How membrane proteins travel across the mitochondrial intermembrane space

Carla M. Koehler, Sabeeha Merchant and Gottfried Schatz

A newly discovered family of small proteins in the yeast mitochondrial intermembrane space mediates import of hydrophobic proteins from the cytoplasm into the inner membrane. Loss of one of these chaperone-like proteins from human mitochondria results in a disease that causes deafness, muscle weakness and blindness.

MITOCHONDRIAL PROTEIN IMPORT has been studied intensively for the past two decades and, therefore, it was assumed that the basic mechanisms of the import machinery were known. However, new components that play a role in the import of integral proteins of the inner membrane have now been discovered in the mitochondrial intermembrane space.

Import of cytoplasmically synthesized precursors starts with their binding to chaperones in the cytoplasm, followed by binding to import receptors on the mitochondrial surface (Fig. 1)[1–3]. The precursors then pass through the translocase of the outer membrane (the TOM complex)[4]. The TOM complex consists of the receptors Tom20p, -22p, -37p and -70p (which have overlapping roles in recognizing mitochondrial precursors), the pore-forming component Tom40p and the three small Tom proteins, Tom5p, -6p and -7p. After passing through the TOM complex, the precursor engages the translocase of the inner membrane (the TIM complex), in which Tim17p and Tim23p form a pore. ATP hydrolysis by a translocation motor, composed of Tim44p, mitochondrial heat shock protein 70 (mhsp70) and the nucleotide exchange factor mitochondrial GrpE (Mge1p), drives the translocation process to completion. An arrested translocation intermediate can span both the TOM and TIM complexes simultaneously[2,5]. Finally, the targeting sequence is removed by the matrix processing peptidase and a battery of chaperones in the matrix mediates the folding of the imported protein.

It seemed reasonable to assume that mitochondrial membrane proteins follow a similar import route. Studies on protein import in the endoplasmic reticulum had suggested that protein insertion into a membrane is mechanistically identical to protein transport across a membrane except that, for insertion, further transport is prevented by a hydrophobic stop–transfer sequence in the transported protein[6]. Why should this principle not also hold for mitochondrial protein import? The proteins would follow the same import pathway as outlined above but remain in the TIM complex because of the stop–transfer sequence, and then escape laterally into the lipid bilayer of the inner membrane. Because the 'matrix import' pathway involves temporary linking of the TOM and TIM complexes, there was no need to explain how a hydrophobic inner-membrane protein would negotiate the aqueous intermembrane space. Indeed, such an explanation would have been difficult, given that all efforts to find conventional chaperones in the intermembrane space had failed. The import pathway of the inner-membrane ADP/ATP carrier (AAC) had been characterized biochemically more than ten years ago[7] but only in the past two years have some of the key components of this pathway been defined. The newly discovered components are located in the intermembrane space and the inner membrane.

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A protein import machinery in the intermembrane space

The important role of the intermembrane space in mitochondrial protein import came to light in an unrelated study. Schweyen and colleagues identified two essential proteins of similar sequence, mitochondrial RNA splicing protein 5 (Mrs5p) and Mrs11p, of which overexpression suppressed respiratory defects caused by the loss of mitochondrial RNA splicing (Fig. 2)[8,9]. Both proteins were located in the intermembrane space. Because most essential mitochondrial proteins participate in protein import[10], Mrs5p and Mrs11p were attractive candidates for novel components of a protein import system. Indeed, inactivation of these small proteins, either through the use of temperature-sensitive versions[11] or by downregulation of the wild-type proteins[12], resulted in mitochondria that were depleted specifically of several integral inner-membrane proteins, including AAC, the inorganic phosphate carrier (P_iC) and the dicarboxylate carrier (D_iC). The two proteins proved to belong to a second TIM system that specializes in the transport of hydrophobic proteins into the inner membrane. Accordingly, Mrs5p and Mrs11p were renamed Tim10p and Tim12p.

