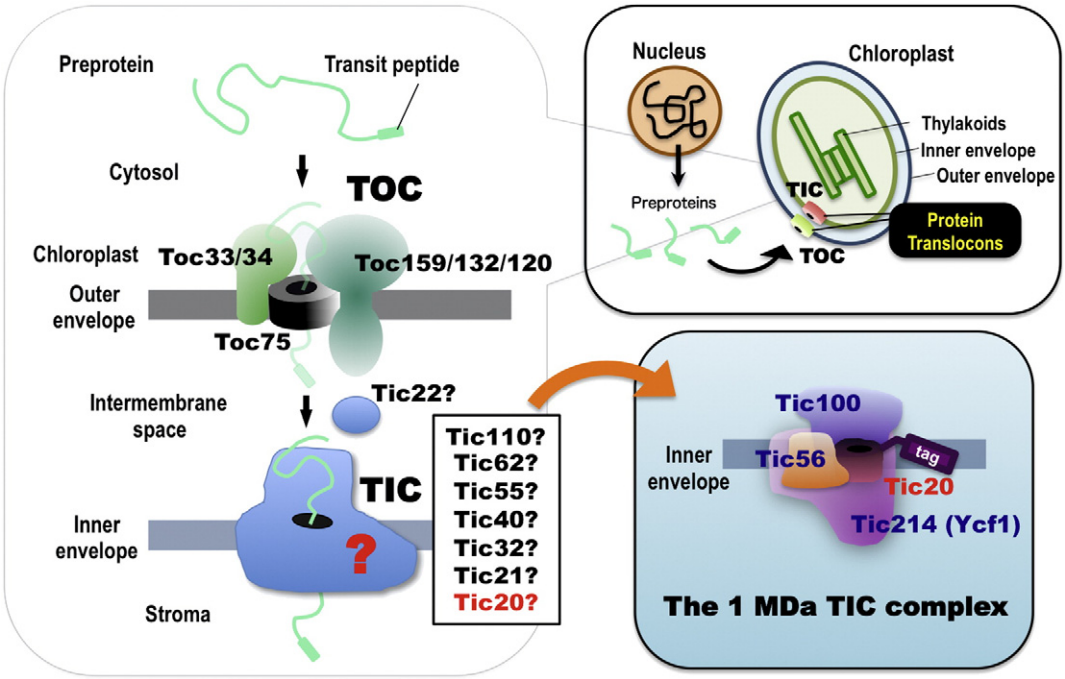

Fig. 1. The TOC and TIC transport system. Most nuclear-encoded chloroplast proteins are synthesized in the cytosol as a larger preprotein carrying an amino-terminal transit peptide. They are then translocated across the outer and inner envelope membranes through TOC (translocon at the outer envelope membrane of chloroplasts) and TIC (translocon at the inner envelope membrane of chloroplasts), respectively. While several TIC candidate proteins including Tic 110, Tic62, Tic55, Tic40, Tic30, Tic22, Tic21, and Tic20 were identified, the core TIC complex remained unclear. Recently, the 1 MDa TIC complex consisting of Tic214 (formerly Ycf1), Tic100, Tic56, and Tic20 has been identified and characterized as a general TIC translocon using transgenic *Arabidopsis* plants expressing a tagged-form of Tic20.

3. The classical view of TIC transport system

3.1. Discovery of Tic110

Tic110 was the firstly proposed TIC component. Tic110, a highly abundant inner envelope membrane protein, had been initially recognized as and also has long been utilized as a marker protein of the inner envelope [49]. One key finding in the history by which this protein was started to be regarded as a main TIC component was the presence of a 100 kDa protein seemingly associated with translocation intermediates which was observed during a series of elegant *in vitro* import experiments performed in the mid 90s of the past century [50]. These outstanding pioneering studies carried out at the very dawn of this research field could identify all core TOC components, namely Toc34, Toc75, and Toc159 (formerly Toc86), as well as Tic22 and Tic20, besides the above mentioned 100 kDa protein [21,22,32,50–53]. In the series of studies, model preproteins consisting of an entire preprotein of Rubisco small subunit or of ferredoxin fused with an IgG-binding domain of Protein A at their carboxyl-terminus were used in *in vitro* import reactions with isolated chloroplasts [51,52]. Because the IgG-binding domain had a tendency to be folded, preprotein translocation across the envelope membranes was retarded so that translocation intermediates were efficiently accumulated under certain conditions such as in the presence of limited amount of ATP or under low temperature. Then, two distinct well-deliberate approaches were taken to identify translocation intermediate-associating proteins.

The first approach was to simply purify the translocation intermediate complexes using IgG-Sepharose beads after solubilization of chloroplasts by detergent Triton X-100 without any pretreatment with chemical crosslinker [21,22,50]. Using this approach with isolated pea chloroplasts, three core TOC constituents, namely Toc75, Toc159, and Toc34, were specifically recovered in the purified fraction [50]. A protein band appeared additionally around 100 kDa in size upon SDS-PAGE of the eluted fraction. The apparent size of this protein was very similar to that of the well-known abundant 100 kDa marker protein of the inner envelope membrane, so that a cDNA clone corresponding to the abundant 100 kDa protein was obtained [50] and then the abundant protein was renamed Tic110 later. While the presence of Tic110 in the original eluted fraction was confirmed with the specific antibodies raised against the recombinant Tic110 protein, there seemed to be somehow only marginal enrichment of Tic110 in the purified fraction as compared with those of TOC components. Besides the 100 kDa protein band, a 36 kDa protein band was observed in the purified fraction and was named IEP36, which was found to be not identical to Tic40 or p36 protein. While the identity of the IEP36 has remained unclear in the literature, this protein might be a Toc34 isoform derived from the outer envelope membrane rather than the inner envelope. By this first method, other TIC protein bands corresponding to Tic20 or Tic22 were not detected, probably because that solubilization by high concentration of Triton X-100 caused dissociation of actual TIC components from the translocating preproteins [54].

