行政院國家科學委員會補助專題研究計畫成果報告

運用螢光方法研究 Rab3A 的訊息傳遞機制 II

計畫類別：■個別型計畫　□整合型計畫

計畫編號：NSC89－2311－B－040－011

執行期間：89 年 8 月 1 日至 90 年 8 月 1 日

計畫主持人：林崇智　中山醫學院生命科學系

共同主持人：高閬仙　陽明大學生命科學系

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執行單位：

中　華　民　國　　　年　　　月　　　日

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Studies in Signal Transduction Mechanism of Rab3A by Fluorescence Techniques II

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一、中文摘要

Rab3A 被認為是一種參與調控式胞吐的小 G 蛋白。根據目前的研究認為 Rab3A 的作用是在囊泡的導引與膜融合，但是沒有直接證據證明其作用的位置與實際參與的生化反應。為了解決這個問題，首先一定要了解 Rab3A 在活體細胞中的位置與其在胞吐作用時的分佈變化。

本年度已完成（1）構築 DsRed (紅螢光蛋白)-ChromograninA 與 DsRed-VAMP (突觸囊泡摩蛋白)，此兩種蛋白將用於了解胞吐位置，分泌囊泡與 Rab3A 在胞吐時的相對位置。（2）為了偵測 Rab3A 活化狀態，Rab3A 結合蛋白(Rabphilin-3A)，接上黃螢光蛋白。此融合蛋白將配合 FRET 顯微影像法，偵測活細胞中 Rab3A 的活化狀態與胞吐之間的關係。（3）初步建立細胞內鈣離子動態影像分析方法，將來將用於了解鈣離子與 Rab3A 動態分布的關係。

關鍵詞：Rab3A，調控式胞吐

Abstract

Rab3A is a small G protein involved in regulated exocytosis, but mechanism that Rab3A regulates exocytosis is unclear. We want to track Rab3A and its activation state in living cells during secretion, and hope to know the exact physiological role of Rab3A in exocytosis.

In this year's project, DsRed-chromograninA and DsRed-VAMP expression vectors are constructed, and they will be indicators to track different exocytosis stages to know what stage Rab3A does regulate. To know activation of Rab3A in living cells, rabphilin3A, which binds GTP-bound Rab3A, is fused to YFP. This fusion protein will be used in FRET imaging to know the activation state of Rab3A. Basic calcium imaging is also established, and this will be used to know effect of calcium on localization and activation state of Rab3A.

Keywords: Rab3A, regulated exocytosis

二、緣由與目的

Rab3 proteins are members of small G proteins. Four Rab3 proteins have been identified so far, Rab3A, Rab3B, Rab3C and Rab3D (Baldini et al., 1992). Rab3 proteins are preferentially localized in neuronal or secretory cells. Several lines of evidence suggest that Rab3 proteins are involved in docking and fusion steps of regulated secretory pathway (Holz et al., 1994; Johannes et al., 1994; Geppert et al., 1994). However, there is very little direct evidence about the exact molecular roles of Rab3 proteins in regulated exocytosis, especially the place where they locate and when they interact with their target proteins during secretion. If Rab3A involves in docking and fusion, it must locate near vesicles and plasma membrane. Tracking Rab3A in living cells during secretion provides important direct evidence to explain the exact place where it acts. Thus, a working system to track Rab3A in vivo is setup first in the first year project.

There are several methods to make particular proteins visible, including fluorescent dyes and immunohistochemistry. But these methods have limitations, ex. immunohistochemistry is used in permable or fixed cells and fluorescent dyes are not very specific. Recently, people found that GFP (green fluorescent protein) is a good tool to label proteins for studying protein translocalization. GFP is isolated from jelly fishes, Aequorea victoria, and its 65-67 amino acid residues are cyclized and oxidized to form the fluorophore, which adsorb 475nm light to emit 507nm fluorescence. There are three GFP