Early evidence for a second TIM system

Sirrenberg *et al.*[13] caught the first glimpse of a new import pathway two years ago when they identified Tim22p, an essential 22 kDa inner-membrane protein, on the basis of its similarity to Tim23p. Downregulation of the intracellular level of Tim22p led to a loss of AAC and P_iC from the mitochondrial inner membrane. Although the new protein seemed to participate in mitochondrial import, it was not part of the well-characterized Tim17p–Tim23p complex. Rather, Tim22p was recovered from detergent-solubilized mitochondria in a separate high-molecular-weight complex[13].

Tim54p, the first partner of Tim22p, was identified by Kerscher *et al.*[14] Inactivation of Tim54p in a temperature-sensitive *tim54* mutant inhibited import of AAC into isolated mitochondria, and Tim54p interacted genetically and physically with Tim22p (Ref. 14).

These discoveries demonstrated that import of AAC and other inner-membrane carriers requires a complex comprising

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Tim22p and Tim54p. It was also suggested that carrier import bypasses the Tim17p–Tim23p system of the general protein import pathway[13]. Three pieces of evidence showed directly that Tim23p is not required for import of AAC. First, saturation of the Tim17p–Tim23p complex with an arrested precursor did not significantly inhibit AAC import[15]. Second, inactivation of Tim23p in a temperature-sensitive *tim23* yeast mutant did not block import of AAC but did block that of proteins imported by the matrix import pathway[14,15]. Finally, whereas precursors with an N-terminal targeting sequence bound to the intermembrane space domain of Tim23p, AAC did not[16]. Interaction of the presequence with Tim23p presumably allows docking of the TOM complex in the outer membrane to the TIM complex in the inner membrane[17]. The absence of an interaction between AAC and Tim23p raised the possibility that transfer of AAC from the TOM complex to the inner membrane necessitated either some other linker molecules or transport of the hydrophobic AAC precursor across the aqueous intermembrane space.

Different roles of Tim10p and Tim12p

The functions of Tim10p and Tim12p were deduced by two slightly different approaches that yielded similar conclusions[11,12]. Fractionation of yeast mitochondria showed that most of Tim10p was located in the soluble intermembrane space, whereas Tim12p was peripherally bound to the outer surface of the inner membrane. Both proteins could be crosslinked chemically to a partly imported AAC precursor, which indicates that they interact directly with the imported protein. However, the different intramitochondrial locations of Tim10p and Tim12p reflect their different functions in the import pathway. Inactivation or depletion of Tim12p did not interfere with import of AAC into the intermembrane space but prevented insertion of AAC into the inner membrane. In contrast, inactivation or depletion of Tim10p blocked import of AAC, P_iC and Tim22 across the outer membrane. Thus, Tim10p functions before Tim12p, probably by binding the incoming precursor as it emerges from the TOM complex.

A 300 kDa 'insertion complex' in the inner membrane

Partner proteins (i.e. interacting proteins or components that are in the same complex) for Tim10p and Tim12p were found by genetic and biochemical approaches[12,18]. A multicopy suppressor screen with the temperature-sensitive Tim12p mutant identified Tim22p (Ref. 11), the very same protein that had already been implicated in the import of AAC (Ref. 13). The genetic interaction between Tim12p and Tim22p corresponded with a physical interaction as shown by co-immunoprecipitation[12] and affinity purification with hexahistidine-tagged Tim10p (Ref. 11). Further analysis showed that the majority of Tim10p was present as a 70 kDa soluble complex in the intermembrane space and a minor fraction was found as a 300 kDa membrane-bound complex, together with Tim12p, Tim54p and Tim22p (Refs 18,19). This membrane complex is thus a distinct TIM complex specialized in the insertion of imported proteins into the inner membrane. The exact composition of the 300 kDa 'insertion complex' is not yet known.