The second approach was to mark nearby proteins with translocating preproteins *in situ* by label-transfer crosslinking upon UV light irradiation after accumulating the translocation intermediates but before solubilization [32,52,53]. To this end, a cleavable photoactivatable radio-labeled crosslinker was introduced site-specifically to the above mentioned model preproteins. The marked radio-labeled (*i.e.* photoaffinity-labeled) proteins were able to be detected easily by simple SDS-PAGE followed by autoradiography without any purification. Using this truly superb approach, Toc75, Toc159, and Toc34 were again detected as expected, as well as two distinct inner envelope membrane proteins around 20 kDa in size, which was later determined as Tic20 and Tic22 by solid biochemical methods [32]. An additional 14 kDa inner envelope protein was also observed as affinity-labeled, which unfortunately remains unidentified yet. However, any labeled 100 kDa protein corresponding to Tic110 seemed to be hardly detected. The similar approach was taken by another group later and again was able to detect Toc75, Toc159, Toc34, Tic20, and Tic22, as well as the 14 kDa protein but no 100 kDa protein [55]. Such label-transferred Tic110 was declared to have been able to be observed using this method just only once in the literature [56]. However, the corresponding observed band seemed to be rather faint and might need some additional assessments to determine the identity of the labeled protein.

Two other research groups started to characterize Tic110 protein biochemically in the same mid '90s, but the conclusive proof for direct involvement of Tic110 in protein translocation across the inner envelope membrane as a core component of TIC translocon was not sufficiently demonstrated [57–60]. For example, experiments by combined extensive chemical crosslinking with immunoprecipitations were performed after *in vitro* import reactions did detect crosslinked products containing preproteins which appeared to be immunoprecipitated by antisera raised against Tic110 [59]. However, the crosslinked complex represented only a small minor fraction which was completely distinct from the major crosslinked entities. Most unfortunately, it was not demonstrated whether the observed minor crosslinked products actually represented a chase-able translocation intermediate complex nor whether there was actual direct interaction (crosslink) between Tic110 and preproteins [60]. Thus, while Tic110 has long been proposed to be central to protein transport across the inner envelope membrane, critical strong evidence which can support the presence of direct 1:1 interaction between a translocating preprotein and Tic110 protein has been surprisingly scarce. Because of this reason, soon after the initial discovery of Tic110 as a proposed core TIC translocon component directly interacting with a translocating preprotein, function of Tic110 has been emphasized as a scaffold for the binding of some stromal molecular chaperones such as Hsp93 or cpHsp70 [56].

The primary sequence of Tic110 shows the presence of two typical amino-terminal transmembrane segments. While some arguments remain, Tic110 have been repeatedly shown to be anchored in the inner envelope membrane *via* the two transmembrane helices with the large carboxyl-terminal soluble domain exposed to the stroma by several different research groups [61–64]. Recent structural prediction of the soluble domain of a red algal Tic110 homolog suggested the presence of several HEAT-repeats with an entirely elongated shape which might serve as a scaffold for interacting partners such as stromal chaperones as mentioned above. The membrane-embedded domain of Tic110 might form a cation-selective channel in the inner envelope [62,65].

3.2. Discovery of Tic20

As mentioned above, Tic20 is the best established inner envelope membrane protein whose direct interaction with translocating preproteins has been demonstrated repeatedly, importantly, by several different research groups and irrespective of slightly distinct crosslinking approaches [32,52–55]. Tic20 is a mostly hydrophobic integral membrane protein consisting of four predicted transmembrane helices [32]. Because of its weak sequence similarity with the Tim23 protein, a central component of the mitochondrial TIM inner membrane protein translocon, Tic20 was firstly proposed to have some evolutionary link with the mitochondrial Tim23 [66,67]. However, the later detailed phylogenetic analysis revealed that this sequence similarity was most likely due to a sort of convergent evolution [68]. The purified Tic20 alone was demonstrated to form a membrane channel [69].

Because of its highly hydrophobic nature and because of lack of usable specific antibodies, thorough biochemical analyses of the Tic20-containing complex had not been carried until recently. Several research groups have confirmed that Tic20 forms a fairly large membrane protein complex in the inner envelope membrane which is clearly distinct from the Tic110-containing entity [32,54,69]. Frequently in the literature including recent review articles by others [70,71], one earlier

study published in 1998 was cited to exemplify a direct association of Tic110 with Tic20 [32]. However, the actual data shown in that study does not seem to support this statement, since marginal co-elution of Tic110 with Tic20 in a same fraction was accompanied with many other proteins and might be possibly due to inevitable non-specific contamination of abundant Tic110 and/or very hydrophobic Tic20 in this fraction. There has been no other solid evidence which can support the presence of physical interaction between Tic110 and Tic20.

3.3. Discovery of Tic40

Tic40 consists of a single transmembrane helix near the amino-terminus and a stromally-exposed carboxyl-terminal soluble domain containing a TPR (tetratricopeptide repeat) domain and a Hip/Hop/Sti1 domain [72–74]. The TPR domain and the Hip/Hop/Sti1 domain have been proposed to be a binding site for Tic110 and for Hsp70/Hsp93 molecular chaperone proteins, respectively, based on *in vitro* experiments using a yeast two-hybrid system or pull-down assays with *Escherichia coli*-expressed proteins as well as *in planta* BiFC analysis. However, a stable complex formation between Tic40 and Tic110 or Hsp93 in the wild-type chloroplasts has not been demonstrated thoroughly in a quantitative manner, while some transient complex formation was observed after extensive chemical cross linking followed by immunoprecipitation experiments.

