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REVIEW

Chaperna: linking the ancient RNA and protein worlds

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ABSTRACT

As a mental framework for the transition of self-replicating biological forms, the RNA world concept stipulates a dual function of RNAs as genetic substance and catalyst. The chaperoning function is found intrinsic to ribozymes involved in protein synthesis and tRNA maturation, enriching the primordial RNA world with proteins of biological relevance. The ribozyme-resident protein folding activity, even before the advent of protein-based molecular chaperone, must have expedited the transition of the RNA world into the present protein theatre.

Introduction

Most of metabolic catalysis is carried out by proteins in all kingdoms of life. The biological functions of proteins are governed by their three-dimensional conformations. Molecular chaperones are involved both in facilitating protein folding and preventing protein aggregation, which would otherwise lead to cytotoxic consequences [1–3]. Conventionally, these pivotal functions have been considered as unique properties of chaperones and chaperonins, which are themselves proteins.
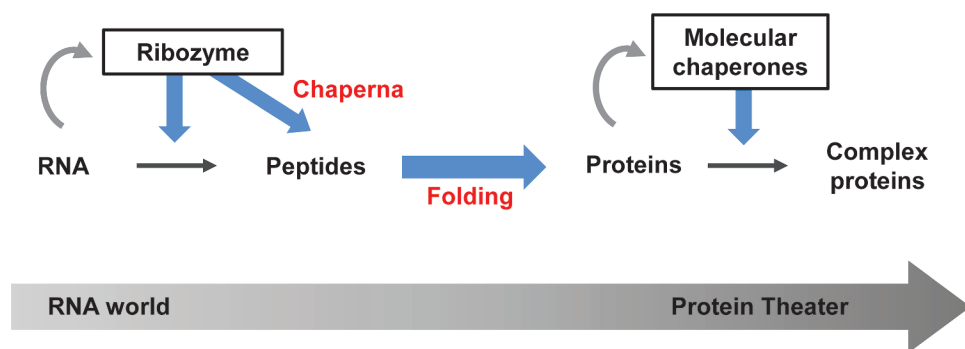
The RNA world concept of the molecular evolution of life forms is based on the dual function of RNAs as genetic materials and catalysts [4,5]. Several fields have contributed to assembling a full picture of the RNA world – better defining early earth environments, prebiotic chemical synthesis under simulated conditions, finding functional ribozymes, refining evolutionary theory and modelling, and artificial protocells [6]. Recent reports suggest that RNA molecules also have a role in assisting protein folding and preventing protein aggregation as molecular chaperones, and are extremely efficient for the folding of a variety of proteins both *in vivo* and *in vitro* [7–12]. The chaperoning activity also arises in ribozymes, which are responsible for the catalytic turnover of RNA or peptide substrates, and also involved in facilitating the folding of variety of client proteins into biologically relevant conformations [8–11,13]. It is likely that the activity of RNA-based chaperones, newly coined as Chaperna (or the more lengthy-term chaperone RNA) [14], greatly facilitated the folding of early proteins, even before the availability of the protein-based chaperones, expediting the transition of the RNA World into the current protein theatre. One note on terminology before proceeding: 'Chaperna' may reduce

potential confusion between 'RNA chaperone'- a protein that assists the folding of interacting RNA, and 'chaperone RNA' – an RNA that helps folding of proteins.

RNA-protein world view and Chaperna

The RNA world concept posits that in primitive Earth's history, RNA served as the primary life substance [4,5]. A life form requires at least two elements: a genetic material as the blueprint, and a catalyst to multiply the genetic material into progeny. RNA molecules exist as a polymer of four distinct subunits, and as various conformations, to serve both roles. A dual function as genetic substance and catalyst was considered as necessary attributes of a self-replicating biological form, although there may never be direct physical evidence of an RNA-based organism. Although the 'RNA-first' view of the origin of life should be given with caution, and must consider other types of self-replicating substances preceding the RNA molecules [15], it presently serves an intellectual framework for understanding molecular evolution of life forms. Molecular biological approaches have uncovered artefacts of this ancestral era in laboratories [16].

