行政院國家科學委員會專題研究計畫　成果報告

蛋白質移位至內質網膜的反應機制之研究

計畫類別：個別型計畫
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計畫主持人：陳瑞彰

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本成果報告包括以下應繳交之附件：
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□國際合作研究計畫國外研究報告書一份

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中英文摘要

最近在蛋白質由細胞質移位到內質網膜上的分子過程的研究已經有相當的進展，但是由信號序列所引導移位到內質網膜上的分子機制並沒有被完全瞭解，部分是由於與糖質體結合的新生蛋白鍊的巨大與組成之複雜性，而一部分是因為移位時各個過程須經歷多種不同的環境，因此造成在移位過程中對信號序列的結構之研究的困難度。我們之前已利用將螢光或光反應探針放在剛被糖質體所製造出來的新生蛋白鍊中的信號序列來研究新生蛋白移位的反應機制，並將完整而且具有功能的移位複合體接上探針加以純化來中做研究，以此方法我們已經解決了新生蛋白要從細胞質移位至內質網膜這個過程一些具爭議性的重要問題。現在我們提出的研究計畫要再度利用這個螢光技術以及螢光共振能量轉移的技術來研究有關由信號序列所引導的蛋白移位之下列幾個問題：

(一) 連接糖質體的信號序列的結構為何？它的結構在糖質體的通道裡與在暴露在糖質體外時的結構有不同嗎？這樣的結構若與信號認定分子結合時會改變嗎？而它進入內質網膜時其結構又為何?

(二) 信號序列與信號認定分子結合後其相關幾何位置為何？信號序列裡的厭水區段會被信號認定分子完全包在裡面嗎?

關鍵詞：蛋白移位，信號序列，信號認定分子，螢光共振能量轉移

Considerable progress has been made recently in our understanding of proteintranslocation into endoplasmic reticulum. However, the molecular mechanisms that accomplish signal sequence dependent protein translocation across andintegration into the endoplasmic reticulum (ER) membrane has not been clarified,partly because of the huge and complicated composition of the ribosome-associated nascent chain complex and partly because each process must be involved in several different environments. Very little is now known about the environment and conformation of the signal sequence as it is synthesized by the ribosome, associated with the signal recognition particle, and incorporated into the ER membrane. We have previously investigated protein trafficking from the point of view of the signal sequence by incorporating fluorescent dyes into the ribosome-associated nascent chain as it is being synthesized by the ribosome. By examining functional, fully assembled, and intact translocation complexes with probes in the signal sequence, we have elucidated several important molecular mechanistic aspects of these processes. We now propose a project to extend our unique fluorescence and fluorescence resonance energy transfer (FRET) investigation of protein translocation by addressing questions that include:

1. What is the conformation of the signal sequence of a nascent peptide? Are they different in the ribosomal tunnel and exposed to the cytosol? Is the conformation altered as it associated with the signal recognition particle? Does it become helix like conformation when it inserts into ER membrane?
2. What is the topology the signal sequence when it associates with the signal recognition particle? Is the hydrophobic segment of the signal sequence completely buried into M-domain of SRP54 proteins as it binds to SRP?

Key words: protein translocation , signal sequence, signal recognition particle, FRET,

計畫緣由與目的

In eukaryotic cells, secretory proteins are translocated across the membrane of the endoplasmic reticulum (ER), and membrane proteins are integrated into the ER membrane. One of the most complicated processes carried out in the cells is to translate DNA-derived mRNA into proteins and then to transport the newly synthesized proteins to their final destinations inside or outside of cells where these proteins can perform their unique functions. The molecular mechanisms for protein targeting to and integrating into the ER membrane have been well documented (for review see 1, 2, &3); however, very little is known about many fundamental structural and mechanistic details of these processes at molecular level. In