It should be noted that there was a serious confusion when the cDNA clone of Tic40 was firstly isolated, which was mostly caused by a fairly complicated history in the process of initial identification of a translocation intermediate-associated 44 kDa protein and also by a complicated following cDNA cloning procedures. Originally, antibodies raised against a tomato 44 kDa envelope membrane protein was described to recognize two envelope membrane proteins from pea chloroplasts, each of which seemed to be localized to the outer and inner envelope membranes and thus were named Com44 and Cim44, respectively [75]. These two proteins were described to be immunologically related so that they were proposed to be homologous proteins. The antibodies were also found to be able to immunoprecipitate translocating model preproteins after extensive chemical crosslinking and solubilization with detergents. Thereafter, Com44/Cim44 proteins were started to be proposed as translocon components bearing close physical contact with translocating preproteins. In the literature, it has remained unclear how the original tomato 44 kDa envelope membrane protein was initially picked up at the very beginning. Then, the same research group reported the cloning of some partial cDNA fragments from the *Brassica napus* cDNA library which were proposed to encode the above mentioned Com44 or Cim44 [76], but the protein encoded by this cDNA clone was somehow renamed Toc36, a 36 kDa TOC component at the outer envelope membrane, by this group [77]. However, unfortunately, conclusive evidence which could support the actual sameness between the cloned cDNA product and the initially observed Com44 or Cim44 protein was surprisingly scarce in these series of experiments [74–76]. Later, two other research groups succeeded in obtaining the full length cDNAs corresponding to the partial cDNA clones from pea and *Arabidopsis* plants, respectively, which were shown to encode a 40 kDa inner envelope membrane protein, and finally termed this protein Tic40 [72,73]. Therefore, it might be said that the actual molecular identities of the initially observed two immunoreactive 44 kDa proteins in the outer and inner envelope membranes have still remained unclear. Physical association between Tic40 and translocating preproteins was detected but only faintly even after extensive chemical crosslinking [73]. It should be noted that, while the initially observed 44 kDa protein was proposed to be located in close proximity with a translocating preprotein, function of Tic40 also has been somehow changed to serve as a scaffold for the binding of stromal molecular chaperone Hsp93 and Tic110 [74,78].

3.4. Discovery of Tic21

Tic21, a 21 kDa integral inner envelope membrane protein of chloroplasts, was initially identified by an elegant well-thought-out forward genetic screen [79]. An *Arabidopsis* mutant exhibiting a defect in protein import into the chloroplast was positively selected and was found to possess a point mutation in a nuclear gene encoding Tic21. Although an alternative function of Tic21 has been proposed as an iron transporter across the inner envelope membrane, namely PIC1 for iron permease in chloroplasts [80,81], a small fraction of Tic21 was biochemically shown to be associated peripherally with a large complex containing Tic20 (see below) [54]. Interestingly, Tic21 contains four predicted transmembrane helices just like Tic20, although there is no sequence similarity between the two proteins. However, whether or not Tic21 participates directly in preprotein translocation remains an open question.

3.5. Discovery of other so-called "redox regulator" components

Three proteins have been proposed to function for modulation of preprotein translocation across the inner envelope membrane as redox regulators. Tic55 is an inner envelope membrane protein which has a Rieske-type Fe–S cluster binding site as well as an additional iron-binding motif and belongs to certain oxygenase superfamily [82]. Tic62 seems to be mainly localized on the surface of thylakoid membranes and belongs to a short-chain dehydrogenase family and has a role for tethering of FNR on the thylakoid surface [83,84]. Tic32 carries an NADP(H)-binding domain and a calmodulin-binding domain [85]. All these TIC candidate proteins were identified as interacting proteins with Tic110. While proposed redox regulation of protein translocation across the inner envelope membrane is a truly fascinating hypothesis that should be expected to exist in concert with other redox control events known to occur inside the chloroplast [86], actual involvement of these TIC candidate proteins in such redox regulation of protein import needs further investigations.

3.6. The classical model of TIC transport system

Based on these historical studies as summarized above, a classical model for protein translocation across the inner envelope membrane was constructed and, until recently, has long been believed among not only most researchers in this research field but also other general readers who had a chance to take a glance at such model in many review articles or in many textbooks [14–17]. The most widely believed classical model of TIC transport system was as follows: When the amino-terminal segment of a preprotein emerges from the TOC complex to the intermembrane space-side of the outer envelope membrane of chloroplast, it interacts with Tic22 and then with Tic110 either directly or indirectly at the inner envelope membrane which likely forms a TIC translocation channel transiently together with Tic20 and Tic21. The stromal soluble domain of Tic110 serves as a trans-side recognition site for a transit peptide of preprotein and also interacts with Tic40. Both Tic110 and Tic40 recruit stromal molecular chaperones, Hsp93 and/or cpHsp70, and docking of Tic110 with Tic40 triggers transfer the preprotein bound on Tic110 to stromal chaperones. Finally, these molecular chaperones, either cooperatively or independently, consume ATPs in order to drive complete translocation of the entire preprotein across the inner envelope membrane.

3.7. Critical problems underlying the classical model

However, the most critically controversial issue has remained completely unresolved in this classical model since the very beginning of the identification of firstly proposed TIC component, which was Tic110; virtually no one have been able to detect actual tight association between any pair of these proposed TIC candidate components. Because

of this, a sort of transiently assembly model has been frequently proposed, in which an incoming preprotein triggers the assembly of actual TIC translocon each time by these TIC candidate proteins [14–16,71]. However, such transiently assembled large complexes composed of these proposed TIC components have not been sufficiently demonstrated in a quantitative manner in the literature. Generally speaking, such entirely transiently assembled protein translocation machinery might be somewhat peculiar for multisubunit translocons.