The RNA world concept posits a canonical view that catalysis proceeded from ribozyme to RNP-enzyme to protein-based enzyme. Although catalytic functions are predominantly carried out by protein enzymes rather than RNA enzymes, RNA enzymes play a pivotal role in cellular system, despite being in the minority. Among the four most outstanding processes in information transfer systems, DNA replication and transcription are governed by purely protein enzymes, whereas the other two – mRNA splicing and protein synthesis – are catalysed by ribozymes. Furthermore, RNAs

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Figure 1. Extended RNA world view. Upon RNA world view, the catalytic functions of RNAs (ribozymes), could expedite to shift primordial RNA-based machinery to protein-based theatre. In this review, we suggest the RNA world view more extended, that is; RNAs serve not only as catalysts, but also play a role in protein stability and folding, fulfiling solely or harmonizing with molecular chaperones.

are crucially involved in RNA processing events [17], viral replication of RNA genomes [18,19], and peptide-bond formation in ribosome-assisted protein synthesis in all living organisms [20,21]. These persistent and pivotal contributions of the RNA-based catalysts in modern organism suggest that the RNA world is not extinct, but extant in the present protein theatre, as an RNA-protein world [22,23]. In the RNA world of contemporary biology, RNAs more often act in concert with proteins [22,24].

A crucial interdependence between RNA and protein could suggest a mutual transfer of energy or information [25]. When RNA interacts with a protein, RNA could contribute to or at least have an effect on how the bound protein folds. In cases where RNA is able to interact with multiple proteins, the RNA could affect the stability and/or folding of many proteins. Thus, the RNA-mediated chaperone effect would be important especially for the function of early proteins, in the absence of sophisticated molecular chaperones enjoyed by contemporary life forms. It is interesting to ask what factors, in the primordial RNA world, could provide chaperoning function to the early peptides/proteins produced by peptide-bond forming ribozymes. The simplest answer would be that early proteins were small, simple, and able to fold independently of chaperones. Alternatively, it is tempting to speculate that between a ribozyme and peptide as a product of the enzyme reaction, the ribozyme could also serve a chaperoning function to its own product (Fig. 1). Thus, it is a natural corollary that ribozymes possibly have an intrinsic moonlighting activity like as a molecular chaperone. Surprisingly, however, the presence and function of RNA-based chaperones are still largely veiled.

New discoveries

Dual function of RNA as ribozyme and Chaperna

Ribonuclease (RNase) P claims a special place among many RNA-based catalysts. As one of the first example of RNA-catalysed reactions, the enzyme is responsible for endonucleolytic cleavage of tRNA precursors to mature tRNA molecules [26]. RNase P is present in all three domains of life, strongly suggesting that, as an ancient enzyme that was present in the last common universal ancestor, it is a remnant of the prebiotic RNA world [27–29]. RNase P remains a universal RNA-based enzyme, only with a potential exception in endosymbiont organelles where protein-based enzyme is found [30,31]. The RNase P holoenzyme is a ribonucleoprotein complex: for instance, the RNase P complex in *E. coli* is composed of M1 RNA, which functions as a true catalyst, and C5 protein that supports the catalytic efficiency and tRNA substrate specificity [17,26].

Recent study unveiled a new function of the RNA component of RNase P in assisting protein folding, in addition to its well-known ribozyme activity [8]. The M1 RNA crucially provided a chaperoning function to the folding of its partner C5 protein *in vivo* and *in vitro*. It could be postulated that the nascent polypeptide of C5 protein, of the ribosome, interacts with M1 RNA, and folds into relevant conformation into RNP complex. Interestingly, M1 RNA also provided a quality control for the C5 protein; if C5 protein goes aberrant by mutations, M1 RNA facilitates its degradation with expedited clearance from the cytoplasm. Thus, from cradle to deathbed, M1 RNA is crucial for the fate of C5 protein. The activity is reminiscent of protein-based molecular chaperones that provide both folding and proteostasis to the client proteins [32].

