Functional dissection of the signal recognition particle

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Biochemical mutagenesis – alteration or removal of specific domains within a biological structure – followed by functional analysis, gives insight into structure–function relationships. We describe the analysis of the signal recognition particle, a ribonucleoprotein known to be required for the entry of most proteins into the secretory pathway, as an example of the strength of this approach.

Signal recognition particle (SRP) has been identified as a component involved in protein targeting to and translocation across the endoplasmic reticulum (ER) membrane (for review, see Ref. 1). Extensive analysis of the effects of SRP on the synthesis, targeting and translocation of the secretory protein prolactin in an *in vitro* reconstituted system, has led to a model in which SRP serves as an adaptor between the cytoplasmic protein synthesis machinery and the membrane-bound protein translocation machinery.

In the *in vitro* system, it has been possible to dissect the activity of SRP into a number of steps, which could be separated from each other temporally (see Fig. 1a). First, it was found that SRP had a measurable affinity (~ micromolar) for all ribosomes. Upon emergence of the signal sequence of a translated protein, the affinity of SRP for the ribosome was found to increase by three or four orders of magnitude. Concomitant with this 'signal recognition' activity, SRP blocks or slows elongation of the presecretory nascent chain. The 'elongation arrest' is released upon interaction of SRP with a component of the ER membrane known as SRP receptor (or docking protein). This interaction (here referred to as 'translocation promotion') leads to the release of SRP from the nascent chain–ribosome complex, and the translocation of the secretory protein across the ER membrane. The events at the membrane can be further dissected into individual steps: (1) the interaction of the signal sequences with membrane proteins[2]; (2) the formation of the ribosome–membrane junction[3], and (3) translocation, which can be experimentally detected by the newly acquired accessibility of the translocated protein to modifying enzymes within the lumen of the ER and by its protection from exogenously added proteases. However, since SRP is released after targeting, these events will not be considered here.

Many of the steps of the protein targeting reaction involve SRP, and so the purification of this single complex has resulted in the clarification of a number of different activities. One approach to understanding the dependency relationships between the different activities mediated by SRP is to study the phenotype of mutant particles. Mutational analysis in other systems has distinguished events which occur in a particular temporal sequence from those that are causally related (e.g. the elegant dissection of cell cycle mutations in yeast[4]). However, because SRP was purified initially from canine pancreas, it has not been feasible to generate mutant particles by classical genetic methods, although with the recent identification of the yeast SRP RNA genes in two yeast species[5–7] such analyses should soon become possible. Yet in spite of this apparent handicap, it has been possible through strictly biochemical manipulations to generate a variety of 'mutant' particles in biochemical quantities by direct alteration of the gene product, i.e. SRP itself.

SRP is a ribonucleoprotein (RNP) composed of six distinct polypeptides and an RNA molecule of 300 nucleotides. Because the molecular interactions between proteins and RNA appear operationally distinct from those by which proteins interact with one another (or those that govern the proper folding of individual proteins), it has been possible to extract selectively the proteins from the RNA without denaturing the protein components[8]. This has been achieved by incubation of the particle with chelating agents, which remove the divalent cations found to be important for the specificity of protein–RNA interactions (for examples, see Refs 9–11). In the absence of the RNA, the polypeptides sediment in a sucrose gradient as monomers and dimers (see below), rather than as a single complex, indicating that SRP is held together both by protein–protein interactions and by protein–RNA interactions.

Biochemical mutagenesis

Analysis of the protein fraction revealed that the six polypeptides of SRP are organized into four protein units[8,12], which, when starting with the protein fraction, can be separated from each other by standard chromatographic methods[12]. Two of these proteins are monomeric (referred to as p19 and p54) and two of these proteins are heterodimeric (referred to as p9/14 and p68/72). The two heterodimeric proteins have not yet been resolved into single polypeptides without the loss of their ability to be reconstituted into an active SRP. For this reason they will be treated as single entities in this analysis.

After disassembly, neither proteins (individually or in combination) nor RNA exhibit any measurable activity.