Most of previous biochemical studies by which these TIC candidates were proposed to be involved in the import process largely relied on the essentially common methodology using extensive crosslinking with non-specific chemical crosslinkers such as DSP (dithiobis (succinimidyl propionate)) in combination with immunoprecipitation with polyclonal antibodies [26,40,59,60,65,72,73,75]. We should be aware that such chemical crosslinkers are widely used not only for fixing specific protein–protein interactions *in situ* but also for making artificial conjugates between proteins even without any specific interaction. Undoubtedly, this conventionally utilized methodology should be useful to detect molecular interactions especially if the interaction occurs only transiently. However, this method sometimes causes significant levels of non-specific or indirect co-immunoprecipitation (or false co-immunoprecipitation) of unrelated (not directly interacting) proteins especially if the protein of interest exists high abundance in the starting materials and/or if the polyclonal antiserum used for immunoprecipitation is not so specific to the antigen.

In addition, after extensive chemical crosslinking, especially hydrophobic membrane proteins or membrane protein complexes and their crosslinked products tend to stuck to the affinity resins used for immunoprecipitation even after solubilization with detergents and cause significant amounts of inevitable non-specific co-immunoprecipitations in the final samples. This sometimes happens even when immunoprecipitation is performed without any prior chemical crosslinking, most probably due to insufficient solubilization of certain hydrophobic membrane proteins or complexes. It is widely accepted that significantly high enrichment of a certain protein of interest in an immunoprecipitated fraction or in a purified fraction obtained from such experiments should be the one most important criterion in order to distinguish specific associations from non-specific ones. However, in some cases, such enrichment factor seems to have not been adequately monitored or have not been considered thoroughly. Thus, without careful evaluations including a sort of such quantitative assessment, data obtained from those analyses therefore sometimes cause misinterpretation of the results and lead premature erroneous conclusions.

4. The revised view of TIC transport system

4.1. Discovery of the 1-megadalton (MDa) translocation complex at the inner envelope membrane that contains Tic20 as a core

Because of the obvious absence of observable tight association among known Tic candidate proteins as mentioned above and because of remaining serious uncertainty in the process of identification of some of proposed Tic proteins, my group has focused on identifying a genuine inner envelope membrane protein complex involved in preprotein translocation across the inner envelope [54].

To avoid the above-mentioned unsteady but, in some cases, inevitable contamination of non-specific proteins which might occur by extensive chemical crosslinking, blue-native PAGE (BN-PAGE) analyses in combination with *in vitro* import experiments were applied to directly detect translocation intermediate complexes at the inner envelope membrane after solubilization by detergents but without any prior chemical crosslinking [54]. To this end, firstly *in vitro* import experiments using radio-labeled preproteins and isolated intact chloroplasts were performed under limited concentration of ATP to accumulate translocation intermediates at the inner envelope membranes. By subsequent BN-PAGE separation of membrane protein complexes solubilized by a mild detergent, digitonin, without any prior chemical crosslinking, radioactive signals containing the accumulated preproteins were observed around the 1 MDa area. Very importantly, the radioactive 1 MDa signal was demonstrated to represent a chase-able genuine translocation intermediate complex. Further biochemical analyses confirmed the inner envelope localization of the translocation complex as well as the inclusion of Tic20 protein as a core of this 1 MDa complex. Even in the absence of preproteins, Tic20 was found to form a stable 1 MDa complex at the inner envelope membrane. Tic21 was only a peripherally associated protein of this 1 MDa complex because the presence of high salts during solubilization caused complete dissociation of Tic21 from the complex but left the remaining 1 MDa complex containing Tic20 seemingly unaffected significantly. The most important point of this experiment was the absence of other well-known Tic proteins such as Tic110 or Tic40 in the 1 MDa translocation complex. In contrast, Tic20, a small integral membrane protein of 20 kDa, appeared to form such huge 1 MDa complex stably at the inner envelope membrane and was found to be located in close proximity with translocating preproteins in the 1 MDa complex.

It should be emphasized that, even before these recent observations, the presence of the Tic20-containing large complex clearly apart from other Tic candidate proteins and the direct 1:1 physical contact between Tic20 and a translocating preprotein have been repeatedly reported by different research groups as mentioned above [32,52–55]. All these findings support the conclusion that Tic20 is a genuine central core component of the general TIC translocon. Phylogenetic evidence indicating the presence of well-conserved Tic20 homologs among virtually all plastid-containing lineages is very compelling [87]. For noteworthy examples, the apicoplast, a relict non-photosynthetic organelle of apicomplexan parasites of red algal origin still retains Tic20 but lacks an obvious Tic110 ortholog [88,89].

Tic20 protein has been frequently pointed out to be considerably less abundant than the other translocon components [69,90]. However, based on the recent biochemical analysis, the stoichiometry of Tic20:Toc75 was estimated as 1:2.5 [91]. Moreover, this stoichiometry is presumably still underestimated, since, due to their high hydrophobic properties, Tic20 and also Tic20-containing huge membrane protein complexes are hard to be solubilized completely and tend to escape from biochemical detections.

4.2. Discovery of the constituents of the 1 MDa complex containing Tic20

To determine the actual composition of the observed 1 MDa Tic20-containing complex, a transgenic *Arabidopsis* plant expressing a tagged-form of Tic20 was constructed and the tagged Tic20-containing complex was able to be purified (Fig. 1) [91]. The purified complex retained its 1 MDa size and stoichiometrically contained three previously uncharacterized essential proteins of 214, 100, and 56 kDa in size together with Tic20 protein, which were named Tic214, Tic100, and Tic56, respectively. All these proteins including Tic20 form a stable 1 MDa complex in the wild-type *Arabidopsis* chloroplasts.