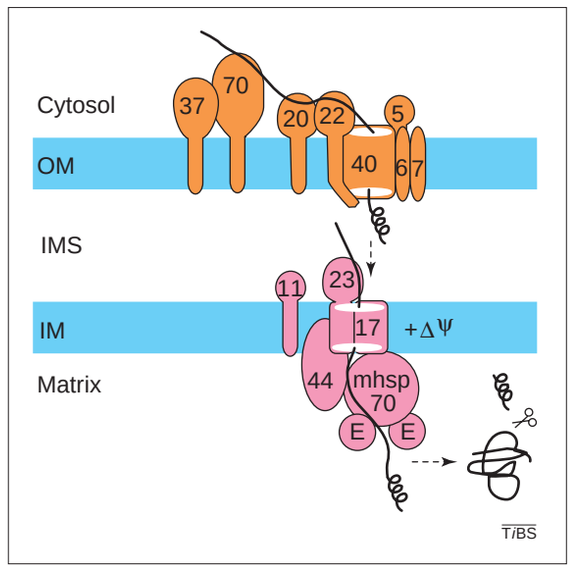

Figure 1

Import of proteins across the inner membrane into the matrix. This pathway is mediated by the Tim17p–Tim23p complex (and adjacent Tim11p), a membrane potential ($\Delta\psi$) and an associated, ATP-driven protein transport motor on the inner face of the inner membrane. The latter complex comprises Tim44p, mitochondrial heat shock protein 70 (mhsp70) and the nucleotide exchange factor mitochondrial GrpE (indicated as E). As the precursor with an N-terminal, basic, matrix-targeting signal (helical line) emerges from the translocase of the outer membrane (TOM) complex (which consists of the receptors Tom20p, -22p, -37p and -70p, the pore-forming component Tom40p and three small Tom proteins, Tom5p, -6p and -7p), it binds to an acidic Tim23p domain in the intermembrane space and thereby induces transient docking of the TOM- and the Tim17p–Tim23p system. A consequence of docking is that the precursor is not released into the intermembrane space. In the matrix, the matrix-processing protease (scissors) removes the matrix-targeting sequence and a battery of chaperones might aid in the folding process to generate the mature protein. IM, inner membrane; IMS, intermembrane space; OM, outer membrane.

Two 70 kDa TIM complexes in the soluble intermembrane space

A partner protein for Tim10p was found as a spontaneous, extragenic suppressor of the temperature-sensitive *tim10* mutant[18] and, in a separate study, by co-immunoprecipitation[19]. The yeast genome encodes three small proteins that resemble Tim10p and Tim12p (Fig. 2). Tim9p is closely related to Tim10p and Tim12p and is, like those two proteins, essential for viability. Tim9p is located primarily in the mitochondrial intermembrane space as a soluble 70 kDa complex that contains approximately equimolar amounts of the Tim9p and Tim10p (Refs 18,19); the rest is present in the 300 kDa insertion complex. A single Ser→Cys mutation in Tim9p allowed the protein to suppress the temperature-sensitive mutation in Tim10p (Ref. 18).

The other two yeast proteins related to Tim10p and Tim12p, Tim8p and Tim13p (Refs 20,21), were found in the intermembrane space as part of a distinct 70 kDa complex that could be separated from the Tim9p–Tim10p complex by ion-exchange chromatography[20]. Co-immunoprecipitation and purification experiments suggested that the Tim8p–Tim13p complex also contains loosely associated Tim9p. Deletion of Tim8p or Tim13p, alone or in combination, had no notable effect on cell growth and did not significantly affect import of AAC or P_iC into isolated mitochondria. However, deletion of Tim8p in combination with a temperature-sensitive Tim10p mutation was lethal[20]. This genetic interaction suggests a functional interaction between the two 70 kDa complexes.