general, most proteins destined for integration into the membrane such as plasma membrane or for secretion are initially translated by ribosomes in the cytosol. The process of these proteins targeting to the ER membrane are mostly cotranslational. In mammalian cells, when the newly synthesized signal sequence of a nascent chain emerges from the peptide exit of the ribosome, the signal recognition particle (SRP), a cytosolic ribonucleoprotein, binds to the signal sequence and the translating ribosome, thus constituting the SRP-RNC complex. This action leads to transiently pause or slow down the nascent chain elongation while the SRP-RNC complex diffuse to the ER membrane. Following a series of GTP-mediated events, a tight ribosome-membrane junction is established and the nascent peptide chain moves through the membrane via the interaction that the SRP of SRP-RNC complex associates with the SRP receptor, an integral membrane protein that is found only at the rough ER membrane. GTP hydrolysis of the GTP-dependent interaction of SRP with SRP receptor leads to the release of SRP and SRP receptor, the binding of the ribosome to a protein conducting channel (translocon) in the membrane, the resumption of protein synthesis, and the initiation of protein translocation or integration. The SRP, SRP receptor, and ribosomes are thus recycled for another run of translocation process, thereby completing the targeting stage of the protein trafficking process. The signal sequence and SRP play important roles on the translocation of a newly synthesized protein into the ER membrane. To mediate protein targeting and facilitate translocation, signal sequences have a characteristic tripartite structure, but no consensual sequence is identified (4). Upon being synthesized by the ribosome, the signal sequence is associated with SRP and targeted to the ER membrane where it is digested by the signal peptidase before it completed its task facilitating RNC translocation. Most critical region is the central hydrophobic segment generally consisting of 7-15 hydrophobic amino acids that is required for targeting and translocation into ER membrane. In addition, a subsequent short stretch of 29 residues, usually small and polar, comprises a consensus motif for cleavage by signal peptidase. The third region contains the highest diversity in amino acid that is located on the N-terminus. During the translocation event, the signal sequence is cleaved off by signal peptidase, and the free signal sequence, which spans the ER membrane with the hydrophobic segment, is eventually processed by signal peptide peptidase (SPP) in the ER membrane (5). Many of the features of the signal sequence are not necessarily important for the function of the respective signal sequence in protein targeting and translocation. This leads to the question that the hydrophobic segment may carry features that are relevant for function beyond protein targeting after cleavage by signal peptidase and liberation of the signal sequence from nascent protein. Furthermore, Binding of SRP to the signal sequence is believed to be through the hydrophobic interaction since the signal sequence usually contains a sequence of hydrophobic 15 to 30 amino acids at their N-terminal ends while the M-domain of SRP54 protein (SRP54p) of SRP contains a hydrophobic groove as well. The environment of the SRP-associated signal sequence may be quite different from it is exposed to the cytsol. From being synthesized by a cytosolic ribosome to being digested off in the ER membrane, however, the signal sequence has to encounter four different environments: (i) in the tunnel of ribosome (ii) exposed to the cytosol (iii) association with SRP and (iv) in the translocon of ER membrane. Interestingly, the length of the hydrophobic segment is not consensual. Some could be as long as 25 aa’s and some could be only 7 aa’s in length. How the structure of the signal sequence is folded in these four different environments, particularly how it spans in the EM membrane before it is digested off by SPP, is intriguing. In the past few years, the subsequent steps in nascent chain ribosome complex (RNC) translocation and integration into ER membrane have been revealed tremendously by an experimental approach in which fluorescent or crosslinking probes are site-specifically incorporated into nascent peptides to directly report the environment of a nascent protein during targeting, translocation or integration. This approach elicited a major change in perspective of the sorting process. The most direct way to monitor the conformation change of the signal sequence of a nascent chain is to detect what the signal sequence experiences

during the targeting by placing a probe in the signal sequence. But one can not selectively label signal seqiuences in a complex sample containing ribosomal proteins and ER membranes using standard protein modification techniques because the nascent chains comprise much less than 1% of the total protein in such a sample, leading to place probes nonspecifically into ribosomes. Instead, probe carrying amino acids must be incorporated into a signal sequence from modified aa-tRNA analogs during in vitro translation. Thus, I have used the approach in which a chemical procedure for attaching a probe to the amino group on the lysine sidechain of Lys-tRNA (6, 7, 8,). The resulting N-labeled Lys-tRNA derivatives were functional, and this class of aa-tRNA analogs therefore provided a unique mechanism for incorporating probes into the signal sequence of a ribosome-associated nascent chain. We also wish to examine how SRP associates with the signal sequence. The signal sequence-dependent targeting of the translating ribosomes to the ER membrane requires an ordered steps of interactions involving both cytoplasmic and membrane-bound molecular species. SRP mediates the targeting, since it both selects the ribosome-nascent chain (RNC) complexes in the cytoplasm and then interacts with the SRP receptor to position these complexes on the ER membrane. The RNA and protein components of SRP have been identified and characterized, and functions have been assigned to specific domains of SRP (9). The binding of SRP to the signal sequence of RNC not only leads to the RNC targeting to the ER membrane but also suggests that the M-domain of SRP may protect or alter the conformation of the signal sequence to adapt the environment of the translocon and the ER membrane. What is the orientation of the signal sequence when it binds to SRP? We have determined that the GTP binding site of SRP54 for the signal sequence is within the FRET-detected distance with the signal (14). Yet, while many aspect of translocation and integration are agreed upon, several issues are still controversial.

Methods and Materials

Preparation of tRNA derivatives

Yeast Lys-tRNA and N-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)- aminohexanoyl]-Lys-$tRNA^{Lys}$ (NBD-Lys-$tRNA^{Lys}$) were purified and prepared following the procedures described (Crowley et al.). *E. coli* $tRNA^{Lys}$ and a derivative with a single base change in the anticodon that converted the $tRNA^{Lys}$ into a tRNA that recognizes the amber stop codon (here termed $tRNA^{amb}$) were synthesized *in vitro* using T7 RNA polymerase as described elsewhere for SRP RNA. The resulting RNA samples were purified by anion exchange chromatography using a Pharmacia FPLC equipped with a Mono Q HR 10/10 column. Generally, the RNA was eluted in 10 mM NaOAc (pH 4.5), 5 mM $MgCl_2$ with a 115 ml linear gradient of NaCl from 0.48 M to 1.0 M. The fractions containing functional $tRNA^{amb}$ were detected by aminoacylation assays , except that $MgCl_2$ was at 6 mM and no KCl was added; most of the $tRNA^{amb}$ eluted near 0.55 M NaCl. The fractions with the highest $tRNA^{amb}$ content were aminoacylated with [^{14}C]Lys, chemically modified with NBD, purified, and stored as described previously with the above changes (Crowley et al). The NBD modification has no detectable effect on the ability of Lys-$tRNA^{amb}$ or Lys-$tRNA^{Lys}$ to function in protein synthesis. For photocrosslinking experiments, photoreactive N-(5-azido-2-nitrobenzoyl)-Lys-tRNA (ANB-Lys-tRNA) will also synthesized as the procedures for NBD-Lys-tRNA except N-(5-azido-2-nitrobenzoyl)-succinimide will be used instead of 6-(7-nitrobenz-2-oxa1,3-diazol-4-yl)aminohexanoyl ester.