Tic214 is surprisingly encoded by the previously enigmatic chloroplast gene *ycf1* (*h*ypothetical *c*hloroplast open reading *f*rame *1*) and is predicted to contain at least six transmembrane helices in the amino-terminal domain. Tic100 is nuclear-encoded protein and contains three short so-called MORN (Membrane Occupation and Recognition Nexus) motifs which might be responsible for interaction with membranes. Tic100 is a peripherally associated component of the 1 MDa complex at the intermembrane space-side. Tic56, a nuclear-encoded protein, has no predicted transmembrane helices but seems to be deeply embedded in the complex. All these constituents are indispensable for the function of the 1 MDa complex in *Arabidopsis*. Electrophysiological analyses confirmed that, when reconstituted into planar lipid bilayers, the purified 1 MDa TIC complexes formed membrane channels, where preproteins specifically interact with and plug the channel pore. Based on the electrophysiological data and observed

stoichiometry of the four components, a trimeric assembly of Tic214, Tic100, Tic56, and Tic20 with 1:1:1:1 stoichiometry was proposed to form three identical channels gathered as the 1 MDa complex [91].

It should be noted that, while the electrophoretic mobilities of Tic100 and Tic56 are somehow similar to those of Tic110 and Tic55, respectively, they are all completely different proteins.

4.3. Decisive evidence of direct involvement of the 1 MDa complex as general TIC translocon

To obtain more compelling evidence to prove the direct involvement of the 1 MDa complex as a general TIC translocon, the historically well-established approach was taken as already mentioned in this review paper (Fig. 2) [91]. This involved using purified model preproteins carrying a carboxyl-terminal tag of the IgG-binding domain of Protein A in *in vitro* import experiments to isolate translocation intermediate associated proteins. The model preprotein constructs used for this sake were essentially similar to ones that had been used when Tic110 protein was declared to be identified for the first time [50]. Slight but critical modifications were introduced; i) digitonin instead of Triton X-100 was used throughout purification to stabilize the translocation intermediate complexes; and ii) after binding to IgG-Sepharose beads, instead of using SDS-containing buffer for elution of bound proteins under denaturing conditions, highly specific elution of the translocation intermediate complexes was conducted under non-denaturing conditions by cleavage with TEV protease at the TEV cleavage site introduced between preproteins and the Protein A-tag. Both modifications were important to detect genuine TIC translocon components, because that solubilization of chloroplasts with high concentration of Triton X-100 causes almost complete dissociation of TIC complex from the translocation complex [54] and that incubation of the affinity beads with a SDS-containing buffer under denaturing conditions results in massive elution of non-specifically bound proteins or inevitably contaminated abundant proteins together with actual TIC components.

This improved procedure was able to identify the well-known TOC components including Toc159, Toc75 and Toc33 as well as the 1 MDa TIC complex components including Tic214, Tic100, Tic56, and Tic20 as translocation intermediate-associated proteins [91]. Importantly, the use of two different model preproteins resulted in essentially similar profiles of associated proteins, which strongly supports the direct involvement of the 1 MDa complex in preprotein translocation as a general TIC translocon at the inner envelope membrane in concert with the well-established TOC translocon at the outer envelope. By contrast, however, Tic110 or Tic40 was hardly observed in these specifically eluted fractions.

The obvious advantageous point of the improved procedure is that it can be easily applied to chloroplasts obtained from various different plants. As a matter of fact, essentially similar translocation complexes containing likely counterparts of Tic214, Tic100, Tic56, and Tic20 together with TOC components (but without Tic110 or Tic40) have been purified from chloroplasts isolated from pea, spinach, and also tobacco plants, indicating that this TOC–TIC transport system is ubiquitous among these plants (unpublished). This is well consistent with broad phylogenetic distributions of Tic214, Tic100, Tic56, and Tic20; they are highly conserved among most land plants (see below) [91].

4.4. Considerations on in vivo mutational analyses

In vivo mutational studies have been carried out using mainly *Arabidopsis* plants to elucidate physiological significances of proposed TIC candidate proteins [33,34,61,68,73,79,92,93]. Such genetic analyses must be unquestionably powerful tools. However, it should be emphasized that, in such mutational analyses especially performed in plants, interpretation of observable phenotypes need precautions not only because of possible functional redundancy among homologous proteins but also because of possible abilities for various kinds of compensations. For example, as described above [33,34], the double knock-out for two Tic22 genes in *Arabidopsis* causes no severe phenotype but this might not necessarily mean no participation of this protein in chloroplast import in the wild-type chloroplasts [32]; certain other factor(s) might be able to compensate the absence of Tic22 in the mutant. Conversely, pleiotropic defects have been frequently observed in various mutant plants so that inefficient protein import capacity in certain mutant chloroplasts does not necessarily mean the presence of primary defect on the protein import machinery. For example, while defects in chloroplast protein import was reported in the *Arabidopsis tic40* null mutant

