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作 品 名 稱：Mechanism of the subcellular localization of the actin binding protein adducin

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作者簡介

我是鄭經倫。我是一個平凡人，一個對自然科學多了熱誠的學生。喜歡在閒暇時，看些科普的書籍、參加科學教育的演講。在種種的機緣下，我有幸能進入實驗室，一睹研究者們的辛勞，並體驗科學研究的歷程。在這過程中，即使我懂得不是很多，只要我一直抱持對未知事物探索的好奇心與熱誠，我仍能成為一位小小科學家。

Mechanism of the subcellular localization of the actin binding protein adducin

Keywords: adducin, cytoskeleton, subcellular localization

1. Abstract

Adducin 蛋白在細胞骨架的調節上扮演著重要的角色。然而，近來有許多研究指出，骨架蛋白也會出現在細胞核並參與轉錄調控，因此本研究的目的即在探討 adducin 蛋白是否會進入細胞核中，並參與轉錄調控或具有其他功能。在本研究中，我們將綠色螢光蛋白(GFP)標示的 adducin 質體 DNA，利用轉染技術送入老鼠纖維母細胞株 NIH3T3 中表現。NIH3T3 細胞原本並無 adducin 蛋白的表現，在共軛焦顯微鏡下觀察，野生型的 GFP-adducin 蛋白會表現於細胞核與細胞質中。由於 adducin 蛋白尾端序列攜有可能往核內運輸的訊號，於是將位在此一訊號中的離胺酸 718 及離胺酸 719 進行突變，結果發現此一突變株只能在細胞質中表現。此外，蛋白磷酸酶 C (protein kinase C) 已知能磷酸化 adducin 蛋白在絲胺酸 716 及絲胺酸 726 的位置，於是假設其磷酸化是否與其在細胞內的分布有關。將 adducin 的絲胺酸 726 置換成丙胺酸，並不影響其在細胞內的分布。然而將絲胺酸 716 置換成丙胺酸後，則完全只在細胞核中表現。

由於 adducin 可分布於細胞核，因此我們懷疑 adducin 蛋白可能與細胞分裂有關，於是本研究利用流式細胞儀分析 adducin 轉染後 NIH3T3 細胞的細胞週期。流式細胞儀的分析結果顯示，攜有 GFP-adducin 或其突變株的細胞與未經轉染的 NIH3T3 細胞的細胞週期並沒有顯著差異。其次，為了避免因轉染的效率不高而造成統計上的誤差，我們利用顯微鏡追蹤技術觀察攜有 GFP-adducin 的細胞株，結果顯示攜有 adducin 突變株的 NIH3T3 細胞株仍能正常分裂。再者，因為 adducin 能與細胞骨架中的肌動蛋白結合，所以 adducin 不同的分布位置可能影響細胞附著與細胞展延的效率。細胞展延試驗的結果顯示， adducin 及其突變株對細胞附著與細胞展延的效率並無明顯的影響。

本研究的結果證明，adducin 的確帶有往核內運輸的訊號，其在細胞質中的分布可能也同時受到絲胺酸 716 磷酸化的影響。然而 adducin 的功用似乎與纖維母細胞的分裂與展延無明顯的關聯性。

Adducin, an actin binding protein, is known to play an important role in the regulation of the membrane cortical cytoskeleton. More and more evidence indicates that proteins involved in the cytoskeletal regulation could also reside in the nucleus and participate in gene regulation. Thus, the goal of this study is to examine whether adducin is expressed in the nucleus and involved in certain nuclear events. In this study, adducin and its various mutants were fused with green fluorescent protein (GFP) and transfected into mouse NIH3T3 fibroblasts which do not have endogenous adducin for monitoring their subcellular distribution under a laser scanning confocal

microscope. The wild-type GFP-adducin was found to be present both in the nucleus and in the cytoplasm. The COOH-tail of adducin contains a motif analogous to the nuclear localization signal (NLS). Mutation of two lysine residues (lysine 718 and lysine 719) located within this motif abolished the nuclear localization of adducin. Moreover, adducin is known to be phosphorylated by protein kinase C at serine 716 and 726. Substitution of adducin serine 726 with alanine had no effect on its subcellular localization. In contrast, substitution of adducin serine 716 with alanine led to only nuclear expression.