N-ethylmaleimide treatment of SRP and SRP54p.

N-ethylmaleimide (NEM, 100 mM) was prepared in a solution containing 28 l dimethyl sulfoxide (DMSO) and 472 l double distilled water. A solution for mock NEM treatment was also prepared as above, except that no NEM reagent was included. Three 0.5-ml microfuge tubes (tubes A, B, and C) were used in a reaction. Tubes A and B were loaded with 48 l SRP, whereas tube C was loaded with SRP buffer. Tubes A and C were

treated with 8 l of 100 mM NEM (14 mM in final), while 8 l of mock solution was added into tube B. After mixing, these tubes were incubated at 26°C for 35 minutes. To stop the reaction, 1 l of 1 M DTT (17.5 mM in final) was added to each tube. The tubes were then allowed to incubate for another 10 minutes at 26°C to eliminate the unreacted NEM.

Preparation of GTP-Rh and GDP-Rh.

The procedures for synthesizing GTP-Rh and GDP-Rh have been was carried out on a Mono Q (HR 5/5, Pharmarcia) when larger amount of GTP-Rh is to be purified. The purity of GTP-Rh was also analyzed by HPLC. Concentration of GTP-Rh and GDP-Rh were determined using specific absorption coefficient at 570 nm = 77,000 M^{-1} cm^{-1} for rhodamine moiety. 2'(3')-O-(N-methylanthraniloyl-GTP (mant-GTP) and mant-GDP molecules were synthesized and purified. The concentration of mant-GTP and mant-GDP were determined using specific absorption coefficient at 350 nm = 5,700 M^{-1} cm^{-1}.

Translation Intermediates.

Homogeneous samples of RNCs were prepared by translation of truncated preprolactin (pPL) mRNAs in wheat germ extract as described elsewhere (Crowley et al. 1993). NBD probes were incorporated into the nascent chain wherever there was a lysine codon in the mRNA by doing the translation in the presence of NBD-[^{14}C]Lys-tRNA as before (Crowley et al. 1993). For example, for pPL nascent chains that were 65 amino acids in length (pPL_{65}), NBD-Lys probes were present either in position 4 or position 9, the locations of lysine in the natural pPL sequence. In some experiments, VW202p, a chimera formed by the fusion of a pPL signal sequence to a long Bcl-2-based polypeptide sequence lacking lysines, was used to ensure that probes were incorporated only into the pPL signal sequence and not into the mature region of the protein. (Since the added NBD-Lys-tRNA must compete with the normal complement of Lys-tRNA in the wheat extract, only 25% of the lysines incorporated into protein are NBD-Lys (Krieg et al. 1989). RNC were then purified by gel filtration to remove residual NBD-Lys-tRNAs and deacylated NBD-Lys. In some experiments, truncated pPL mRNAs with an amber codon inserted into the signal sequence were translated in the presence of the modified amber suppressor tRNA, NBD-[^{14}C]Lys-$tRNA^{amb}$ to correct for light scattering and background fluorescence. The total number of ribosomes in a sample was dteremined by absorbance at A_{260} nm (Sperazza et al. 1980). The total number of probes in a sample was determined by its radioactivity content. The total number of nascent chains was equal to the number of incorporated probes in the amber suppressor experiments, and was twice the number of incorporated probes whenever the probes were incorporated into the pPL or VW202p signal sequence using NBD-[^{14}C]Lys-tRNA.

Fluorescence Spectroscopy.

Fluorescence experiments were done at 4°C in 50 mM HEPES (pH 7.5), 140 mM KOAc, 5mM $Mg(OAc)_2$, 1 mM DTT, using the instruments, microcuvettes, and other procedures (e.g., mixing, sample-blank matching) described earlier (Crowley et al. 1993). SRP was titrated into samples by sequential addition of known amounts of SRP (determined by absorbance at 280 nm) to the sample volume. After each addition of SRP and mixing, a constant fluorescence intensity signal was obtained (i.e., equilibrium was reached) within 5 minutes. After blank subtraction and dilution correction, the emission intensity of the sample at any point in the titration (F) was compared to its initial intensity in the absence of any SRP (F_o).In all experiments, F/F_o reached a maximum value as SRP was increased, as expected for SRP saturation of NBD-RNC-binding sites. All titration data were analyzed as competition experiments (since every sample contained both NBD-RNCs and inactive ribosomes) using the equations and analysis methods detailed in Bock et al. (1997).