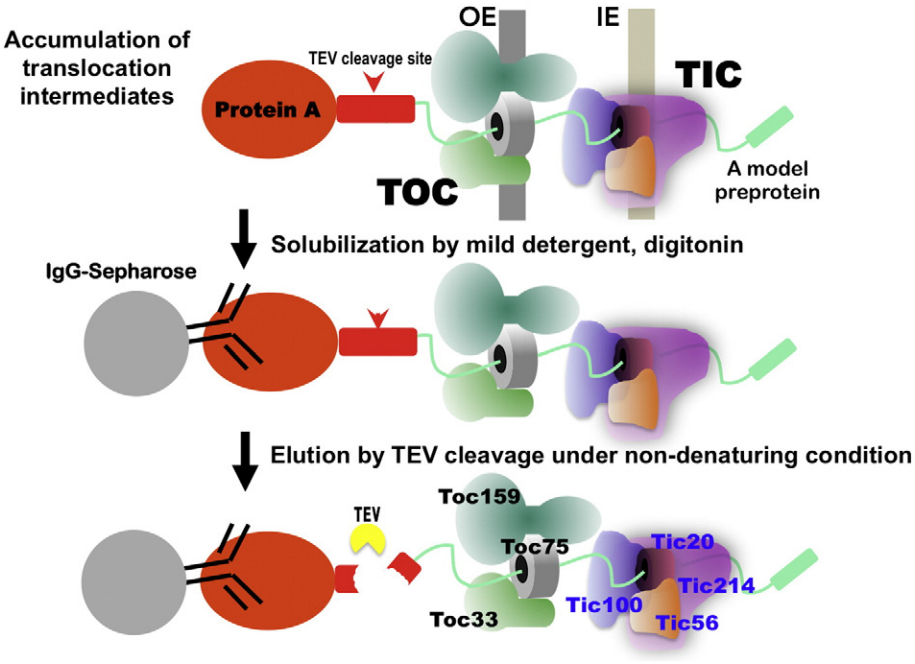

Fig. 2. Decisive evidence of direct involvement of the 1 MDa complex as general TIC translocon. A purified model preprotein carrying a carboxyl-terminal tag of the IgG-binding domain of Protein A and the TEV cleavage site introduced between the preprotein and the Protein A-tag was used to isolate translocation intermediate complexes. Digitonin instead of Triton X-100 was used for solubilization, highly specific elution of the translocation intermediate complexes was conducted under non-denaturing conditions by cleavage with TEV protease.

which is pale green but viable [73,74,94], the same research group also reported the similar import defects in the *Arabidopsis dgd1* mutant, which is known to be deficient in the accumulation of chloroplast lipid digalactosyl diacylglycerol (DGD) [95]. A critical mutation in a key component of protein translocons of chloroplasts should be expected to cause severe lethality in plants, but, needless to say, observed severe lethality does not necessarily mean the involvement of the gene product in chloroplast protein import, because there are many cases in which a defect in various other processes in chloroplasts causes such severe lethality [96]. With regard to the reported mutant phenotypes of the other classical TIC proteins, homozygous *tic110* mutants exhibits embryo-lethality [61,94], whereas none of *tic32* [85], *tic55* [97], or *tic62* [84] *null* mutant display significant defects in plant growth nor in chloroplast protein import.

Examination of synthetic phenotypes in double mutants or double-knock mutants has been widely used for assessment of *in vivo* functional relationship between the two proteins of interest. However, interpretations of such genetic analyses appear to be not so simple. For example, phenotypic "additivity" is sometimes interpreted as the absence of direct functional relationship between the two proteins, whereas synthetic lethality is always interpreted as the presence of strong functional link between the two proteins. Conversely, non-additive phenotypic change, namely epistasis, is often interpreted as the presence of close functional relationship between the two. However, this might not be applicable to all cases; for example, defects in certain lipid supply in the chloroplasts might be reasonably expected to be masked in certain unrelated photosynthetic mutants simply because slowly growing plants due to low capacity of photosynthesis are expected not to require high demand of lipids in the chloroplasts.

4.5. Mutational analyses on the 1 MDa TIC translocon components

Besides these unavoidable ambiguities, substantial amounts of genetic analyses have been accumulated for the 1 MDa TIC translocon components [68,79,91,93,98]. The *null* mutant of *Arabidopsis* Tic20-I (a major Tic20 encoded by chromosome 1) exhibits severe albino and seedling lethality due to a strong defect in chloroplast protein import [68,79,93]. *Null* mutants of Tic56 and Tic100 showed very similar albino and seedling lethality [91]. In addition, *tic20-I tic56* and *tic20-I tic100* double knock-out mutants showed exactly similar phenotypes. Moreover, mutational analyses on the chloroplast *ycf1* gene encoding Tic214 were carried out in *Tobacco* [99] and also in *Chlamydomonas* [100] and concluded that this gene is essential for plant viability. Thus, Tic20, Tic56, Tic100, and Tic214 are essential components of the 1 MDa TIC translocon required for chloroplast protein import and are therefore indispensable for plant viability.

Interestingly, all these TIC translocon component mutants exhibit severe seedling lethality but still can develop at least albino seedlings on sucrose-supplemented synthetic media, in which some "housekeeping" plastid proteins were found to accumulate [91,93]. This residual import ability for non-photosynthetic proteins was demonstrated to be attributed to partial compensation by the elevated expression of Tic20-IV, a minor isoprotein of Tic20 encoded by chromosome 4 in *Arabidopsis*, which was shown to be expressed mainly in roots (Fig. 3) [91,93]. Double knock-out for both *tic20-I* and *tic20-IV* genes were shown to cause most severe embryo-lethality. In addition, *tic20-IV tic56* and *tic20-IV tic100* double knock-outs also exhibit embryo-lethality. All these data suggest there seem to be yet unidentified "non-photosynthetic-type" or "root-type" TIC translocon in which Tic20-IV get involved independently from the main "photosynthetic-type" 1 MDa TIC translocon composed of Tic20-I, Tic56, Tic100, and Tic214. Further detailed investigation regarding such "non-photosynthetic-type" alternative TIC transport system would be highly meaningful to elucidate the entire TOC–TIC protein transport system operating not only in photosynthetic tissues but also in non-photosynthetic tissues of plants. In addition, elucidation of molecular details of the alternative TIC transport system would give some important insights into the evolution of this transport system (see below) [91].