Nuclear localization of adducin renders it possible that adducin may be involved in the regulation of cell division cycle. For cell cycle analysis, flow cytometry was applied. The results of flow cytometry indicated that expression of adducin and its mutants in NIH3T3 fibroblasts did not affect their cell cycle progression. To further examine the effect of adducin on cell division, NIH3T3 cells transiently transfected by adducin were monitored by time lapse video-microscopy. The video clearly showed that the cells with GFP-adducin underwent cell division to generate two daughter cells. Since adducin is well known to bind to actin and thereby regulate microfilaments, we wondered that expression of adducin in NIH3T3 cells might affect their adhesion and spreading onto extracellular matrix proteins. The results of cell spreading assays showed that adducin appeared not to affect cell spreading.

In conclusion, our results demonstrate that the subcellular distribution of adducin is likely regulated by two signals, one is the nuclear localization signal and the other is the phosphorylation status of the serine 716. However, enforced expression of exogenous adducin in fibroblasts such as NIH3T3 cells does not alter their cell cycle or cell spreading on fibronectin.

2. Introduction

The integrity of the plasma membrane relies on the membrane cytoskeletal structure, which is essential for the cell to function normally. The cellular membrane cytoskeleton is a network of fibers composed of actin filaments, spectrin, adducin, and others, as shown in Figure 1. Likewise, the nuclear cytoskeleton is also required for the nucleus to maintain its three-dimensional structure within the cell. Adducin is already known to bind to spectrin at the cytoplasmic face of the plasma membrane. Since actin, spectrin and other proteins involved in the cytoskeletal regulation were recently reported to reside in the nucleus, it is possible that adducin may also localize in the nucleus and participate in certain nuclear activities.

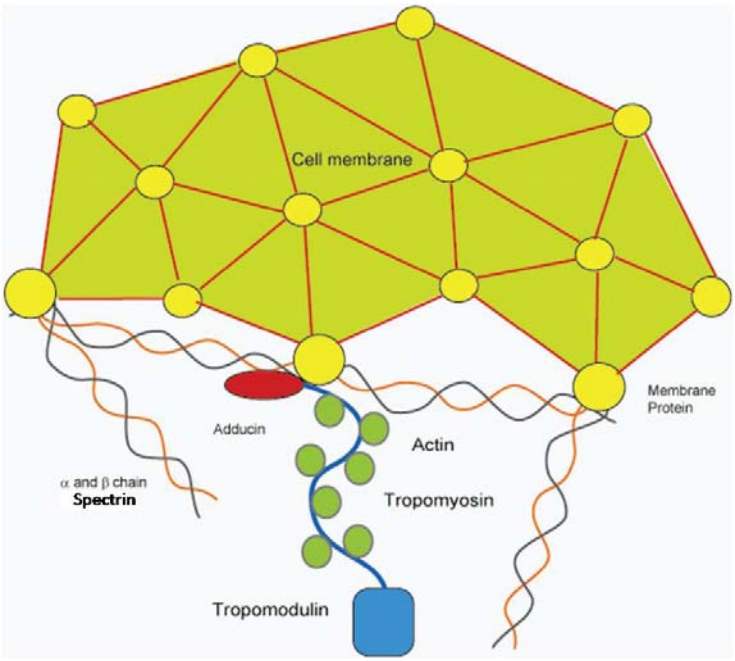


Figure 1: Adducin complexes with spectrin and actin filaments to support the plasma membrane

Adducin is encoded by three closely related genes termed α, β and γ adducin. Each adducin proteins contains an N-terminal globular head domain, a neck domain and a C-terminal protease-sensitive tail domain. At the end of tail domain there is a 22-residue MARKS-related domain that has high homology to myristoylated alanine-rich C kinase substrate (MARCKS) protein. The phosphorylation sites of PKC are resided on the MARKS-related domain of adducin.