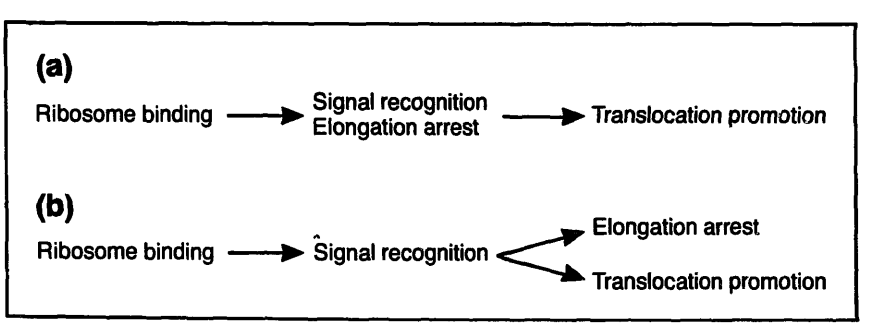

Fig. 1. Sequence of SRP activities. (a) Temporal sequence. Signal recognition and elongation arrest occur simultaneously, and are followed by translocation; (b) Causal sequence. Signal recognition is required for both elongation arrest and translocation promotion. However, the latter two activities can occur independently of each other.

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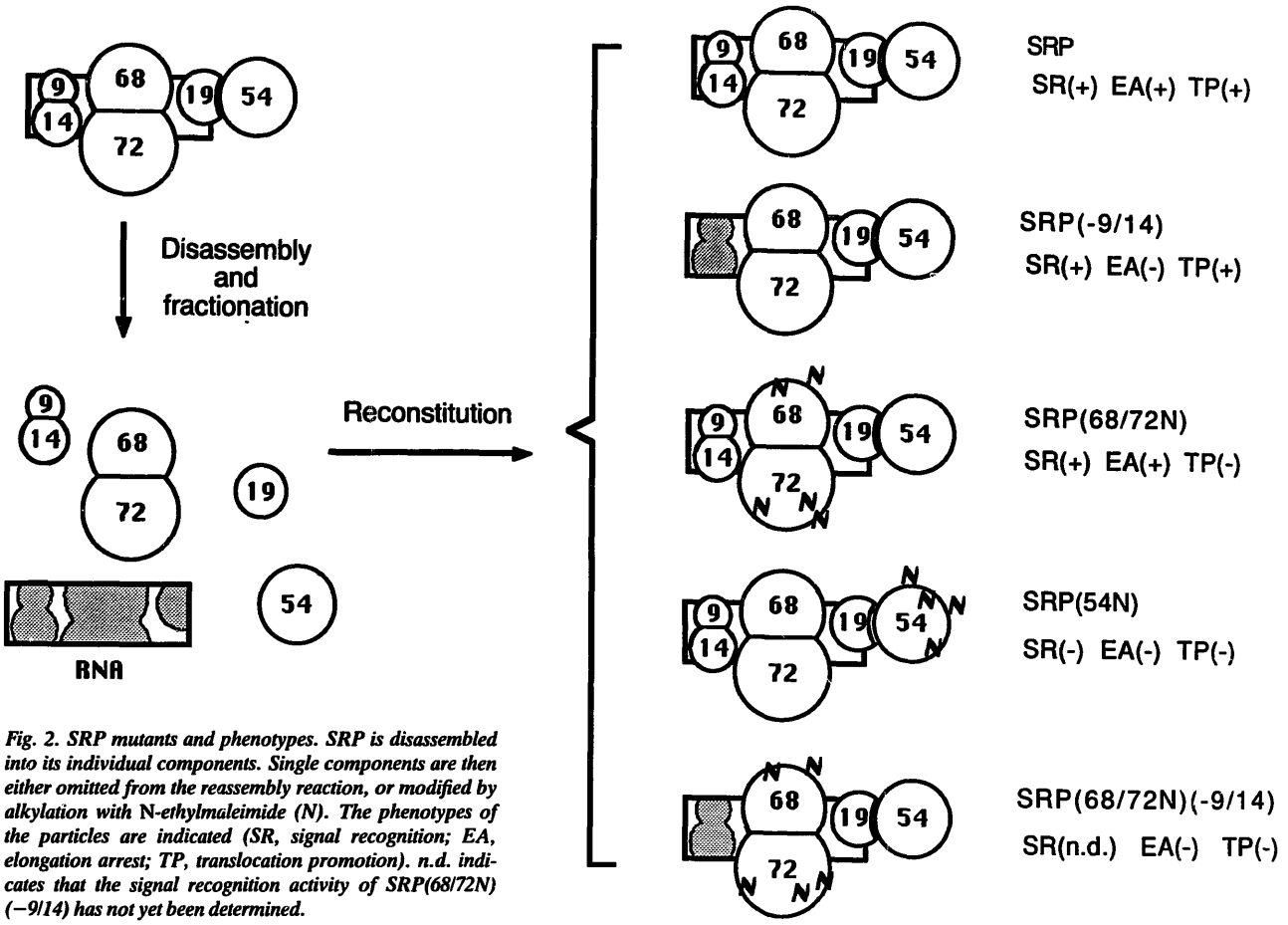