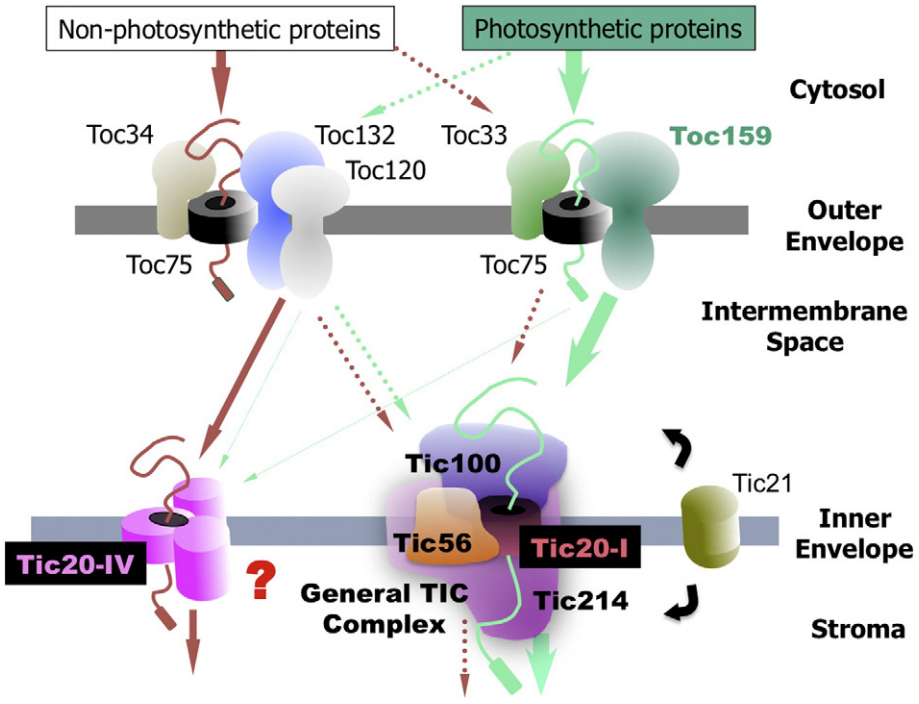

Fig. 3. Model for the coordinated function of the general TIC complex and the hypothetical minor non-photosynthetic TIC complex in substrate-specific protein import in concert with different types of TOC complexes. In *Arabidopsis*, Tic20-IV, an isoprotein of Tic20, is the only identified constituent of such non-photosynthetic TIC complex. The nonphotosynthetic TIC complex might have some direct evolutionary relationships with a distinct Tic20-containing TIC complex functioning in grasses and also with more primitive TIC complexes that should have appeared right after the initial endosymbiotic event and have been possibly retained in all plastid-containing lineages.

4.6. Evolutionary considerations on the 1 MDa TIC translocon components

While all plastids derive from a single endosymbiosis of a cyanobacterium-like organism, no obvious direct homologs for either Tic56, Tic100, or Tic214 could be found in extant cyanobacteria, suggesting that this TIC transport system has evolved largely after the initial endosymbiotic event [91]. The lack of obvious Tic214 homologs in cyanobacteria is particularly surprising since most chloroplast genes are known to be of cyanobacterial origin. A gene for Tic214 appears to have evolved in the chloroplast genome instead of in the nuclear genome before Chlorophyta diverged from Streptophyta. The complete Tic20/56/100/214 complex seems to have been established when Embryophyta (land plants) evolved. Most seed plants including eudicots and monocots possess the Tic20/56/100/214 proteins indicating the general importance of the complex. However, recently diverged unique class of monocot formed by grasses including rice, maize, and wheat have neither Tic56, Tic100, nor Tic214 in their nuclear or chloroplast genomes. Since Tic20 homologs are conserved among these grasses, one may hypothesize that a distinct Tic20-containing TIC complex functions in these species. In this context, noteworthy is the presence of the minor Tic20-IV isoform in *Arabidopsis* which can partly compensate for the absence of Tic20-I independently of Tic56, Tic100, or Tic214 as mentioned above [91,93]. In addition, there might be some evolutionary link between the minor Tic20-IV-containing complex and the grass Tic20-containing complex, both of which might also have some evolutionary relationships with an ancestral primitive type of Tic20-containing complex possibly functioning in Rhodophyta or Glaucophyta. Actually, very recently, we have identified all components of the grass Tic20-containing complex and have found that some of the components including Tic20 are well conserved among virtually all plastid-containing lineages (in preparation). Thus, existence of such alternative TIC transport system may be able to account for other rare eventual loss of the *ycf1* gene from the chloroplast genomes [96,101]. Further analyses on Tic20 homologs and TIC complexes in those other organisms must provide interesting insights into the evolution of this TIC transport system.

4.7. A revised working model of TIC transport system

Based on these recent findings, a working model of TIC transport system might be extensively revised as follows: After passage of preproteins across the outer envelope membrane through the TOC translocon, preproteins become engaged in the general TIC complex consisting of Tic20, Tic56, Tic100, and Tic214, which forms the actual protein-conducting channel at the inner envelope membrane (Fig. 3). Tic20 is located probably in closest proximity to the translocating preprotein as a channel core. The well conserved amino-terminal membrane-embedded domain of Tic214 might form a part of the channel. Tic56 and Tic100 are peripheral components at the intermembrane space-side and might have functions for recognition of incoming preproteins and/or for interaction with the intermembrane space domain of the TOC complex.

The general TIC complex composed of Tic20/56/100/214 is likely responsible for major photosynthetic protein transport and therefore might have preferential physical and functional interaction with the known photosynthetic-type TOC complex consisting of Toc159, Toc33, and Toc75 [102–105]. The above-mentioned alternative TIC complex containing the Tic20 isoform and yet unidentified components might be mainly involved in translocation of house-keeping proteins in non-photosynthetic tissues such as roots and might have preferential interaction with the non-photosynthetic-type TOC complex composed of Toc132/120, Toc34, and Toc75.