Measurement of Anisotropy

The excitation and emission wavelength were determine each time a new probe was used. For NBD-RNC, the samples were excited at 468 nm, while the emission was monitored at 530 nm. The excitation band pass (slit) was set at 4 nm, while the emission band pass was set

at either 4 or 8 nm. For steady-state anisotropy measurements, two Glan-Thompson prism polarizers were placed in the excitation and emission light beams. The anisotropy (r) was calculated using the following equation

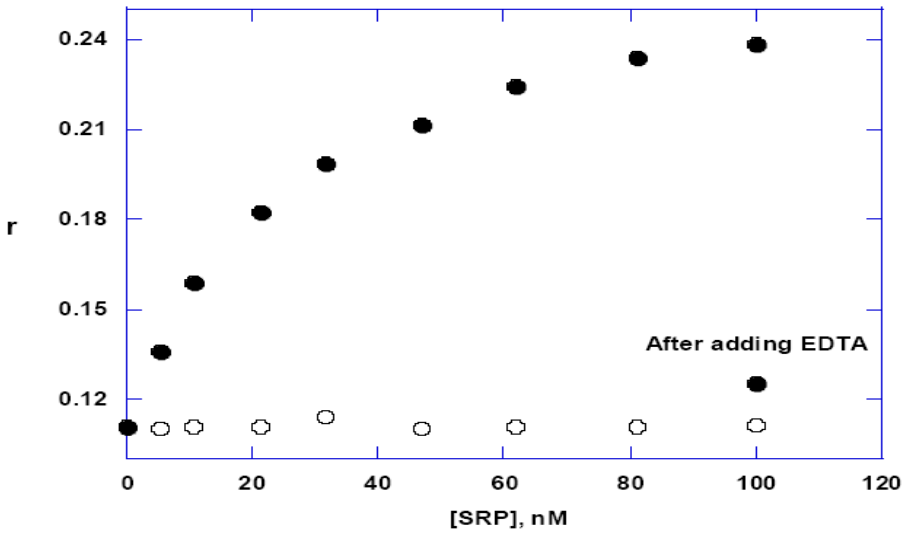
$$r = (I_{VV} - G I_{VH}) / (I_{VV} + 2 G I_{VH})$$

where G is the grating factor and is equal to I_{HV}/I_{HH}. I_{HV} represents the emission intensity observed when the excitation polarizer was horizontal and the emission polarizer was in the vertical position. I_{VV}, I_{VH}, and I_{HH} are defined analogously. To determine the net fluorescence intensity of a sample, the signal of an equivalent sample lacking fluorescent probes was subtracted from the signal of the sample. The intensities of I_{VV}, I_{VH}, I_{VV}, and I_{VH} in the above equation were not corrected for dilution due to the addition of titrants, since the anisotropy is independent of the dilution

結果與討論

Association of fluorescent-labeled GTP with SRP and SRP54 requires magnesium.

SRP consists of six SRP proteins and one SRP RNA. Of the SRP proteins, SRP9/14p, SRP68/72p, SRP19p, and SRP54p, the SRP54p contains a GTP binding site. To determine whether the nucleotide associates with SRP using fluorospectroscopy, GTP-Rh was synthesized and purified as described previously. Fluorescent intensity and anisotropy were detected when GTP-Rh was titrated with purified SRP. The increase in the fluorescent intensity was minimal after the titration of GTP-Rh with SRP, indicating that the environment of the rhodamine moiety on the GTP-Rh is not tremendously altered after GTP-Rh binding to SRP. On the other hand, a significant increase in anisotropy was observed (from 0.11 $\pm$ 0.01 to 0.23 $\pm$ 0.02) when SRP was titrated into GTP-Rh in the presence of magnesium (Figure 1). Each datum was taken after the binding of two particles in each titration reached to equilibrium in which the fluorescent anisotropy is stable. The anisotropy reached to plateau after an excessive SRP was added into the solution. To determine whether the binding of GTP-Rh to SRP requires magnesium as the most GTP-binding proteins do, EDTA was added into the mixture to chelate the magnesium. The anisotropy of the fluorescent probe was returned to near the original. In addition, titration of GTP-Rh with an excessive SRP in the absence of magnesium did not show the increase in anisotropy, indicating magnesium is required for the binding (data not shown). The increase in anisotropy was also returned to near the original when an excessive unmodified GTP or GDP was added into the mixture in other experiments (data not shown), indicating that GTP-Rh occupied the GTP/GDP binding



site on **Figure 1. GTP-Rh binding to SRP**
SRP.