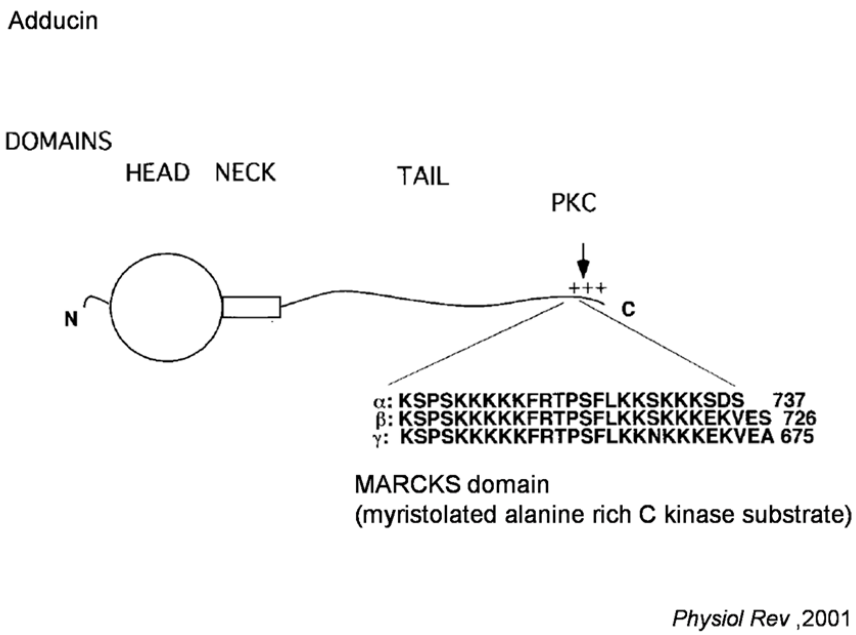


Figure 2. The domains of adducin monomer

Our laboratory recently found that adducin was localized in both the cytoplasm and the nucleus of Madin-Darby canine kidney epithelial cells. The goal of this study is to examine the mechanism by which adducin shuttles between the cytoplasm and the nucleus. In addition, the potential function of adducin in fibroblasts will be examined as well.

3. Materials and Methods

(1) Materials

The plasmids pEGFP-C1-α-adducin was kindly provided by Vann Bennett (Duke University, Durham, NC). All mutagenesis was carried out using QuikChange site-directed mutagenesis kit (Stratagene).The mutagenic primers used are:

α-adducin S716A:
5'-GGTCTCCAGGCAAGTCCCCGGCCAAAAAGAAGAAGAAGTTCCG-3';
α-adducin S716E: 5'-GGTCTCCAGGCAAGTCCCCGGAGAAAAAGAAGAAGAAGTTCCG-3'; α-adducin K719A:
CAAGTCCCCGTCCAAAAAGGCGAAGAAGTTCCGTACCCCG-3' ;
α-adducin K718A/719A:
5'-GGCAAGTCCCCGTCCAAAAAGGCGAAGAAGTTCCGTAC-3'.
α-adducin S726A:
5'-GTTCCGTACCCCGGCCTTTCTGAAGAAGAG-3';

The positions of substituted codons are underlined. The desired mutations were confirmed by dideoxy DNA sequencing, a service provided by the Biotechnology Center of National Chung Hsing University, Taiwan.