Although intrinsic enzymatic activity resides in the RNA subunit of RNase P, the protein subunit is known to increase the catalytic efficiency and the substrate specificity to the pool of pre-tRNA substrates [33–36]. It should be recalled that tRNAs, as adaptor molecules for translating genetic information encoded in mRNA into amino acids, are an integral part of ribosome-based protein synthesis. tRNA molecules, specific for each triplet codon in mRNA, are essential for the quality of protein synthesis and the whole protein theatre. The RNA subunit of RNase P enables itself to generate properly folded RNase P holoenzyme efficiently by providing chaperoning function, further facilitating the provision of efficient machinery for the pool of mature tRNAs. Correct creation of tRNAs was likely an important early bridge point between the RNA and protein worlds, which enabled adaptor-mediated protein synthesis (Fig. 2A).

23S rRNA as peptidyl transferase and Chaperna

According to the RNA world view, ribozymes that once dominated a primitive metabolism were gradually supplanted by more efficient protein-based enzymes. It is
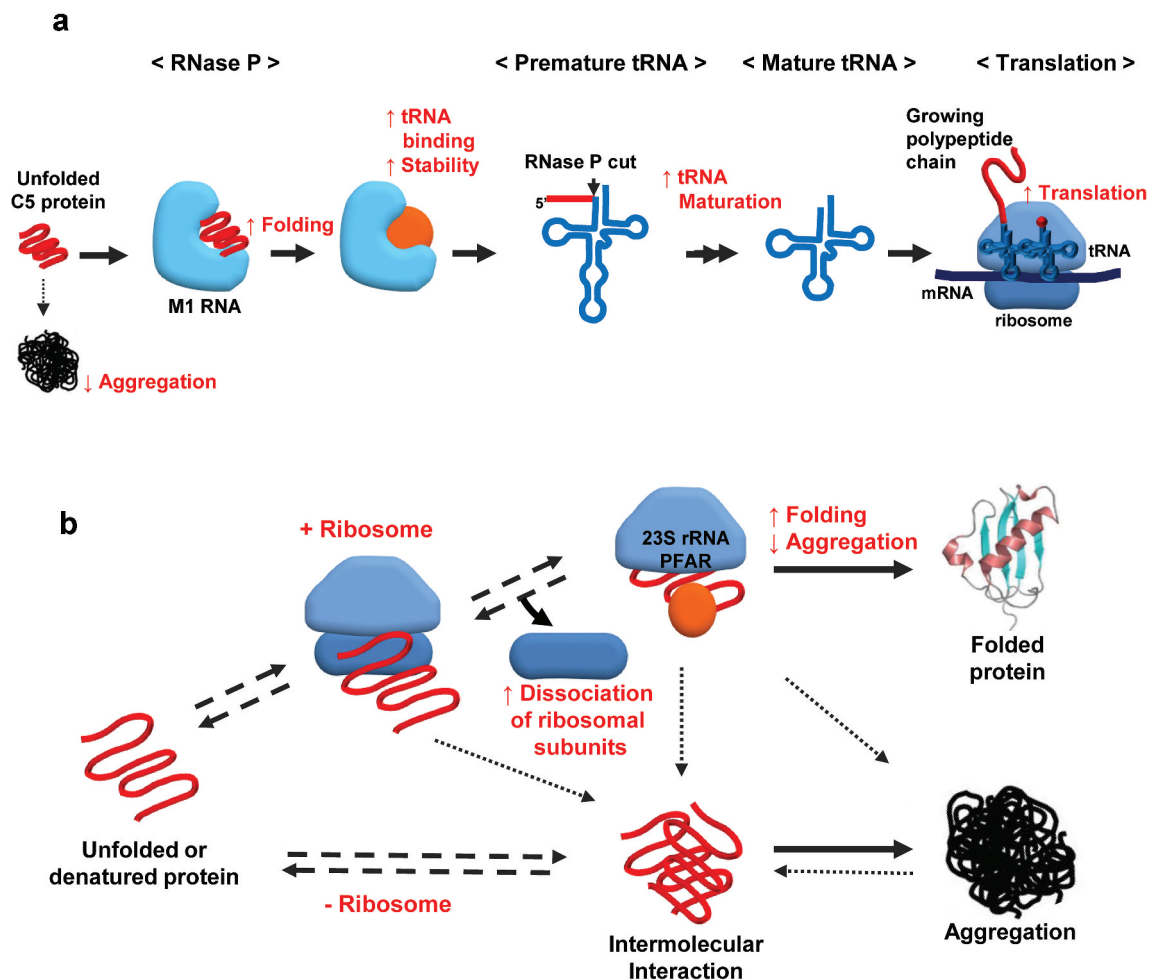
Figure 2. Moonlight Chaperna function of RNAs in adaptor-mediated protein synthesis. (A) Dual function of RNase P as prototype ribozyme and Chaperna. In addition to previously known catalytic function, M1 RNA (RNA component of RNase P), facilitates the folding of its cognate protein, C5 protein. The folding of C5 protein enhances the overall stability of RNase P complex, binding ability to tRNA, and consequently tRNA maturation, further stimulating translational efficiency. (B) Schematic view of the function of 23S rRNA as peptidyl transferase and Chaperna. As a holdase, 23S rRNA prevents misfolding of *de novo* synthesized polypeptides. The chaperoning role of 23S rRNA subunit of ribosome -the protein folding activity of ribosome (PFAR)- lends credence to the intrinsic function of ribozymes as Chaperna, which expedited the transition from RNA-based world into protein-based (and/or RNA and protein-based) world.

