行政院國家科學委員會補助專題研究計畫成果報告

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※ 海星卵母細胞成熟期間 PRK2 調節機制之探討[1/3] ※
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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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計畫編號：NSC 90-2313-B-002-257
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一、中文摘要

未成熟動物卵母細胞其減數分裂暫停於第一次減數分裂之前期，直到被激活後，減數分裂才能繼續進行。海星卵母細胞受其成熟激素(1-methyladenine, 1-MA)之刺激卵內蛋白磷酸脢 C 二號相關磷酸脢(protein kinase C-related kinase 2, PRK2)活性會升高，繼之以成熟促進因子(maturation promoting factor, MPF)及蛋白質新生成機制之活化。蛋白質之新生成主由真核細胞起始因子 4E (eukaryotic initiation factor 4E, eIF4E)之磷酸化來調控，最近我們更証明了 PRK2 可在活體外將 eIF4E 磷酸化，且 PRK2 之活性並受到 PI3 kinase 及 adneylyl cyclase 之調節，因此 PRK2 極可能在 PI3 kinase 及 adenylyl cyclase 下游來調控卵母細胞之成熟過程。但因以上所述 PRK2 相關試驗均在活體外完成，為證明 PRK2 真是扮演我們所推測之角色，此本計劃將以活體的試驗來測試各種 PRK2 激活及抑制劑，包括自體活化(constitutively active)及負面優勢(dominant negative)之 PRK2，對海星卵母細胞 PRK2 活性及卵母細胞成熟之影響。因台灣地區無法取得先前試驗用之海星品種(*Pisaster ochroceus*)，因此本試驗乃改用澎湖地區特有之飛白楓海星(*Archaster typicus*)及由澳洲 Tasmania 進口之海星(*Asterias amurensis*)，測試其基礎卵母細胞成熟之活性以供後續之研究。同時利用 *Pisaster ochroceus* 之 PRK2 抗體我們可由 *Asterias amurensis* 卵母細胞中以免疫沉澱法萃取出 PRK2 蛋白質，而飛白楓海星卵母細胞中雖可以西方墨點法偵測到 PRK2，但其結果卻不是很恆定，現正以免疫沉澱法測試中。

關鍵詞：海星，卵母細胞成熟，蛋白磷酸脢 C 二號相關磷酸脢，真核細胞起始因子 4E，PI3 kinase，adneylyl cyclase 和 Rho

Abstract

Animal oocytes grow and acquire all the required mRNA and proteins for the early embryonic development. After growth oocytes arrest in the prophase of meiosis I until the stimulation of maturation hormone. In starfish, maturation hormone, 1-methyladenine (1-MA), triggers the activation of maturation promoting factor, which is a major driving force for the resumption of meiosis. At the mean time, the protein synthesis machinery is also activated for translating stored mRNA. 1-MA is known to induce the activation of inhibitory G protein that leads to a bifurcate pathway including the activation of PI3 kinase and inactivation of adenylyl cyclase. Although, we know both the increase in PI3 kinase and the decrease in adenylyl cyclase activities are required for activation of MPF and protein synthesis, the downstream effectors of these signaling pathways are unclear. Shortly after the induction of 1-MA, there is an increase in protein kinase C like activity, which precedes the activation of MPF and initiation of protein synthesis. Later, this PKC-like activity is proved to be mainly due to the protein kinase C-related kinase 2 (PRK2). The initiation of protein synthesis is activated by phosphorylation of eukaryotic initiation factor 4E (eIF4E). Recently, I further demonstrated that PRK2 phosphoryaltes eIF4E *in vitro*. (Lee *et al.*, 2000. Dev Biol. **228,** 166-180.). In addition, PRK2 activity is regulated by 1-MA-induced PI3 kinase and adenylyl cyclase-dependent pathway. These observations suggest PRK2 is the key molecule mediating this G-protein-induced event during meiotic maturation in starfish. However, the

definite proof awaits PRK2 functional study *in vivo*. This study aims to elucidate the role and regulation of PRK2 during meiotic maturation in starfish oocytes. Specifically, I will ask *whether the role of PRK2 in vivo can be determined using known PRK2 activators and inhibitors, including a constitutively active and a dominant-negative PRK2.* Due to the lack of the same starfish species used in previous studies, our laboratory is trying to use a native starfish, *Archaster typicus*, collected at the beach of PenHu and *Asterias amurensis* from Tasmania, Australia. We have established a system for testing the responses of *Archaster typicus* and oocytes to maturation hormone. We also demonstrated the presence of PRK2 protein in *Asterias amurensis and possibly Archaster typicus* oocytes by Western blotting or immunoprecipitation against *Pisaster ochraceus* PRK2 antibody. In addition, we also generated (1) a constitutively active PRK2 by removing the regulatory domain and (2) a dominant-negative mutant by changing PRK2 amino acid 666 from a lysine to an arginine. The effects of PRK2 mutants described on the maturation responses of *Archaster typicus* and *Asterias amurensis* oocytes are underway.