With regard to the identity of the TIC translocon associated import motor required for the completion of preprotein translocation across the inner envelope membrane, as mentioned above [35,37,39], significant amounts of studies have been carried out to analyze the molecular interactions between stromal molecular chaperones and Tic110 and/or Tic40 (Fig. 4) [36,38,40]. However, as described above, neither Tic110 nor Tic40 seems to be directly involved in the preprotein translocation step across the inner envelope membrane [91]. Hence, the genuine TIC translocon associated import motor should be reexamined carefully in relation to the uncovered 1 MDa TIC complex composed of Tic20/56/100/214. Actually, an as-yet-undescribed huge ATPase complex seems to physically and functionally interact with the TIC complex (in preparation). Thus, while various stromal chaperone proteins should have certain important functions at the final steps of chloroplast protein import as proposed, the molecular model for protein import propulsion across the inner envelope membrane might still need extensive revision in the future.

With regard to the genuine functions of Tic110 and Tic40, these Tic proteins might be possibly involved in preprotein translocation across the inner envelope at the very later stage as scaffolds for stromal molecular chaperones as have long been proposed [14,16,18,71]. However, other possibilities might be that these TIC proteins have other essential important functions for chloroplast biogenesis rather than direct roles on chloroplast protein import but indirectly affect the import process. The structural study on Tic110 clearly suggests that this protein surely provide a sort of large interaction surface "scaffold" at the stromal side of the inner envelope membrane [64]. Hsp93 is the best-known interacting partner of Tic110 [56,59,60]. Recent quantitative analysis of Hsp93 demonstrated that most of envelope-localized Hsp93 forms a Clp protease complex with proteolytic core subunits suggesting its

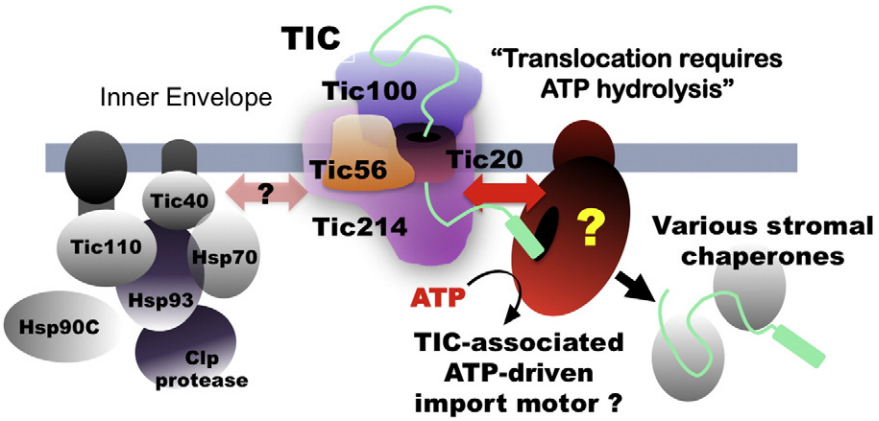

Fig. 4. The hypothetical model for the coordinated function of the general TIC complex and a tentative membrane-bound TIC-associated import motor complex in ATP-driven preprotein translocation across the inner envelope membrane of chloroplasts. Tic 110 or Tic40 might function in more later or alternative stages during the import process. Various molecular chaperone proteins also should have important roles in various subsequent stromal events.

primary function of proteolysis [106]. Thus, Tic110 might be also involved in such proteolytic or quality control events at the inner envelope membrane together with the Clp protease, possibly relevant to certain protein import processes. Moreover, other recent intriguing studies concerning Tic110 and Tic40 proteins should provide us further important insights for their alternative functions [107]. Interestingly, overexpression of Tic40 in *Tobacco* chloroplasts resulted in massive proliferation of the inner envelope membrane [108]. In addition, Tic40 in *Brassica* has been proposed to be a key factor in controlling lipid accumulation [109]. All these findings may simply indicate a possible direct role of Tic40 in a sort of lipid metabolism at the inner envelope membrane. In relation to this, it should be noted that another protein of approximately 44 kDa in size seems to be associated with translocation intermediates, which is clearly distinct from Tic40 [91]. This protein might possibly be an alternate candidate for Com44 or Cim44 which was originally identified at the very beginning of the history of Tic40 (in preparation).

5. Concluding remarks

Recent identification of the novel 1 MDa TIC complex and its actual constituents, Tic20, Tic56, Tic100, and Tic214, has clarified the presence of stable multisubunit protein translocon at the inner envelope membrane. However, not only the precise roles of these constituents in the complex but also the interplay between the TIC complex and the TOC complex as well as that between the TIC and the as-yet-undescribed TIC-associated import motor complex remain totally open questions. Especially, examinations on preferential interaction between different types of TOC complexes and different types of TIC complexes should probably be important to elucidate how plant cells control efficiency of protein import of various different substrates in various different plant tissues. Another exciting challenge is to understand the evolutionary history of this TIC transport system. This includes many fascinating questions: What are the constituents of more primitive form of TIC translocon functioning in Rhodophyta or Glaucophyta? Why were Tic56, Tic100, and Tic214 required to be added during the evolution of green algae ad land plants? Why does Tic214 remain chloroplast-encoded? What is the evolutionary origin of the chloroplast *ycf1* gene encoding Tic214? Why did grasses lose all the TIC constituents except for Tic20? What is the actual TIC translocon functioning in grasses? And what was the origin of this grass TIC system? By clarifying these questions and further more related questions, we will surely gain more clear-cut pictures regarding the molecular mechanisms and evolution of protein translocation system across the double envelope membranes of chloroplasts.

Transparency Document

The Transparency document associated with this article can be found, in the online version

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