therefore intriguing that the catalyst responsible for the synthesis of virtually all proteins is a ribozyme. This remains a universal feature in the translation procedure, only with a few exceptions in specialized peptide antibiotics that are synthesized by protein enzymes [37,38]. One of the main functions of the ribosome in translation is peptidyl transfer for the extension of peptides. Although other ribosomal functions, including aminoacyl-tRNA selection and translocation, merit attention from mechanical point of view, peptide-bond formation by a ribozyme is the sole chemical event that moves amino acids into the protein world.

Various biochemical analyses as well as the crystal structure of the ribosome and its subunits unambiguously placed 23S rRNA as the enzyme responsible for the peptide-bond formation [39–41]. The domain V of the rRNA of the large subunit of the ribosome (23S for bacteria, 25S for yeast, and 28S for mammals) was identified as the domain bearing the peptidyl-transferase centre (PTC) responsible for the peptidyl-transferase activity [39]. The universally conserved adenine 2451, for *E. coli* numbering,

in domain V of 23S rRNA has been known to have the central role in the catalysis of peptidyl transfer [21,42].

Perhaps eclipsed by the canonical function of ribosome in protein synthesis, the protein folding activity of ribosome (PFAR) has not been given full attention until recently. Initially identified two decades ago in bacterial ribosomes *in vitro* [43], the activity was shown to be conserved among bacteria, eubacteria, eukaryotes, and even mitochondria [44]. The biological relevance of PFAR with protein synthesis remained controversial but could be deduced from various biochemical observations. First, the folding activity is inherent to the rRNA of the large subunit of the ribosome, and more specifically, to domain V where universal peptidyl-transferase activity resides [45]. And yet, the nucleotides involved in PTC and PFAR do not physically overlap within domain V, allowing the execution of distinct biological activities [45]. Second, the nucleotides for PFAR are placed at the interface of the small and large subunits [46]. Although the whole ribosome as a complex of small and large subunits is engaged in protein synthesis, the dissociation of ribosomal subunits is triggered by unfolded polypeptides, rendering the