mutants with different colors, blue, yellow and cyan. Fluorescence quantum yield is very high (0.72-0.85); fluorescence intensity is very strong and lasts over 24hr. (Cubitt et al., 1995; Tsien et al., 1993). Because of stability and strong intensity of GFP, GFP is a good tool to label proteins to study protein translocalization. Besides, proteins fused to different GFP variants are also very useful for protein-protein interaction studies, because of FRET (fluorescence resonance energy transfer). When two fluorescent groups is close enough (below 10nm), fluorescence emitted from one fluorescent group will be transferred to the other to emit fluorescence of the other fluorescent group. Adam and their colleagues label PKA (protein kinase A) regulatory and catalytic subunits with fluorescein and rhodamine. In absence of cAMP, regulatory and catalytic subunits are associated, thus excited fluoresceins in regulatory subunits of PKA will tranfer energy to rhodamine to emit fluorescnece. Once cAMP increases, regulatory and catalytic subunits are dissociated, and rhodamine fluorescence decreases (Adams et al., 1991). Miyawaki and their colleagues fuse BFP and GFP to calmodulin and M13 (one of calmodulin-binding proteins), and these fusion proteins are tagged with organelle-specific localization signal peptides to express these proteins in specific organelles. Because binding calmodulin with M13 is calcium-dependent, fluorescence by FRET will reflect calcium concentration in particular organelles (Miyawaki et al., 1997). Rab3A is fused to GFP and GFP fluorescence indicates where Rab3A is.

Because of fast exocytosis and small secretory vesicles, imaging system needs both high spatial resolution and temporal resolutions. For high spatial-resolution and time-resolution, evanescent wave fluorescence microscopy will be a better choice than conventional confocal microscopy, because confocal microscopy needs time for high spatial-resolution and it is not a good idea to apply to measure kinetics of exocytosis. Evanescent wave only illuminates a thin layer of interface between the cell and medium, about 300nm, thus this system avoid interference from out-of-focus fluorescence to give confocal microscopic grade resolution. According to previous investigations, evanescent wave fluorescence microscopy can detect single exocytosis of chromaffin granule (Steyer and Almers, 1999). Because of limited budget, we modify Steyer's setup method, only put an annular mask and high NA objective to regulate incident angle of the light beam to generate evanescent wave, and replace laser and CCD by mercury lamp and conventional camera. Although our imaging system is not as perfect as Steyer's, it is enough to detect Rab3A translocalization and provide some basic results.

In report of last year's project, we have setup a system to track Rab3A translocalization in living cells, this system will be applied to know the exact locations of Rab3A during exocytosis and find out the role of Rab3A in regulation of exocytosis.

Based on these results, we have construct DsRed-chromograninA and DsRed-VAMP expression vectors this year, and they will be indicators to track different exocytosis stages to know what stage Rab3A does regulate. To know activation of Rab3A in living cells, rabphilin3A, which binds GTP-bound Rab3A, is fused to YFP. This fusion protein will be used in FRET imaging to know the activation state of Rab3A. Basic calcium imaging is also established, and this will be used to know effect of calcium on localization and activation state of Rab3A.

三、結果與討論

Construction of expressing vectors of DsRed-ChromograninA and DsRed-VAMP.

Optic method can provide enough spatial and temporal resolution to detect movement, docking and fusion of a single exocytotic vesicle (Neher, 1998 and Murphy, 1999). Therefore, optic method is a good method to clarify where and what step Rab3A acts during exocytosis. To establish optical method to detect steps exocytosis needs to construct fluorescent secretory proteins and membrane proteins of secretory vesicles. Chromogranin A is a soluble protein of dense core vesicles and can be secreted by increasing cytosolic calcium concentration. VAMP is a synaptic vesicle membrane protein. Therefore, both of proteins will be fused to DsRed, red fluorescent protein, and red fluorescence will locate where vesicles and exocytotic events are.

VAMP cDNA is a gift of Dr. Horng at Chang-Gung University, and Chromogranin A cDNA is from Dr. Gorr at University of Kentucky. Restriction sites for cloning are added to both ends of genes by PCR and genes connect to N-terminus of DsRed because targeting signals to membrane of these proteins are their N-terminus. Constructs are subject to orientation check and sequenced. (Fig. 1)

Construct YFP-Rabphilin3A and DsRed-syntaxin

The activation state of Rab3A during exocytosis is still unknown. Because Rabphilin3A intereacts GTP-bound Rab3A, interaction of Rab3A and Raphilin3A will indicate activation state of Rab3A. FRET imaging is a very powerful tool to detect protein/protein interaction. Therefore, we fuse Rab3Ato CFP, and fuse Rabphilin3a to YFP.

Rabphilin3A cDNA clones are form Dr. Holz at Michigan University (myc-tagged bovine Rabphilin3A). Restriction sites for cloning are added to both ends of genes by PCR and genes connect to C-terminus of YFP. The construct is subject to orientation check and sequenced. (Fig. 1)

Establishment of basic calcium imaging

From previous investigations, Rab3A is thought to be a molecular brake of exocytosis, and this brake is supposed to be removed by cytosolic calcium. There are several ways to explain how to remove inhibition of Rab3A in exocytosis. One candidate is

Rab3A-GAP that promotes GTPase activity of Rab3A to make Rab3A inactive, because it has calcium binding domain. Second, GDP-bound Rab3A will retrieve from vesicles into cytosol by Rab3A binding calmodulin and dissociating from synaptic vesicles. From these investigations, Rab3A seems to dissociate from synaptic membranes by increasing cytosolic calcium.