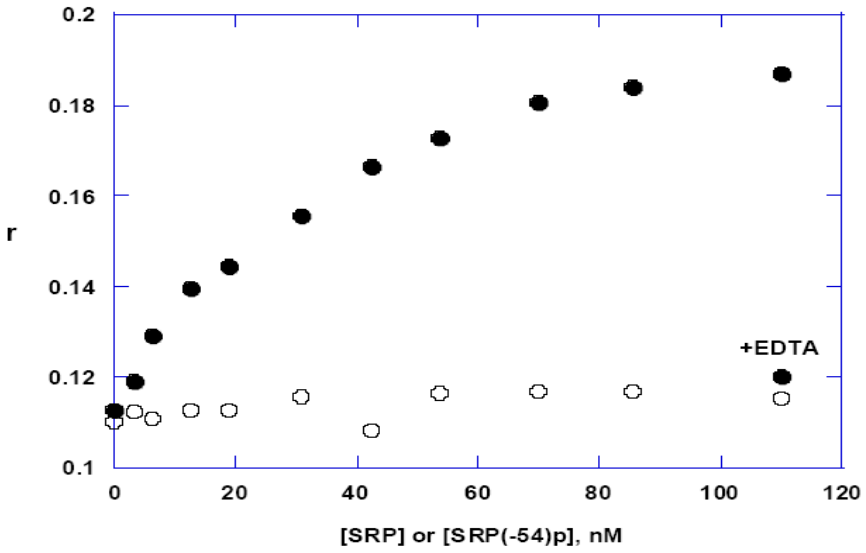


Figure. 2

Whether SRP54 protein (SRP54p) alone binding to GTP-Rh was also determined. SRP54p was purified from the other SRP proteins and SRP RNA as described previously. Titration of GTP-Rh with SRP54 protein (in the absence of other SRP components) increased the anisotropy from 0.11 $\pm$ 0.01 to 0.20 $\pm$ 0.02 (Fig.2), while the intensity decreased by approximately 12% (data not shown). The anisotropy enhancement can be reversed to near the original number after EDTA or excessive GTP or GDP (data not shown) were added into the mixture, indicating that GTP-Rh occupies the authentic GTP/GDP binding site on SRP54p. When GTP-Rh was titrated with excess SRP54p in the absence of magnesium, the anisotropy barely increased. Upon adding magnesium chloride into the mixture, it was observed that the anisotropy of GTP-Rh immediately rose to 0.20 (Fig. 3). Magnesium is required for the binding of GTP-Rh to SRP54 protein, since the anisotropy of GTP-Rh stayed at a similar value after SRP54 was added into the mixture without magnesium. The slightly lower anisotropy of SRP54p•GTP-Rh (0.20) than that of SRP•GTP-Rh (0.23) probably reflect the larger size of the elongated SRP upon binding toGTP-Rh.

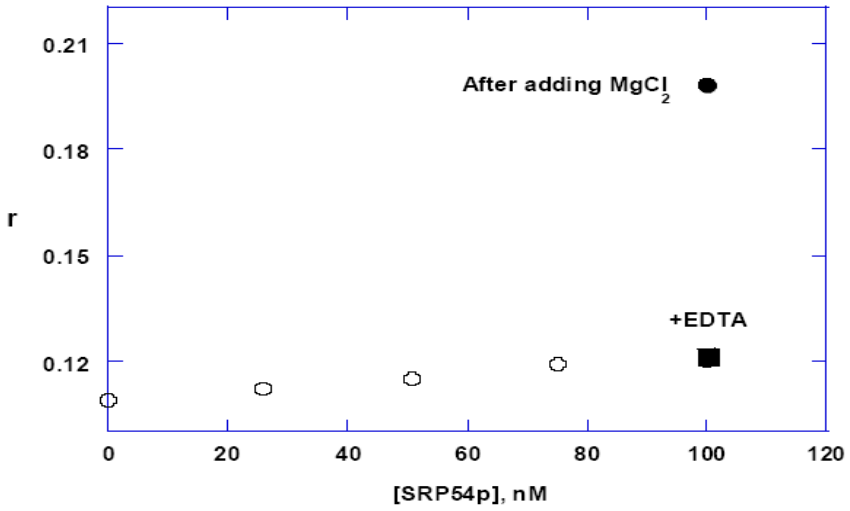


Figure 3. Magnesium Ion Dependence of GTP-Rh Binding to SRP54p

Thus, the binding of GTP-Rh to SRP and SRP54p requires magnesium. GTP-Rh was also titrated with the other SRP proteins (SRP19, 9/14, and SRP68/72) in the absence of

SRP54. Neither the fluorescence intensity nor the anisotropy of the GTP-Rh was changed after a large amount of these proteins was added.

The emission intensity of GTP-Rh was increased by approximately 6% when GTP-Rh was titrated with an excess of SRP. Since the intensity increased by about 12% when GTP-Rh was titrated with SRP54p, it appears that the binding of SRP RNA to SRP54p in the presence of the other SRP proteins alters the environment of the GTP-Rh without altering the affinity of SRP54p for GTP-Rh.

The SRP-dependent change in GTP-Rh anisotropy was used to measure the affinities of SRP and SRP54p for GTP-Rh. The Kd value (25 $\pm$6 nM) for the SRP•GTP-Rh complex was average from seven titrations using five different SRP preparations (Fig. 2), and the Kd value (29 $\pm$ 4) for the SRP54p•GTP-Rh was obtained from three titration. Thus, the affinities of GTP-Rh for SRP54p and for SRP are very similar. Mant-GTP and mant-GDP were also synthesized and used for determining the binding to SRP. Similar results were obtained as the affinity of mant-GTP for SRP is approximately 22 nM, while the affinity of mant-GDP for SRP is about 3.5 nM.