PFAR centre physically accessible to the guest proteins for folding (Fig. 2B) [44,47]. Thus, PFAR and the protein synthesis are choreographed to be independent but synergistic to each other as an integral ribosome function; when PTC is engaged for elongation of peptide bonds, PFAR is silenced, but PFAR becomes operational only when the peptide synthesis is halted. Finally, anti-prion compounds were found to be potent PFAR inhibitors [48,49], highlighting the close link between the ribosome-resident protein folding activity and the proteostasis and quality control of cellular proteins, which, if aberrant, results in protein-misfolding disease consequences [13,50,51].

Accumulating biochemical evidence suggests that the moonlighting activities in protein folding should be in the limelight as an integral function of the ribosome [52]. The dual function of ribozyme as peptidyl-transferase and Chaperna for protein folding could be a vestige of the ancient RNA world, but powerfully in operation in all life forms (Fig. 2B). Conceivably, before the advent of protein-based chaperones, the RNA-based catalysts for peptide-bond formation could also serve as protein-based chaperones for the folding of their own peptide products. This property could have expedited the transition from the RNA into protein-based cellular machineries.

Protein-based Chaperones-Chaperna network

Similarities and mechanical distinctions

Protein-based chaperones have evolved to fold a variety of cellular proteins differing in primary, secondary and tertiary structure by recognizing and binding to folding intermediates. How chaperone proteins recognize client proteins is still being discovered. Based on obvious structural characteristics of all unfolded proteins, it is a common belief that the exposed hydrophobic residues must be shielded from water molecules by interaction with chaperone proteins [32].

Currently, there are two types of protein-based chaperones classified: the 'holdase' type of chaperone proteins that function without ATP, and the 'foldase' type of chaperone proteins that utilize ATP for recycling chaperone proteins from cargo proteins [53–55]. Small heat shock proteins (sHsps), for example, are ATP-independent holdases, which help prevent protein aggregation, maintain proteins in a folding-competent state for eventual refolding by foldase machinery, or direct them for degradation by proteosomal or autophagy pathways. It should be noted that sHsps can exist in two states, with a low (monomeric structures) and high (oligomeric structures) affinity, respectively. In response to heat shock, the structures of sHsps shifts strongly towards oligomeric structures, thereby allowing them to more efficiently interact with target proteins and to form a stable complex, preventing irreversible aggregation and re-solubilizing proteins. Of note, electrostatic interactions have been suggested to be an important component of protein-based chaperone function [56]. Similarly, polyanions such as RNA, DNA and polyphosphates can serve as effective ATP-independent chaperones [12,57], and in the case of both DNA and polyphosphate, oligomerization appears to be an important component of their

chaperone activity [58,59]. Thus, Chaperna is expected to function as 'holdase', rather than 'foldase'.

Although the exact mechanism of RNA-dependent folding still needs to be elucidated, it could be proposed that client proteins would be initially attracted to RNAs by long-range electrostatic interactions between highly negative charges on the phosphate backbone of RNA and positive-charged residues on the client proteins. The charge-charge repulsion of RNA among RNA-protein complexes can help proteins maintain a monomeric state, encouraging proper (intramolecular) folding and preventing (intermolecular) aggregation among misfolded intermediates (Fig. 3). Then, hydrophobic interaction follows between the water-exposed non-polar side chains of client proteins and the purine/pyrimidine bases, or even the ribose ring of RNA backbone [60]. A variety of interactions of hydrophobic nature between protein and RNA are well documented in the crystal structure of RNP complexes [61]. Similar interactions may also operate in the binding of folding intermediates, shielding hydrophobic residues from water molecules. The entropic exchange at the interface of binding may drive the hydrophobic collapse leading into stable folding and subsequent release of client protein from the RNA molecule. Possibly, a folding-friendly chemical interface at the binding site obviates the need for client-specific folding instructions, allowing the clients to direct their own folding. This strategy may have preceded more elaborate ATP-dependent chaperones. Chaperna thus could represent the primordial type of chaperone before licencing ATP as universal cofactors for energy requirement [6,16,62].