Keywords: starfish, oocyte maturation, PRK2, eIF4E, PI3 kinase and adenylyl cyclase.

二、緣由與目的

As animal oocytes grow, they store proteins and messenger RNAs (mRNAs) that are required for early embryonic development. Meiotic arrest in starfish oocytes is relieved by a maturation hormone, 1-methyladenine (1-MA, Kanatani *et al*., 1969; Kanatani and Hiramoto, 1970). 1-MA stimulates a cascade of signals that initiates the resumption of meiosis, activates the protein synthesis machinery, and prepares the plasma membrane for interaction with sperm. Stimulation of oocytes by 1-MA leads to the activation of components of the protein synthesis machinery. The subsequent initiation of translation of stored mRNAs and the increase of protein synthesis are required for the completion of meiosis and the rapid cycles of mitosis that follow fertilization (Houk and Epel, 1974; Xu *et al*., 1993). The increase in the rate of protein synthesis during meiosis in starfish oocytes is due in part to the activation of eukaryotic initiation factor 4E (Xu and Hille, 1990). Xu *et al*. (1993) showed that eIF4E is phosphorylated in the starfish oocyte 20 minutes after the addition of 1-MA. Coincidentally or slightly before the phosphorylation of eIF4E there is an increase in a protein kinase C (PKC)-like activity in cell-free extracts from starfish oocytes (Xu *et al*., 1993). Since PKC can phosphorylate eIF4E in mammalian cells (Tusan *et al*., 1990; Morley *et al*., 1991), it is likely that PKC-like kinase may also phosphorylate eIF4E in starfish oocytes. One candidate in starfish oocytes for this early PKC-like activity is an abundant kinase homologous to human protein kinase C-related kinase 2 (PRK2). Starfish PRK2 is expressed in immature oocytes, is activated prior to eIF4E phosphorylation, and persists at least until GVBD. PRK2 is present in the cytoplasm, but is translocated to the germinal vesicle before GVBD (Stapleton *et al*., 1998). Using an *in vitro* kinase assay, we have recently demonstrated that the eIF4E glutathione-S-transferase fusion protein (GST-4E) can be phosphorylated by immunoprecipitated PRK2 in the presence of the unsaturated phosphatidic acid, 1,2 dioleoyl-glycerol-3-phosphate.
Site-directed mutagenesis of GST-4E shows that starfish eIF4E has as its putative phosphorylation site the serine residue analogous to serine-209 in mammals. More importantly, *in vitro*-phosphorylated GST4E contains the same phosphopeptides as *in vivo*-phosphorylated eIF4E as revealed by two-dimensional phosphopeptide mapping. These results suggest that PRK2 mediates the initiation of protein synthesis during maturation by phosphorylating eIF4E (Lee *et al.,* 2000). However, definite proof awaits PRK2 functional studies *in vivo*.

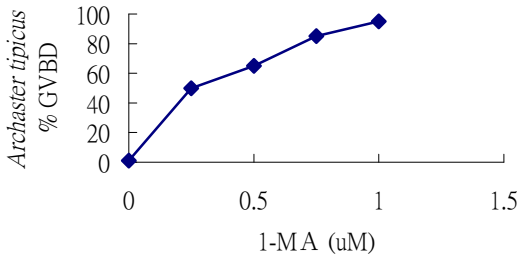
Sadler and Ruderman (1998) demonstrated that the phosphoinositide 3

kinase (PI3 kinase) inhibitors, Wortmannin and LY294002, block the activation of MPF and GVBD in starfish oocytes. PI3 kinase can be activated by the Gβγ in mammalian cells (Stephens *et al.,* 1994; Tang and Downes, 1997). These observations suggest that 1-MA induces the release of $G_i\beta\gamma$ from G_i in starfish oocytes. $G_i\beta\gamma$ then activates PI3 kinase to transmit the hormone signal through the cell. We showed that Wortmannin and LY294002 also block PRK2 activation and eIF4E phosphorylation. PI3 kinase could thus be a potential regulator of PRK2 and eIF4E (Lee *et al.,* 2000). Furthermore, the other subunit of G_i, $G_i\alpha$, is known to inhibit adenylyl cyclase, thus decreasing cAMP production and the resultant protein kinase A activity. The maturation of starfish oocytes is inhibited by cytoplasmic cAMP as artificially elevating cAMP level by treating the oocytes with the phosphodiesterase inhibitor IBMX prevents GVBD. The phosphorylation of eIF4E and the increase in protein synthesis rate are also inhibited by cytoplasmic cAMP (Meijer *et al.*, 1989; Xu *et al.*, 1993). We demonstrated that the increase in PRK2 activity is also inhibited by cytoplasmic cAMP (Lee *et al.,* 2000). It appears that PRK2 is the key mediator that relays the G-protein-dependent bifurcate pathways. However, whether PRK2 is the direct effecter of PI3 kinase or cAMP-dependent protein kinase is unknown. How does PRK2 integrate the bifurcate pathways that lead to the completion of meiosis? This is a very intriguing question that requires an understanding of PRK2 interacting molecules. We have established the in vitro maturation system of *Archaster typicus* oocytes. To investigate the role of PRK2 *in vivo* during oocyte maturation, we have made (i) PRK2 N-terminal antibody, (ii) constitutively active PRK2, (iii) PRK2 regulatory domain for manipulating PRK2 activity *in vivo*.