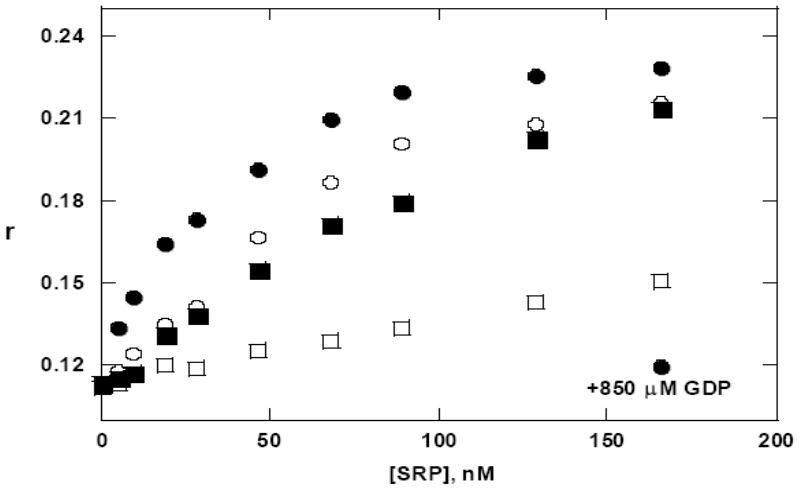


Figure 4. Competition of GTP and GDP with GTP-Rh for Binding to SRP

Free SRP has higher affinity for GDP than for GTP.

The affinity of GTP for SRP could be altered after the 2'- or 3'- hydroxyl group on the sucrose of GTP was modified. To determine the affinity of SRP for unmodified GDP and GTP, competition experiments were done in which the affinities of GDP and GTP for SRP were determined by their ability to compete with GTP-Rh for binding to SRP. More SRP was required to saturate the GTP-Rh binding in the presence of GDP than of the same amount GTP, indicating the affinity of SRP for GDP is highter than that for GTP. Using the formalism of Bock et al., the Kd values for SRP•GTP and SRP•GDP complexes were approximately 2000 nM and 200 nM, respectively (Fig. 4). To confirm the accuracy of the calculated Kd values based on the GTP-Rh•SRP complex. Another GDP derivative (mant-GDP) was also used in the competition experiment. Mant-GDP was titrated with SRP in the presence and absence of certain amount of GTP or GDP, and anisotropy was monitored during the titration. It was observed that more SRP have to be added into the mixture to reach the maximum value of the mant-GDP anisotropy when same concentration of GTP and GDP were added in the mixture (Figure 5). It would not be suitable to calculate the affinity of SRP for GDP since the final anisotropy of the titration curve had not reached to the maximal value, approximately 0.30. A similar titration was carried out except that the concentration of GDP was reduced to 0.4 μM. A lower concentration of GDP will require lower amount of SRP to

saturate for the binding of mant-GDP. At the end of titration, all of the final anisotropy reached the plateau when enough concentration of SRP was added into the mixture (Figure 6). Using the same formalism as mentioned above, the Kd values for GTP•SRP and GDP•SRP are 2287 nM and 190 nM, respectively (Figure 6). These two set of data are very similar, indicating that the affinity of SRP for GDP is approximately ten times as much as for GTP in the absence of a \ signal sequence or ribosomes.

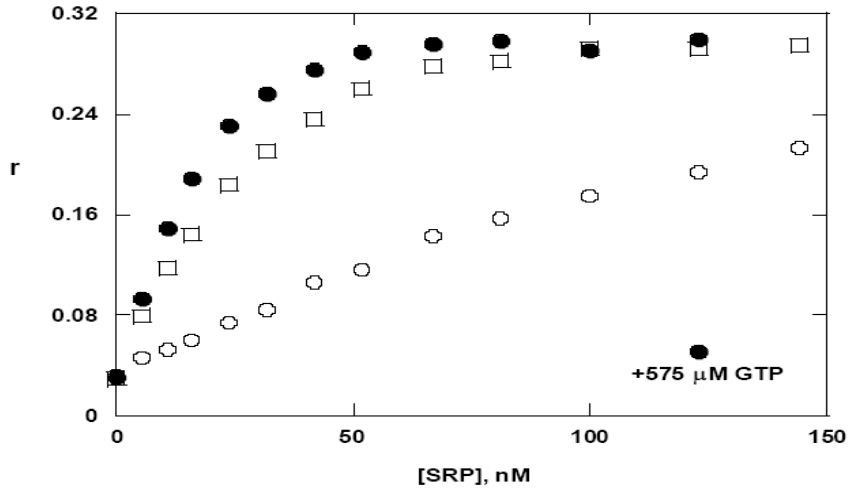


Figure 5. Competition Binding of Mant-GDP with GTP and GDP for SRP

Back titration of SRP•GTP-Rh complex by GTP or GDP were carried out to verify SRP binding to the nucleotides. SRP•GTP-Rh complexes were titrated in parallel with either GTP or GDP. Our data showed that more GTP than GDP was needed to displace GTP-Rh for the SRP•GTP-Rh complex. The addition of GTP or GDP to the sample should have decreased the concentration of the SRP•GTP-Rh complex due to the increase in volume. However, a control sample titrated with SRP buffer showed that the dilution did little effect on the dissociation of SRP•GTP-Rh, probably because the decrease in concentration and ionic strength were insignificant. Therefore, SRP has higher affinity for GDP than for GTP.