(2) Cell culture and transfections

NIH3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum and cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air atmosphere. NIH3T3 cells were transfected with pEGFP-C1, pEGFP-C1-α-adducin, pEGFP-C1-α-adducin-S716A, pEGFP-C1-α-adducin-S726A, pEGFP-C1-α-adducin-S716A/S726A, pEGFP-C1-α-adducin-S716E, pEGFP-C1-α-adducin-K719A, or pEGFP-C1-α-adducin-K718/719A using LipofectAMINE (Invitrogen Life Technologies)

(3) Laser-scanning confocal fluorescent microscopy

Cells were plated on 12-mm collagen-coated glass coverslips for 24 hours, fixed for15 minutes in phosphate-buffered saline containing 4% paraformaldehyde. Coverslips were stained with DAPI for 30 minutes. Coverslips were mounted in anti-fading solution and viewed using a Zeiss LSM510 laser-scanning confocal microscope image system with a Zeiss 63X Plan-Apochromat objective. Wavelengths 488 nm and 359nm were used to excite GFP and DAPI, respectively. A beam path filter (BP 505-550 nm) and a long path filter (LP 465 nm) were used to acquire images for the emission from GFP and DAPI, respectively, in a multi-track channel mode.

(4) Cell spreading assay

NIH3T3 fibroblasts were collected by trypsinization and suspended in serum-free medium at 10^6 cells/ml. 2 ml of cell suspension was added to a 60-mm dish that had been coated with fibronectin (10 ng/ml) and blocked with bovine serum albumin. Cells were allowed to spread, and spread cells were scored under a phase-contrast microscope at indicated intervals. Cells with extended processes and not phase bright were defined as spread cells.

(5) Time lapse video-microscopy

NIH3T3 fibroblasts on the microscope stage were maintained at 37°C with a humid CO_2 atmosphere in a micro-cultivation system (model POC-R, Zeiss) with temperature and CO_2 control devices (tempcontrol 37-2 digital and CTI controller 3700 digital, Zeiss). Cells were monitored under differential interference contrast (DIC) optics on an inverted Zeiss microscope (Axiovert 100) using Zeiss 40X LD Achroplan objective. Time-lapse sequential micrographs were captured every 5 minutes using a cooled CCD camera (CoolSNAP *fx*, Roper Scientific) and analyzed by MetaImaging Series™ software (version 4.5) from Universal Imaging Corporation (WestChester, PA).

(6) Flow cytometry

NIH3T3 fibroblasts (5 x10^6 cells) were collected by trypsinization, washed once with phosphate-buffered saline, and fixed in phosphate-buffered saline containing 2% paraformaldehyde for 60 min. The cells were suspended in 70% ethanol overnight at -20℃ and then stained in a solution containing 40μg/ml of PI and 100μg/ml of ribonuclease at 37℃ in the dark for 30 min before collecting data. Data were collected by Cytomics™ FC500 flow cytometer and analyzed by CXP Analysis software.

4. Results

(1) Distribution of endogenous adducin in the nucleus and the cytoplasm of MDCK cells.

Endogenous adducin of MDCK cells was detected in the nucleus and the cytoplasm by immunofluorescent staining with polyclonal anti-adducin under a confocal fluorescent microscope (Figure 3).

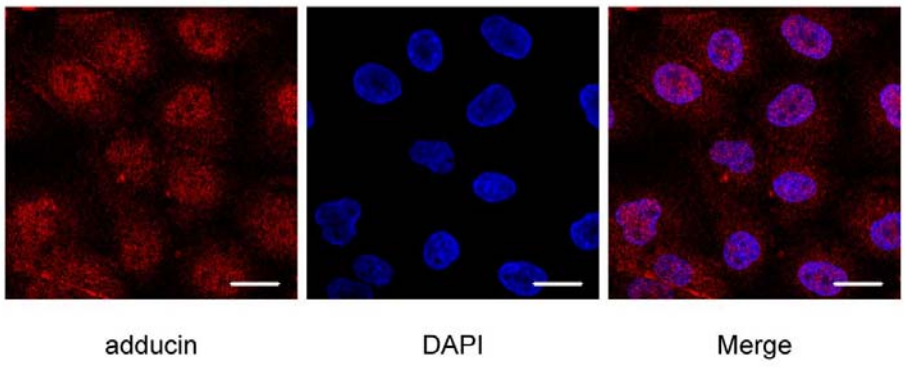


Figure 3. MDCK epithelial cells express endogenous adducin that distributes in the nucleus and cytoplasm