In this year, we have setup a simple calcium imaging for SK-N-SH cells, and hope that this system can be used to detect the effect of calcium on localization of Rab3A in living cells. Cells are load with Fluo-3 for 30min at 37℃, record fluorescence change Fluo-3 by confocal microscope in time-course mode (frame rate: 0.5Hz). After 10s recording, cells are stimulated by 56mM potassium chloride and continue recording for 60s. High potassium has very little effect to evoke calcium increase in SK-N-SH cells, and this is correlated with previous studies, SK-N-SH cell have very low voltage-gated ion channels. This result indicates that our system is sensitive enough to detect small calcium change. (Fig. 3)

四、計畫成果自評

In this year's project, we have already prepared five important marker genes fused with fluorescent protein (YFP-KDEL and YFP-GalT from Clontechnique for detecting ER and Golgi, DsRed-ChromograninA for detecting fusion events and secretory granules, DsRed-VAMP for detecting synaptic vesicles and recycle of synaptic membrane) and these genes will know localization of Rab3A during exocytosis in living cells. Besides, we also have fused YFP to Rabphilin3A, and this gene will be used to know the activation of Rab3A. For understanding effects of calcium on Rab3A localization, we setup a calcium imaging system to detect intracellular calcium in SK-N-SH cells.

Frankly, the progress of this year's project is slow and caused by the following reasons. (1) SK cells grow very bad and cannot express fluorescent protein-tagged proteins (CHO cells are transfected with these genes and express them without problem, thus transfection efficiency is good). So far, we have checked medium content, batches of serum, microplasm contamination and growth condition, and these are not working. We will ask Dr. Liu at Suchow University for healthy stocks of SK cells. (2) Detection system for our imaging system needs long exposure time, and this always causes photodamage of fluorescent proteins. Therefore, this is very hard to track fluorescent proteins fast and long. To solve this problem, we will use filter wheel and shutter to control exposure time, and use highly sensitive cool CCD to reduce exposure time. (3) Because confocal microscope in our school is broken, we cannot detect intracellular calcium in SK-N-SH cells. Therefore, we need to setup another method for calcium imaging. This experiment will be done in Dr. Kao's lab that has a entire advanced calcium imaging system at Yang Ming University. Because SK-N-SH cells have little secretion activity and low calcium increase during stimulation, we will use PC12 for further studies.

In the following project, we will focus on characterization of Rab3A-associated vesicles and dynamic localization by optic method. At same time, we will use DsRed-Chromogranin A to detect exocytosis and DsRed-VAMP to detect biogenesis and recycle of synaptic vesicles by fluorescence method.

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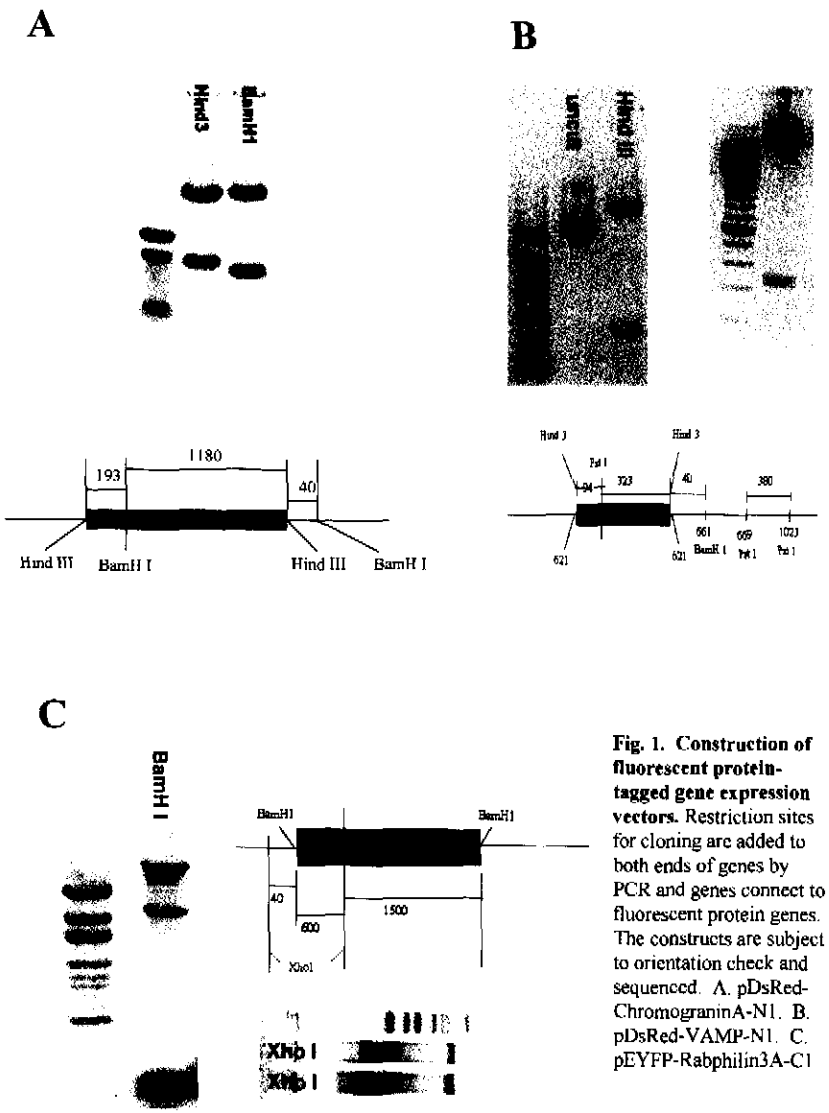