Functional relationships between molecular chaperones and Chaperna

Certainly, Chaperna is not archaeologically extinct as a vestige of an ancient RNA world, but rather still extant as a powerful cellular protein folding machinery supporting adaptor-based protein synthesis [8], and the ribosome-based PFAR. It remains an exciting area of future research to study functional relationships between protein-based molecular chaperone and Chaperna. Are they complementary or even synergistic in *de novo* protein folding in cellular environment? It should be noted that the role of protein-based chaperones is rather limited in *de novo* folding of nascent proteins as evidenced by the proteome-wide analysis or *in vivo* genetic deletion studies. GroEL/ES, a major molecular chaperone in prokaryotes, is estimated to assist the folding of limited number of proteins in *E. coli* [63]. In addition, the physical depletion of GroEL via knockdown approach showed little effect on de novo protein folding in *E. coli* [64]. Consistent with the data, the deletion of either gene of DnaK or Trigger factor has little effect on protein folding [65]. In fact, GroEL is absent in the Mycoplasma [66]. Moreover, bioinformatics analyses suggest that the class of intrinsically disordered proteins (IDPs), which constitutes a significant portion of human proteomes, is probably less dependent on molecular chaperones for their folding [67]. Due to the intrinsic ability to adopt multiple conformational repertoires and interaction with multiple partners, IDPs function as hubs in signalling pathways or in transcription machineries. It remains unanswered how IDPs,
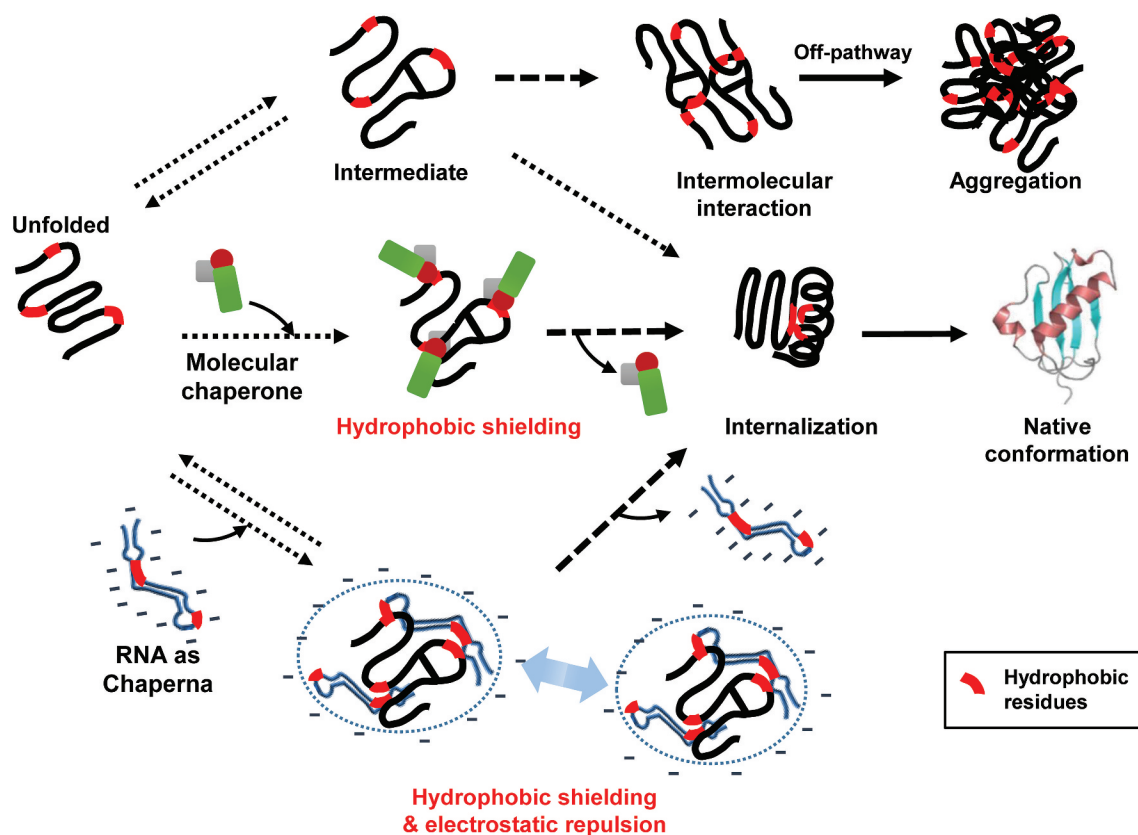
Figure 3. Overviews of the chaperoning mechanisms of RNAs function for protein folding. Unfolded proteins are prone to intermolecular interaction and consequently aggregation. However, in the presence of molecular chaperones, the hydrophobic residues of folding intermediates are shielded, preventing intramolecular interactions into misfolding and aggregation. Due to its highly negative charge of RNP complex, the charge-charge repulsion among monomers resists intermolecular interaction of folding intermediates in favour of intramolecular interactions into native conformation.