(2) Localization of GFP-adducin proteins in NIH3T3 cells

GFP-adducin was transiently expressed in NIH3T3 cells and its localization of was defined into three groups:

a. Expression in the nucleus only.

b. Expression in the cytoplasm only.

c. Expression in both nucleus and cytoplasm

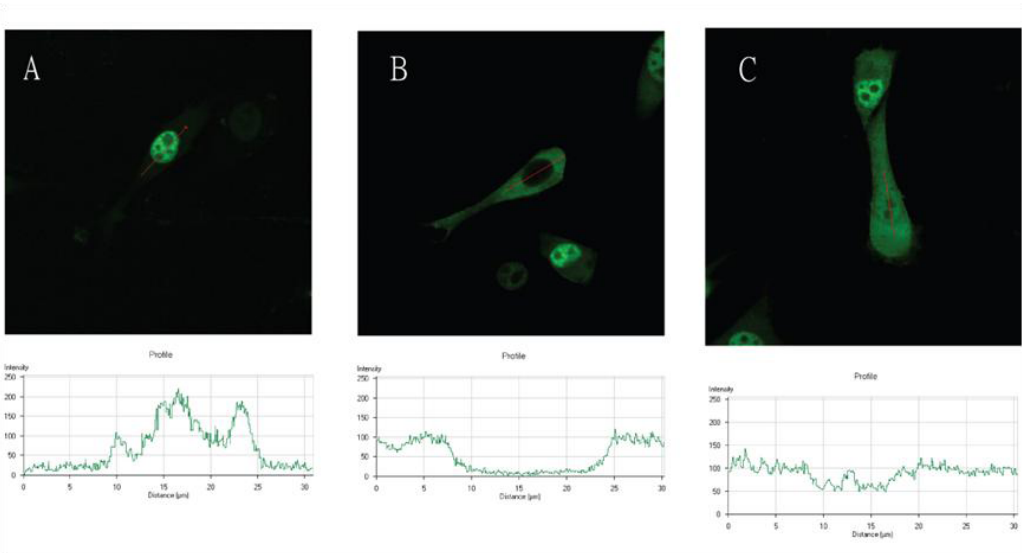


Figure 4. Transient expression of GFP-adducin in NIH3T3 cells

(3) The effect of mutation on the localization of adducin

a. Control GFP, all expressed in both cytoplasm and nucleus.

b. Wild type GFP-adducin, 38% in nucleus and 60% in both cytoplasm and nucleus.

c. GFP-adducin (S716A) mutant, all in the nucleus.

d. GFP-adducin (S716E) mutant, 56% in the nucleus and 40 % cell in both cytoplasm and nucleus.

e. GFP-adducin (S726A) mutant, 48% in the nucleus and 52 % cell in both cytoplasm and nucleus.

f. GFP-adducin (S716/726A) mutant, all in the nucleus.

g. GFP-adducin (K718A) mutant, 68% in the cytoplasm and 28 % cell in both cytoplasm and nucleus.

h. GFP-adducin (K718/719A) mutant, all in the cytoplasm.