The nature of this interaction was revealed by measuring the import of a broader spectrum of integral inner-membrane proteins in strains that lack Tim8p or Tim13p (Ref. 22). Tim23p and Tim11p were imported less efficiently into Tim8p-deficient mitochondria,

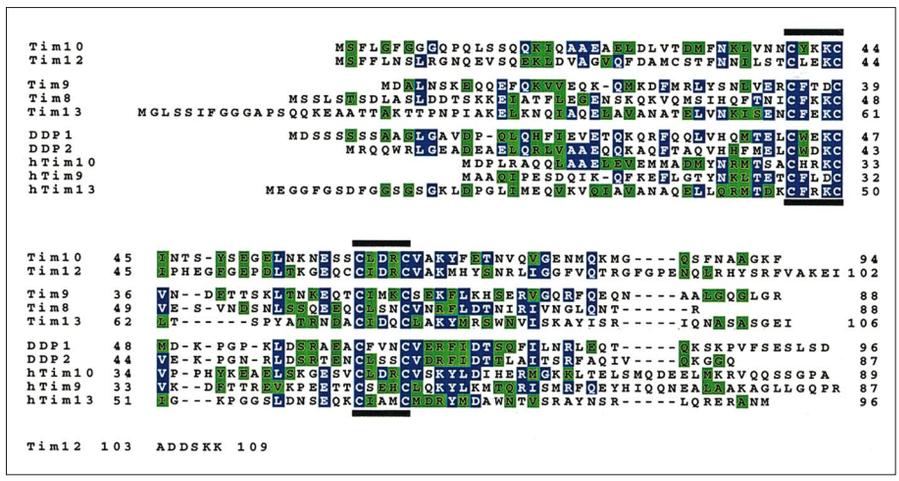

Figure 2

Proteins related to Tim10p of the small Tim (translocase of the inner membrane) protein family in *Saccharomyces cerevisiae* and humans (h). Black bars mark the 'twin Cx_3C' motif. Identical residues are blue, similar ones are green (0.4 was set as the minimum for identity and similarity, using the program Boxshade at the European Molecular Biology net, http://www.embnet.ch).

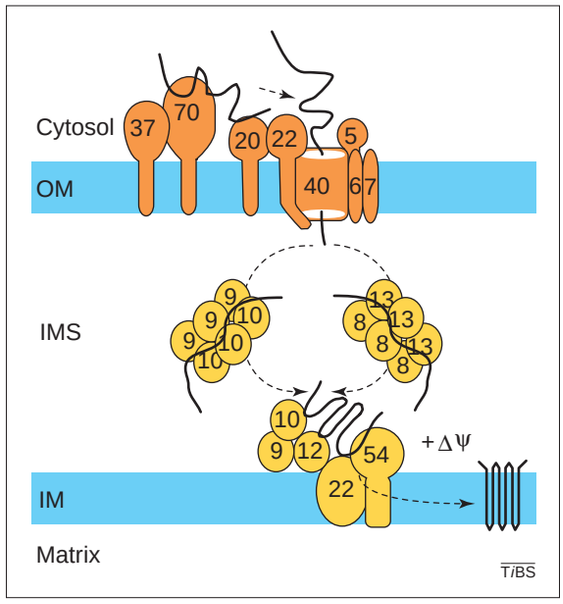

Figure 3

Import of proteins into the mitochondrial inner membrane (IM). As the precursor emerges from the translocase of the outer membrane (TOM) complex (orange), it binds to the Tim9p–Tim10p or Tim8p–Tim13p complex (both in yellow) of the intermembrane space (IMS). The bound precursor is usually delivered to an insertion complex (yellow) composed of Tim9p, Tim10p, Tim12p, Tim22p and Tim54p that catalyzes the membrane potential ($\Delta\psi$)-dependent insertion of the precursor into the inner membrane (IM)[11,22]. An alternative model (not shown here) proposes that the Tim9–Tim10 complex receives the precursor directly from the TOM complex and passes it to the Tim22p–Tim54p complex through formation of a translocation contact site[12,25]. Abbreviation: OM, outer membrane.

which suggests that their import is aided by the Tim8p–Tim13p complex. Indeed, a Tim23p translocation intermediate could be crosslinked to the Tim8p–Tim13p complex. Because Tim23p is an essential protein, Tim8p and Tim13p are therefore also expected to be essential. However, the residual import of Tim23p in the absence of Tim8p and Tim13p seems to be sufficient to keep yeast cells alive[23]. Thus, the Tim8p–Tim13p complex works in parallel with the Tim9p–Tim10p complex by mediating the import of a subset of integral inner-membrane proteins.