intrinsically prone to misfolding into cytotoxic consequences, maintain solubility or folding competence in the crowded cellular environment. Of note, IDPs are highly enriched in RNA-binding proteins [68–70], and mutations in defective RNA binding lead into neurodegenerative or progressive disease consequences [71,72]. It is possible that the folding and quality control of IDPs are controlled in part by RNA interactions.

It should be noted that under stress conditions, mRNAs form stress granules (SGs) or processing bodies (P-bodies), also known as liquid-like RNP granules and membraneless organelles [73,74], and have a pivotal role in guiding proteins to SGs or P-bodies [75,76]. RNA-binding also chaperones DNA-binding proteins from starved cells [77]. The multivalent interactions between RNAs and proteins have a possible role in assisting protein folding and maintaining the stability of the IDPs or proteins which have large intrinsically disordered domains (IDDs), as well as promote membraneless organelles [78,79]. Although further research is needed to elucidate the mechanism of mRNAs as Chaperna, it is now understood that cells exploit this 'RNA buffer' as a mechanism against protein aggregation under stress conditions.

Indirect evidence on potential cooperation between protein and RNA-based chaperones is given in recent *in vitro* studies [12]. Recent findings have shown that chaperones Hsp70, 110, and 60 interact with A + U rich elements (AREs) both *in vitro* and *in vivo*, and that uridine-rich RNAs can be very effective

at preventing protein aggregation [12,80–83]. ~10% of all eukaryotic mRNAs have AREs in their 3′ UTR, suggesting possible mechanisms of AREs in rapid response to stress by stabilizing and assisting protein folding by cooperating with molecular chaperones upon stress conditions [84,85]. Moreover, uridine-rich RNA, in cooperation with the DnaK chaperone, synergized in increasing the refolding efficiency [12]. In this respect, it is also worth mentioning a direct Chaperna function of cellular RNA (5S rRNA) towards DnaK folding *in vitro* [86].