Alkylation of SRP blocked its binding to GTP-Rh, but not to the signal sequence. SRP-dependent protein translocation from cytosols to the ER membrane is sensitive to N-ethylmaleimide (NEM), a sulfhydryl alkylating reagent that causes SRP inactivation. It has been reported that alkylation of SRP by NEM yielded a particle that fail to promote protein translocation into the ER membrane. In addition, it has been detected that alkylated SRP54p failed to recognize signal sequences. GTP-Rh was used to detect the binding to NEM-modified SRP54p. Since neither the anisotropy nor the intensity of GTP-Rh was altered by the addition of NEM-modified SRP54p, it is clear that the GTP binding site on SRP56p is blocked by the alkylation after NEM-treatment. To examine whether SRP RNA and the other SRP proteins can prevent SRP54p from the damage of alkylation on the GTP binding, similar experiment was also carried out when ethylated SRP was used instead of ethylated SRP54p. Neither the anisotropy nor the intensity was observed when ethylated-SRP was added into GTP-Rh. Parallel experiments had been also carried out in which GTP-Rh was still able to associate with the mock-treated SRP, although the affinity of the mock-treated SRP for GTP-Rh (Kd = 28 $\pm$ 4 nM, n =3) is slightly lower than that of untreated SRP for GTP-Rh (Kd = 23 $\pm$ 3 nM). This observation may account for that the ethylated SRP was not able to promote the translocation of secretory proteins into the lumen of the ER. Interestingly, we examined whether ethylated SRP still retains its ability to bind to the signal sequence of a RNC complex. Our data showed that the alkylated-SRP or alkylated-SRP54p is still able to

associate with NBD-RNC (VW202) determined fluorospectroscopically (Figure 5). However, it needs more ethylated SRP than unmodified SRP to saturate the binding to the signal sequence of the NBD-RNC. It appears that the ethylated SRP remains its ability to associate with the signal sequence, but its affinity for NBD-RNC is less than SRP. From the titration curve, but NEM-SRP lost its affinity for NBD-RNC (globin). These results suggest that NEM-SRP may lose its affinity for the ribosome and GTP/GDP, but still hold its affinity for the signal sequence.

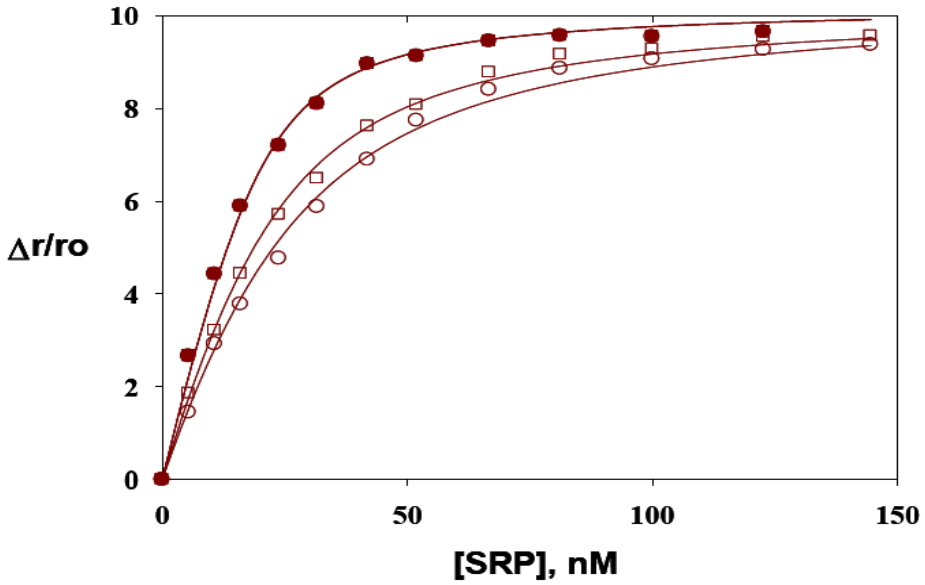


Figure 6. Competitive Binding of Mant-GTP, GTP, and GDP to SRP.

SRP binds to both a signal sequence and GTP-Rh simultaneously. It is arguably believed either that the signal sequence of a nascent chain could block the binding of GTP and GDP to SRP or that the signal sequence promotes GTP binding to SRP. To solve the question, we utilized frequency resonance energy transfer (FRET) approach to detect if SRP simultaneously associates with both a signal sequence and GTP. The requirement for observing FRET between two fluorescent probes, the probes have to be close enough to let one probe (the donor) emit lights (or energy) to the other probe (the acceptor). The distance between GTP-binding domain and the M-domain of SRP54p is less than 50 A. This allows one to determine if SRP54p can hold a signal sequence and GTP/GDP at the same time or one at a time. To answer the question, we placed an NBD probe on a signal sequence and a rhodamine probe on GTP. Since the emission spectrum of NBD on the signal sequence overlaps with the excitation spectrum of GTP-Rh, FRET experiments were carried out to determine whether SRP binds both the signal sequence and GTP/GDP simultaneously prior to interacting with SRP receptor. The emission intensity of NBD in the signal sequence of a RNC complex decreased when more GTP-Rh was added into the NBD-RNC in the presence of SRP till the decrease reached to the plateau. The maximal energy transfer efficiency of about 40% (i.e. 40% decrease in intensity) was observed when excess GTP-Rh was titrated into the sample (67mer or longer) containing SRP. The observed FRET was reversed simply by the addition of excess GTP or GDP. To examine if SRP is a nest for both of the signal sequence and GTP-Rh, a similar experiment was also done as above except that no SRP was added in the mixture. However, no FRET was observed (Figure 5), indicating both the signal sequence and GTP-Rh associate with SRP. A similar result was observed when SRP54p, instead of SRP, was used in the FRET experiment.