Import models

Upon translocation across the TOM complex[24], the specific route taken by a substrate to reach the inner membrane is still uncertain. One possibility is that the small Tim complexes act as chaperone-like molecules to guide the precursor across the aqueous intermembrane space, yielding a soluble intermediate in which the precursor is bound to the 70 kDa complexes in the intermembrane space (Fig. 3). This model is supported by import studies with temperature-sensitive *tim10* and *tim12* mutants and by the fact that an AAC translocation intermediate bound to Tim10p in intact mitochondria is protected from added protease[11,18]. It predicts a transient complex in which Tim9p–Tim10p or Tim8p–Tim13p is bound directly to the precursor. Equally plausible is a model in which the 70 kDa complexes form a link between the TOM and the TIM complexes. In this model, the precursor is not released into the intermembrane space but binds to the small Tim proteins as it emerges from the TOM complex. Further transfer to the Tim22p–Tim54p complex could then occur without release into the intermembrane space. This model is supported by the recent finding that an AAC translocation intermediate is partially degraded by added protease[25]. It predicts a transient complex in which the TOM complex as well as the small Tim proteins are bound to the precursor.

Both models raise intriguing questions. What is the mechanism by which the precursor is handed from one complex to another? Do the two 70 kDa complexes select the imported substrates as they emerge from the TOM complex? Is the transfer of the precursor between the different Tim proteins mediated by conformational changes of those proteins?

The import pathway could be even simpler than shown in Fig. 3. It is possible that the small Tim proteins are not essential for the imported substrates to reach the inner membrane but they might only facilitate the process. This possibility is supported by the finding that Tim8p and Tim13p are not essential for viability even though they mediate the import of an essential protein[22]. It is conceivable that the intermembrane space has a battery of chaperones with overlapping function (like the chaperones in the cytosol) to facilitate import across the aqueous compartment. The essential function of Tim9p and Tim10p might lie at the inner membrane rather than in the intermembrane space. If so, one might expect to find even more chaperone-like proteins in the intermembrane space.

How do the 70 kDa TIM complexes recognize their substrate proteins? The amino acid sequences of the small Tim proteins are 25% identical and 50% similar to each other. They also share a 'twin Cx_3C' motif, in which two cysteine residues are separated by three amino acids and each Cx_3C sequence is separated from its twin by 11–16 amino acids[20]. This motif is reminiscent of a canonical zinc finger but with a longer spacer[26]. Recombinant Tim10p- and Tim12p-fusion proteins bind zinc and interaction between Tim10p and AAC is inhibited by zinc chelators[12]. This

suggests that the small Tim proteins bind zinc and that zinc binding is required for their function *in vivo*. A role for zinc in chaperone function has already been suggested because the molecular chaperones DnaJ and Hsp33 each possess a zinc-finger-like domain. That of DnaJ is required for recognition and binding of denatured proteins[27], whereas the chaperone function of Hsp33 is induced by the release of zinc from the zinc-finger-like domain[28]. The zinc-binding regions of Tim10p and Tim12p might be structurally important, either for formation of the corresponding oligomeric complexes or for the interaction with substrate proteins. The zinc-binding motif of Tim10p and Tim12p could interact with those extramembrane loops of AAC that appear to contain mitochondrial targeting information[12,25]. An analogous study with Tim23p also revealed that positively charged residues in the extramembrane loops are required to mediate insertion into the inner membrane[29].

Although it is possible that the small Tim proteins recognize specific sequence motifs[12,25], it is equally plausible that they simply recognize unfolded proteins. The latter hypothesis is supported by the finding that the Tim9p–Tim10p complex mediates the import of Coq2p, Tim11p and Tim17p, all of which lack sequence similarity to the metabolite transporters[22]. In this context, it could also be relevant that the import receptor Tom70p binds to several different regions of the P_iC (Ref. 30).