Fig. 2. SRP mutants and phenotypes. SRP is disassembled into its individual components. Single components are then either omitted from the reassembly reaction, or modified by alkylation with N-ethylmaleimide (N). The phenotypes of the particles are indicated (SR, signal recognition; EA, elongation arrest; TP, translocation promotion). n.d. indicates that the signal recognition activity of SRP(68/72N)(−9/14) has not yet been determined.

However, when the proteins are combined with the RNA in the presence of magnesium ions, a fully active particle is readily reconstituted indicating that none of the proteins has been significantly denatured by the fractionation procedure[12]. Most interestingly, three of the four proteins can be shown to bind to the RNA independently. The binding sites of each of these three RNA binding proteins is distinct. This has been determined by a combination of protein–RNA footprint analysis of individual reconstitutes[13] and nuclease digestion and sucrose gradient sedimentation analysis of intact SRP[14,15]. Furthermore, it has been shown by sucrose gradient analysis on partially assembled particles that p54 becomes associated with the particle via an interaction with p19 (Ref. 8). This interaction is of special interest, since p19 and p54 show no affinity for one another in the absence of SRP RNA, and, as concluded from footprint analyses, p54 does not directly contact the RNA. Thus binding of p19 to SRP RNA must trigger a conformational change in p19 that now creates a high affinity binding site for p54, or conversely, interference with the p19–SRP RNA interaction by disassembly also interferes, albeit indirectly, with the binding of p19 to p54. The information on the structural relationship of SRP proteins and RNA has been incorporated into the model of SRP shown in Fig. 2.

Because three of the four proteins can bind to the RNA independently, it was possible to introduce a number of different alterations into the particle, which could be considered the biochemical equivalent of mutations in the particle, and to assay the activity of these mutant particles *in vitro*. For example, single proteins have been removed from the assembly mixture[12], generating a series of particles that are the equivalent of a series of null alleles. In other experiments, cysteine residues of single proteins have been alkylated with the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM)[16]. This reaction was performed after binding of the proteins to SRP RNA to protect cysteine residues that may be important for RNA binding, alkylation of which would affect SRP reassembly rather than function.

The functional analysis of mutant particles required that they could be prepared in biochemical quantities as a homogeneous population. In order to achieve this, the protein fractions had to be free of cross-contamination, and the modified proteins had to be saturated with modifications by alkylating all available cysteine residues. Since all SRP proteins contain multiple cysteine residues, it would have been virtually impossible to separate differently modified particles from one another if only a random subset of sulfhydryl groups were modified. Thus, since all accessible sulfhydryl groups of a given protein were modified, the equivalent of multiple – rather than single – point mutations was generated. More subtle alterations in the proteins may become possible once their genes are cloned.

Reconstitution

Given the inherent 'crudity' of this sort of analysis, it is perhaps surprising that many of these mutant particles have interpretable phenotypes. A selection of mutant particles generated and their phenotypes is summarized in Fig. 2.

Three distinguishable SRP activities have been measured: signal recognition, elongation arrest and transloca-

tion promotion. Mutant SRPs could be generated in which each of three activities is missing. SRP(−9/14), the particle which is assembled in the absence of p9/14, has no elongation arrest activity; SRP(68/72N), the particle which contains sulfhydryl modifications on p68/72, is missing the translocation promoting activity; and SRP(54N) is deficient in signal recognition activity. In addition, the phenotype of the double mutant SRP(68/72N)(−9/14) is the sum of the individual phenotypes: both elongation arrest and translocation activities are absent.