Fig. 1. Construction of fluorescent protein-tagged gene expression vectors. Restriction sites for cloning are added to both ends of genes by PCR and genes connect to fluorescent protein genes. The constructs are subject to orientation check and sequenced. A. pDsRed-ChromograninA-N1. B. pDsRed-VAMP-N1. C. pEYFP-Rabphilin3A-C1

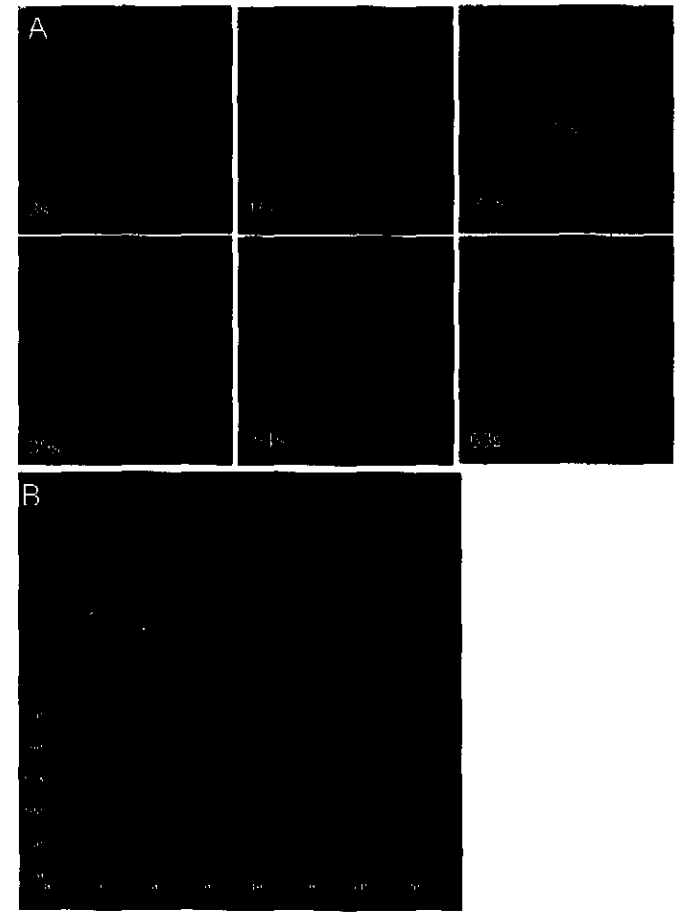

Fig. 2. Calcium imaging of SK-N-SH stimulated by high potassium. Cells are load with Fluo-3 for 30min at 37℃, record fluorescence change Fluo-3 by confocal microscope in time-course mode (frame rate: 0.5Hz). After 10s recording, cells are stimulated by 56mM potassium chloride and continue recording for 60s. A. Frames taken at different time point. B. Calcium imaging of area of interest is located to plot calcium concentration during stimulation.

Fig. 2. Localization of fluorescent protein-tagged fusion proteins in CHO cells.
CHO cells are transfected with different fluorescence protein-tagged fusion constructs, and microphotographs are taken after 48hr transfection.