Biogenesis of this import machinery itself is complicated because individual subunits use different pathways[21]. Tim54p is imported via Tim9p–Tim10p (Ref. 22) and inserted into the inner membrane through the Tim17p–Tim23p machinery[21], whereas Tim22p is imported via the Tim22p–Tim54p complex[11,19,21]. Import of the small Tim proteins bypasses the Tim machinery altogether: it requires Tom5p but no membrane potential[21]. The complex interplay between the different machineries might ensure coordinate regulation of the assembly of the mitochondrial-protein import systems.

Defective protein import: a novel type of mitochondrial disease

Humans contain at least five homologs of the small Tim proteins found in the yeast mitochondrial intermembrane space. One of these homologs had already been termed deafness-dystonia peptide (DDP) because its loss results in the severe X-linked Mohr–Tranebjaerg syndrome, which is characterized by deafness, dystonia, muscle weakness, dementia and blindness[31,32]. The other four homologs are encoded autosomally. Because one of these four homologs is closely related to DDP, the initially discovered isoprotein, DDP, is referred to as DDP1, and the second one as DDP2 (Ref. 20). The remaining three human homologs are named hTim9, hTim10 and hTim13 because of their sequence relationship to the corresponding yeast proteins.

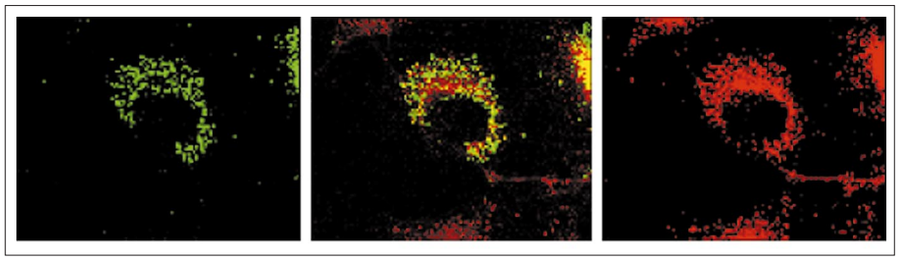

Figure 4

Human deafness dystonia peptide 1 (DDP1) expressed in monkey cells localizes to mitochondria. DDP1, tagged at its C-terminus with a hemagglutinin epitope, was expressed transiently under the control of the cytomegalovirus promoter in cultured COS7 cells. The epitope tag was visualized by indirect immunofluorescence (left panel) and mitochondria were localized by the mitochondria-specific, red fluorescent dye Mitotracker® (right panel). The middle panel shows a superposition of the two side panels. Adapted from Koehler *et al.*[20]

DDP1 is most similar to yeast Tim8p (Fig. 2) and, when expressed in monkey (Fig. 4) or yeast cells, is located in mitochondria. Mohr–Tranebjaerg syndrome is, therefore, almost certainly a new type of mitochondrial disease caused by a defective protein import system of mitochondria. Loss of DDP1 function probably lowers the mitochondrial abundance of some inner-membrane proteins that are crucial for the function, development or maintenance of the sensorineural and muscular systems in mammals. The findings from yeast suggest that DDP1 functions as a complex with related partner proteins. On the basis of sequence homology and isoelectric point, hTim13 might be a partner of DDP1 or DDP2. As mutations in DDP1 partner proteins could also be deleterious and because all potential partner proteins are encoded autosomally, non-X-linked diseases with symptoms resembling those of Mohr–Tranebjaerg syndrome might well have a related etiology. Further, the link between a mitochondrial import defect and a neurodegenerative disease could provide insights into the molecular basis of other, more frequent neurological diseases, such as parkinsonism, that have been correlated with mitochondrial dysfunction.