More importantly, in two cases the loss of one SRP activity had no effect on the other SRP activities. Thus SRP(−9/14) is fully active in signal recognition and translocation promotion, and SRP(68/72N) is fully active in signal recognition and elongation arrest. Therefore, even though translocation promotion follows elongation arrest in a temporal sequence, they are not causally related (Fig. 1b). Both of these activities, however, are dependent on signal recognition. SRP(54N), which has lost the signal recognition activity, has also lost both elongation arrest and translocation promotion activity. Because we know that protein translocation is limited for secretory proteins (as well as other proteins bearing ER-directed signal sequences), the causal relationship between signal recognition and the other activities of SRP makes sense intuitively.

Functional assignments

Furthermore, because mutant particles exist in which a single activity is missing, it has been possible to map the activities onto specific protein domains. Thus p9/14 is required for the elongation arresting activity of SRP, and p68/72 is required for its translocation promoting activity (most likely p68/72 is involved in the interaction of SRP with the SRP receptor[16]). Because of the epistatic relationship between signal recognition and the other activities of SRP, the assignment of the signal recognition activity to p54 is less firm. However, there is other evidence, such as crosslinking between the signal sequence and p54 (Refs 17 and 18), which lends more direct support to this assignment. It is interesting to note that the signal recognition activity, which is most crucial to SRP's function, maps to the protein that is least intimately associated with SRP RNA (see above). Thus we think of its interaction with signal sequences as a true protein–protein interaction, whereas in the other RNP domains discussed here, the RNA may also play an important functional role.

Single omission often had more severe effects on the activity of the particle than sulfhydryl modification. For example, SRP(−68/72) was completely inactive, in contrast to SRP(68/72N), which maintained signal recognition and elongation arrest activities. It seems likely that removal of p68/72 results in an improperly assembled particle, rather than that p68/72 contributes to activities other than translocation promotion. Similarly, it is often difficult to assess the wild-type function of a gene product from the phenotype of the null allele in cases of multiple interacting gene products.

Additional mutant particles can be generated by breaking covalent interactions within SRP[14,15,19]. For example, after nuclease digestion of SRP a subparticle can be isolated, called SRP(S), that has lost p9/14 and about half of the RNA. SRP(S) is identical in phenotype to the particle missing only p9/14 (Ref. 15). Thus p9/14 seems to reside in a separable structural domain, and a full half of the RNA is not required structurally or functionally for signal recognition or translocation promotion function. Protease digestion has revealed that SRP proteins can also be cleaved into distinct domains[19]. However, in the studies described so far, multiple proteins have been affected and no particles with interpretable phenotypes (distinct from a complete inactivation of SRP) have yet been created.

A modular structure

Based on this mutational analysis, SRP seems to be a modular structure: the RNA provides a structural lattice, and each of the proteins contributes a distinct function. Indeed, since SRP appears, by electron microscopic analysis, also to be composed of three distinct structural domains[20], each of the functional domains may have a direct structural correlate. The modular structure of SRP is in apparent contrast to the allosteric behavior of ribosomes, in which several different proteins contribute to a single functional domain, and in which single proteins seem to contribute to a number of different functional domains (for example, see Ref. 21). It is probably because of this modular composition that the individual phenotypes of the mutant SRPs could be so well resolved.

However, it seems almost certain that there are long-range interactions within SRP that have been missed in these analyses. For example, it is known that the assembly of SRP is cooperative[8], which must be due to additional conformational effects of protein binding, or to protein–protein interactions within the particle. Similarly, a conformational change is likely to occur in the signal recognition process *per se*, which then leads to the high-affinity interaction of SRP with the ribosome. Upon interaction with the SRP receptor, the high affinity is lost again and SRP is released. Finally, an important function of SRP has not yet been mapped onto the particle, namely that of ribosome binding. We consider it likely that the binding site is not confined to only one of the domains discussed here and that the interaction with the ribosome could provide thus an additional means for cross-talk between SRP domains. Mutational analysis of the RNA or more subtle perturbations of the proteins are almost bound to uncover additional fascinating properties of SRP.

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