The extent of FRET observed, which is dictated by the distance between the two probes, is largely dependent of the availability of the signal sequence from the ribosome. No FRET

was observed when NBD-RNC37mer•complex was titrated with GTP-Rh, whereas only 15% FRET was observed when NBD-RNC55mer was used. Puromycin was used to release the nascent chain from the p-site, and this action would expose its signal sequence from the ribosome. The fluorescent intensity was gradually decreased by approximately 40% after 90 minutes in both two cases when puromycin was added into the complex. However, the FRET in 86mer-RNC and 67mer-RNC almost was not changed after puromycin was added into the mixture.

From our results shown as above, GTP-Rh can associate with SRP when SRP also binds to the signal sequence. One would curious if the Kd value of SRP•GTP-Rh is affected by the signal sequence. FRET occurs only when GTP-Rh binds to SRP and the signal sequence also associates with SRP. Since the extent of intensity decrease depends on the occupancy of GTP-Rh by SRP•NBD-RNC, one can calculate the affinity of GTP-Rh for SRP•NBD-RNC directly from the titration curve for the energy transfer experiment. Using the calculation described as above, the affinity of GTP-Rh for the signal-sequence-associated-SRP was approximately 50 nM, about two times less than that for free SRP.

GDP binds more tightly to the SRP•RNC complex than GTP. We next examine whether unmodified GDP or GTP bind more tightly to SRP when SRP is signal sequence-bound. Two sets of parallel competition experiments as FRET were carried out. We titrate GTP-Rh into NBD-86merRNC in the presence of SRP and GTP or GDP, i.e., GTP-Rh was employed to compete with GTP or GDP for binding to SRP•NBD-86merRNC, respectively. The rational is that: if one of the unmodified nucleotide (GTP or GDP) binds more tightly to the NBD-86merRNC than the other, one would expect more GTP-Rh is needed to compete out the unmodified nucleotide for the binding than the other. This competition process will be revealed by detecting FRET during the titration. The competition titration curves detected by FRET showed that less GTP-Rh was needed to completely occupy the GTP-binding site on SRP•NBD-RNC in the presence of GTP than that in the presence of GDP (Figure 6). In the other word, GDP still has higher affinity for the signal sequence-bound SRP than GTP does. To confirm the relative affinity of SRP•RNC complex for GTP and GDP, GTP-Rh•SRP•65mer•ribosome complex was back-titrated with GTP and GDP, respectively. To eliminate the effect the association and dissociation rates on the result, we wait enough times to allow the competition is completed. Our data revealed that more GTP was needed to replace GTP-Rh in the complex, indicating that GDP has a higher affinity for SRP•65mer•ribosome complex than GTP.

GDP and GTP does not influence of the binding of SRP to the NBD-RNC complex.

Both the signal sequence and GTP binding sites are located in SRP54p, but in different domains. The above energy transfer experiments show that SRP can bind simultaneously to the signal sequence and GTP/GDP. Although the two domains are connected by a peptide bridge and are separated by a cleft approximately.10 A wide in the free SRP54p, it is not clear whether the binding affinity of the signal sequence for SRP is influenced by the occupancy of the nucleotide binding site on SRP by either GTP or GDP. The same final fluorescence intensities of the NBD-RNC complexes in the presence and absence of GTP or GDP indicate that the environment of the probes is not influenced by the binding of GTP/GDP to SRP in the complex. The affinities of GTP•SRP and GDP •SRP for the NBD-RNC (65mer) complex were 0.6 $\pm$ 0.1 nM (n=2). Thus the binding of GTP and GDP to SRP54 does not detectably alter its affinity for the signal sequence.

計畫成果自評

From our study, we have showed very interesting results on the protein trnslocation into the ER membrane and a manuscript for publication is being prepared. Taking advantages of various fluorescent techniques, we have determined that SRP binds to both a signal sequence

and GTP-Rh simultaneously. We also have calculated the affinities of GTP and GDP to SRP in the presence of SRP and nascent chain ribosomes, a long-stand question that couldn't be solved by using other techniques such as gel filtration and EMSA. The FRET could also be used as a molecular ruler which can measure the distance less than 100 angstroms between two specific sites either on the same molecule or different molecules. I just moved from China medical University to the National Chiayi University, and we just purchased a state of the art fluorescent spectroscopy facility which can do FRET. We need to find enough grant to continue the protein trafficking that is studied by many investigator here. The FRET techniques can also be used on other field. For example, real-time PCR is one of the most known instruments applying the FRET technique on measuring the concentration of cDNAs. A further long-stand question is whether the signal bent after it is associated with SRP. Obviously, FRET is the best way to solve the question. We will find financial support for continue this project, and hence place the important pieces on the puzzle.

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