Chaperna in viral infections

Chaperna also operates at the interface between some viruses and their hosts during infection. For instance, the HIV encoded TAR RNA is responsible for folding competence of HIV-1 Tat protein. The transactivator Tat protein of HIV-1 belongs to the large family of intrinsically disordered proteins (IDPs), and recruits host proteins for the transactivation of viral RNA synthesis. Tat interacts with transactivator response RNA (TAR RNA) and exerts RNA chaperone activity for the structural rearrangement of interacting RNAs. Here, TAR RNA also stabilizes the Tat conformation, and then mediates the transfer of Tat into host transcriptional machinery, establishing a proteome link between virus and the infecting host [14]. Except for such limited studies, the extent of Chaperna

function in the proteostasis – folding of nascent polypeptides, stable maintenance, and disposal of aberrant species through proteosomal pathways – remains to be further elaborated. Although the Chaperna activity of TAR RNA should be further examined, it is tempting to speculate that this could represent part of viral strategies for subverting cellular machineries for viral replication [87]. Chaperna provides a unique view for understanding new therapeutic modalities of pathological consequences of defective proteostasis either by metabolic dysregulation or by infectious agents.

Novel RNA-based technologies

As biotechnology increases, RNA technology relying upon the chaperone activity of RNA becomes increasingly attractive. With the novel RNA technologies in controlling gene expression, e.g., riboswitch, RNA interference and CRISPR, Chaperna is expected to serve as a robust folding vehicle for the 'difficult-to-express' proteins [7]. By using RNA-binding domains to mediate the Chaperna function, nanoparticles (NPs) of Middle East Respiratory Syndrome Coronavirus (MERS)-CoV and trimeric influenza haemagglutinin (HA) were effectively assembled, and upon immunization, elicited strong receptor-binding interference, high titres of a neutralizing antibody, and/or immune response against viral infection [88,89], suggesting the potent role of Chaperna in production of vaccines against pandemic outbreaks such as COVID-19. It should be noted that perturbation of the ratio of ribosomal subunits via tuning BMS1 transcript levels allowed producing high-yielding proteins, consistent with the putative role of PFAR in protein folding [90]. Thus, it is tempting to speculate that combining both Chaperna-mediated folding technology and modulation of PFAR via transcriptome analysis would enhance both protein quality and production yield for better expression of recombinant proteins. The RNA-dependent folding competence of IDPs [14] may also immensely increase the repertoire of IDPs as novel protein drugs. Whether harnessing Chaperna function to the folding of guest proteins has a direct impact on therapeutic, diagnostic and prophylactic applications merit further investigation. Many neurodegenerative diseases are the consequence of misfolding of RNA-binding proteins [70,91]. Confirmation of the aetiology of observed cytopathic outcome – an anomaly of either the protein itself or its RNA partners – would have an immense impact on the therapeutic approaches. For example, 6-aminophenanthridine (6AP) and its derivatives, guanabenz acetate (GA), and imiquimod (IQ), have been identified as anti-prion compounds [48,49,92]. Surprisingly, however, it has been studied that the compounds do not target prion itself, but target rRNA and specifically inhibit domain V rRNA which is the active site for PFAR. Furthermore, there are a variety of small molecules which target RNAs as therapeutic drug targets [93]. Given the fact that RNAs are closely associated with proteinopathies, antibodies and small molecules which target Chaperna could be utilized as probes for unravelling mechanisms of protein aggregation-associated diseases and the role of Chaperna therein.

Conclusions & prospects

As intrinsic to ribozymes, Chaperna function further extends and complements the current RNA world concept of molecular transition of catalysis. RNA-mediated protein folding provides a 'missing link' between the primordial RNA world and the contemporary protein-dominated biology. A dual function of RNA as ribozyme and Chaperna expedited both tRNA molecules as adaptor of amino acids, and rRNA as ribosome-based protein synthetic machinery. The ribozyme-resident protein folding activity, even before the advent of protein-based molecular chaperones, must have facilitated the transition from the RNA world into the present protein theatre within a much shorter time frame than the current RNA world conceives. As the ribozyme is powerful in operational in all life forms, Chaperna likely performs potent protein folding functions. Its strength and extent, and the potential network with protein-based chaperones merit further exploration.

Disclosure statement

The authors report no conflict of interest.

Funding

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