Concluding remarks

In the past two years, new proteins in the intermembrane space and inner membrane have been identified that mediate the import and insertion of inner-membrane proteins. Possible homologs of the small Tim proteins are encoded in many eukaryotic genomes, including those of plants, nematodes, mammals, fungi, plasmodia and ciliates, which suggests that these proteins are ubiquitous in eukaryotes. In plants, they are probably restricted to mitochondria. Although the complete genome sequence of a plant is not yet available, it appears that the number of possible homologs encoded in the *Arabidopsis* genome, so far, is not significantly higher than that encoded in mammalian genomes. This would not be expected if the proteins functioned also in the plastid. Furthermore, all the identified *Arabidopsis* homologs are as closely related to the corresponding yeast proteins as to the human and *Caenorhabditis elegans* proteins, which, again, argues against divergence to a plastid pathway.

Why does overexpression of Tim10p or Tim12p suppress defects in the splicing of mitochondrial RNA? These defects are also suppressed by overexpression of Mrs3p and Mrs4p, two mitochondrial metabolite carriers of unknown substrate specificity[33]. Increasing the amounts of Tim10p and Tim12p might raise the levels of Mrs3p and Mrs4p in the mitochondrial inner membrane and thereby enhance the transport of a crucial cofactor, such as a metal ion that is required to attenuate or bypass the splicing defect.

This protein import pathway still poses many intriguing questions. One of them concerns the interaction between the two 70 kDa complexes and the 300 kDa inner-membrane complex (Fig. 3). Another unresolved question is the role

of the 300 kDa insertion complex. Is this complex a scaffold that facilitates proper folding of the incoming membrane protein before inserting it *en bloc* into the inner membrane? Or is the complex a pore through which a multispanning membrane protein is 'stitched' into the inner membrane? Molecular dissection of this import pathway will provide further insights into the mechanism by which proteins are inserted into a membrane.

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Progress in protrusion: the tell-tale scar

Tatyana M. Svitkina and Gary G. Borisy

The crawling movement of a cell involves protrusion of its leading edge, in coordination with the translocation of its cell body, and depends upon a cytoplasmic machinery able to respond to signals from the environment. Protrusion is now understood to be driven by actin polymerization, and signalling from membrane receptors to actin has been shown to be mediated by the Rho family of GTPases. However, a major gap in our understanding of regulated motility has been how to connect the signalling pathway to the motile machinery itself. Recent structural, biochemical and genetic studies have identified some of the missing links and provided a strong working model for the pathways and mechanisms by which the signals are interpreted and implemented.

TWO MAJOR PROTRUSIVE organelles exist in crawling cells: lamellipodia, which contain a network of diagonally oriented actin filaments, and filopodia, which contain a tight bundle of parallel actin filaments, the filaments invariably oriented with their barbed ends forward. Models that have been proposed to account for polymerization-driven protrusion of lamellipodia involve the nucleation of actin filaments at the membrane and their subsequent release[1], or the continuous treadmilling of actin filaments by growth at their barbed end and shortening at their pointed end[2].

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Recent electron microscopic studies have revealed new details of the supramolecular organization of actin filaments in lamellipodia[3,4]. The leading edge is characterized by a distinctive, extensively branched network of actin filaments. Consistent with previous models for protrusion, barbed ends were numerous near the leading edge but, surprisingly, free pointed ends were not detectable. Instead, pointed ends were involved in structural association with the sides of other filaments at ~70° angles, resulting in Y-junctions and the formation of a diagonal network filling the lamellipodium (Fig. 1a). These structural studies suggested that lamellipodial protrusion needed to be re-interpreted in terms of the formation and recycling of a branched filament network.

Arp2/3 complex

A key component in understanding the origin of the branched actin-filament network is the Arp2/3 complex (reviewed in Refs 5,6). This consists of actin-related proteins 2 and 3 and five other proteins[7–10], it localizes to the leading edge of crawling cells[7–9,11] and to cortical actin patches in yeast[10], and it is sufficient to induce actin polymerization at the surface of Listeria cells[12]. The Arp2/3 complex binds to the sides of actin filaments and to their pointed ends[8] and nucleates actin filaments,

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