二、結果與討論

GVBD in *Archaster typicus* oocytes

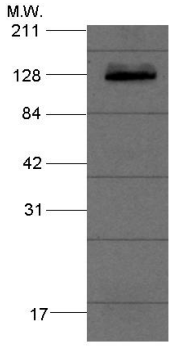
Oocytes were collected from isolated gonads from female *Archaster typicus*. Immature oocytes were washed three times with calcium free artificial seawater, one time with artificial seawater and keep at room temperature until use. Oocytes were treated with different concentrations of 1-MA and the % of germinal vesicle breakdown (%GVBD) was counted at 60 min after the addition of 1-MA. As shown in the following figure, the % of GVBD increased with the increase in the added 1-MA. At 1 μM 1-MA, >90% oocytes underwent GVBD at the end of hour. Therefore, 1 μM 1-MA will be used for *Archaster typicus* oocytes.



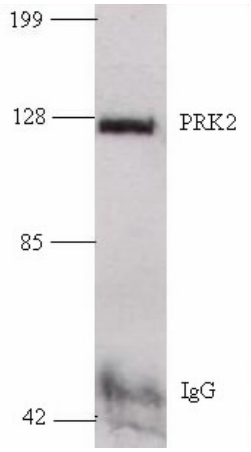
In contrast, a significant lower 1-MA (at 0.25 μM 1-MA) was needed for a full induction of GVBD in *Asterias amurensis* oocytes (data not shown)

The presence of PRK2 in Archaster typicus and *Asterias amurensis* oocytes

Immature *Archaster typicus* oocytes were lysed and processed as described by Lee *et al.* (2000). A polyclonal antibody (2298) generated against *Pisaster ochraceus* PRK2 N-terminal HR1a domain (Stapleton *et al.,* 1998) was used to detect the presence of PRK2 in *Archaster typicus* oocytes lysate by Western Blotting at a dilution factor of 1 to 200. A 130 KDa protein was recognized by antibody 2298 (as the following graph). The molecular weight is exactly the same as *Pisaster ochraceus* PRK2. However, the results was not consistent among trials.



In contrast, we consistently identify PRK2 from *Asteria amurensis* oocytes by using immunoprecipitation against Ab 2298 as shown in the following graph.



Therefore, we used the *Asterias amurensis* for the rest of the experiments.

Effects of PRK2 mutants

We have generated 3 mutants of PRK2 as described as following. A partial sequence (-ELFAIKALKK-) located at the C-terminal catalytic domain is important for the PRK2 catalytic activity. We have synthesized a mutant protein by replacing the red color-coded lysine (amino acid 666) to arginine. This mutation should disrupt the PRK2 activity. The N-terminal of PRK2 contains a pseudosubstrate site, which serve as a negatively regulatory domain for PRK2. We have also synthesized a pseudosubstrate peptide based on the starfish sequence and injected into oocytes. We found the pseudosubstrate peptide had no effects on the %GVBD (data not shown). Whether it would interfere with protein synthesis is still under investigation. To avoid the fact that pseudosubstrate peptide synthesized may be too short to exert its effects, we synthesized a regulatory half PRK2 and catalytic half PRK2 by cutting at the amino acid 623 by using restriction digestion. These mutants have been verified by sequencing. The effects of these mutants on the meiotic maturation are still undergoing. Since these PRK2 mutants are designed based on the PRK2 sequences from Pisaster *ochroceus*. To avoid the possible sequence differences between two species. We used the degenerate PCR approach as described by Stapleton *et al.*, (1998) to clone PKC isoforms from the *Asteria amurensis* oocytes. We found prominent PCR products around 200-400bp, which is consistent to the results by Stapleton *et al.* The PCR products will be sequenced and subcloned to get the complete PRK2 sequence.

Effects of C3 transferase

C3 transferase, a Rho inhibitor was injected into oocytes and the responses of oocytes to 1-MA were examined. Oocytes treated with up to 100 ng/ml C3 transferase showed normal rate of GVBD. It implied that nuclear maturation might be independent of Rho. However, since the high ionic strength of seawater hinders the effectiveness of all chemical inhibitors. Higher concentration of C3 transferase may be necessary.

四、計劃進度自評

Due to the lack of the same animal species and the establishment of a newly started laboratory, the experiment progress is lag behind the expected progress. However, since we demonstrated that PRK2 protein should be very close if not the same. We also got the required PRK2 mutants , the pace should be catch up in the sucereceeding project. PRK2 has gained more and more attention in the recent years in a variety of field. Demonstration of it's potential role during meiotic maturation and even later development is of great importance and worthwhile to be published in international

journals.

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