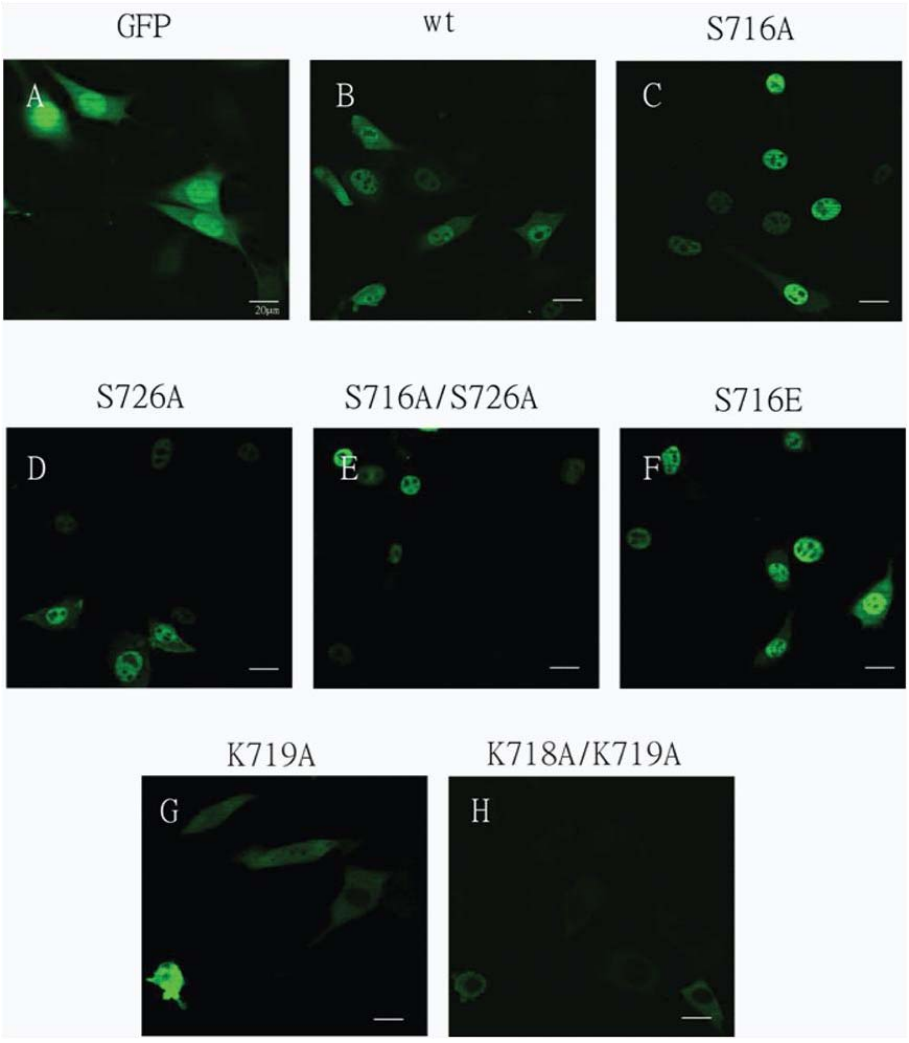


Figure 5. Localization of GFP-adducin and its mutants in NIH3T3 cells

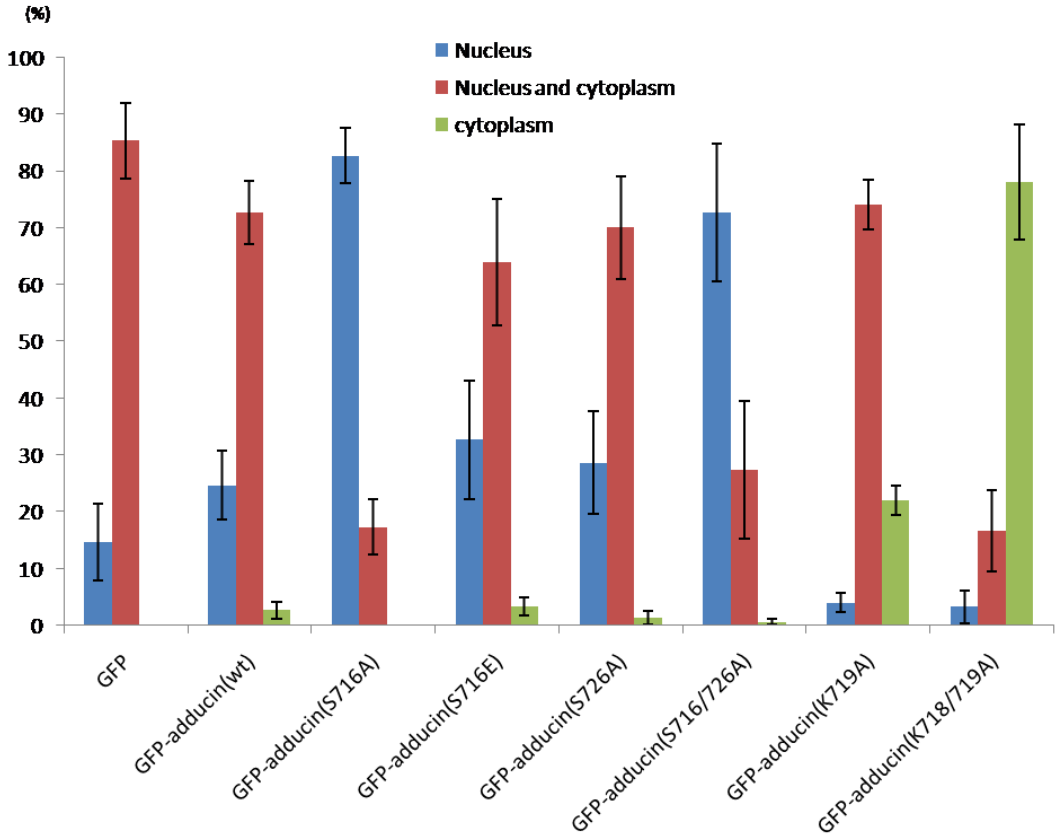


Figure 6. The quantification of the localization of adducin and its mutants in NIH3T3 cells

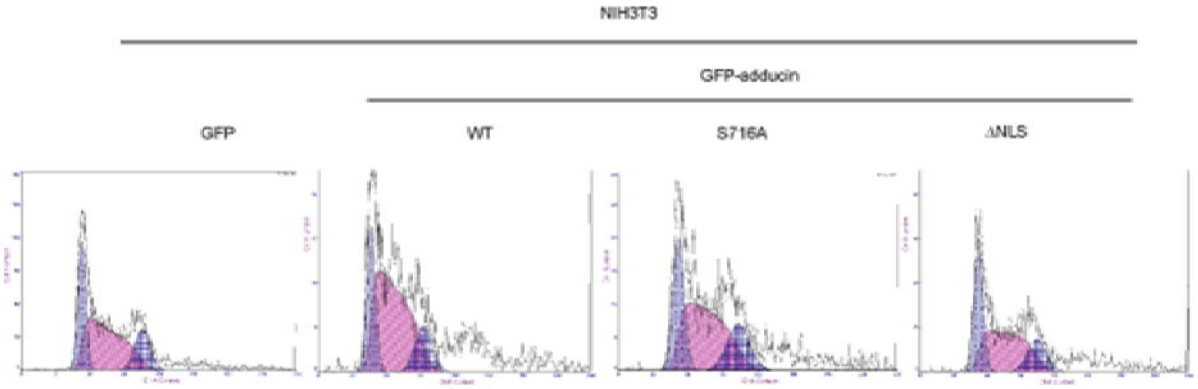
(4) The effect of adducin mutants on the cell cycle

a. NIH3T3 fibroblasts transfected with GFP as the control

b. NIH3T3 fibroblasts transfected with wild type GFP-adducin had a slightly larger portion on S phase

c. NIH3T3 fibroblasts transfected with GFP-adducin (S716A) mutant exhibited a cell cycle profile similar to the control

d. NIH3T3 fibroblasts transfected with GFP-adducin (K718/719A) mutant exhibited a cell cycle profile similar to the control



%Gated	GFP	GFP-adducin	GFP-adducin S716A	GFP-adducin NLS
G0/G1	28.6	22.9	27.6	32.1
S	53.4	62.8	53.1	50.7
G2/M	18	14.3	19.3	17.2
All	100	100	100	100

Figure 7. The cell cycle profile of NIH3T3 fibroblasts transfected with GFP-adducin or its mutants

(5) The effect of different adducin mutants on cell division

a. NIH3T3 fibroblasts transfected with GFP-adducin (S716A) mutant could divide normally. The GFP-adducin (S716A) mutant seemed to gather again in the nucleus after cell division.

b. NIH3T3 fibroblasts transfected with GFP-adducin (K718/719A) mutant could divide normally.

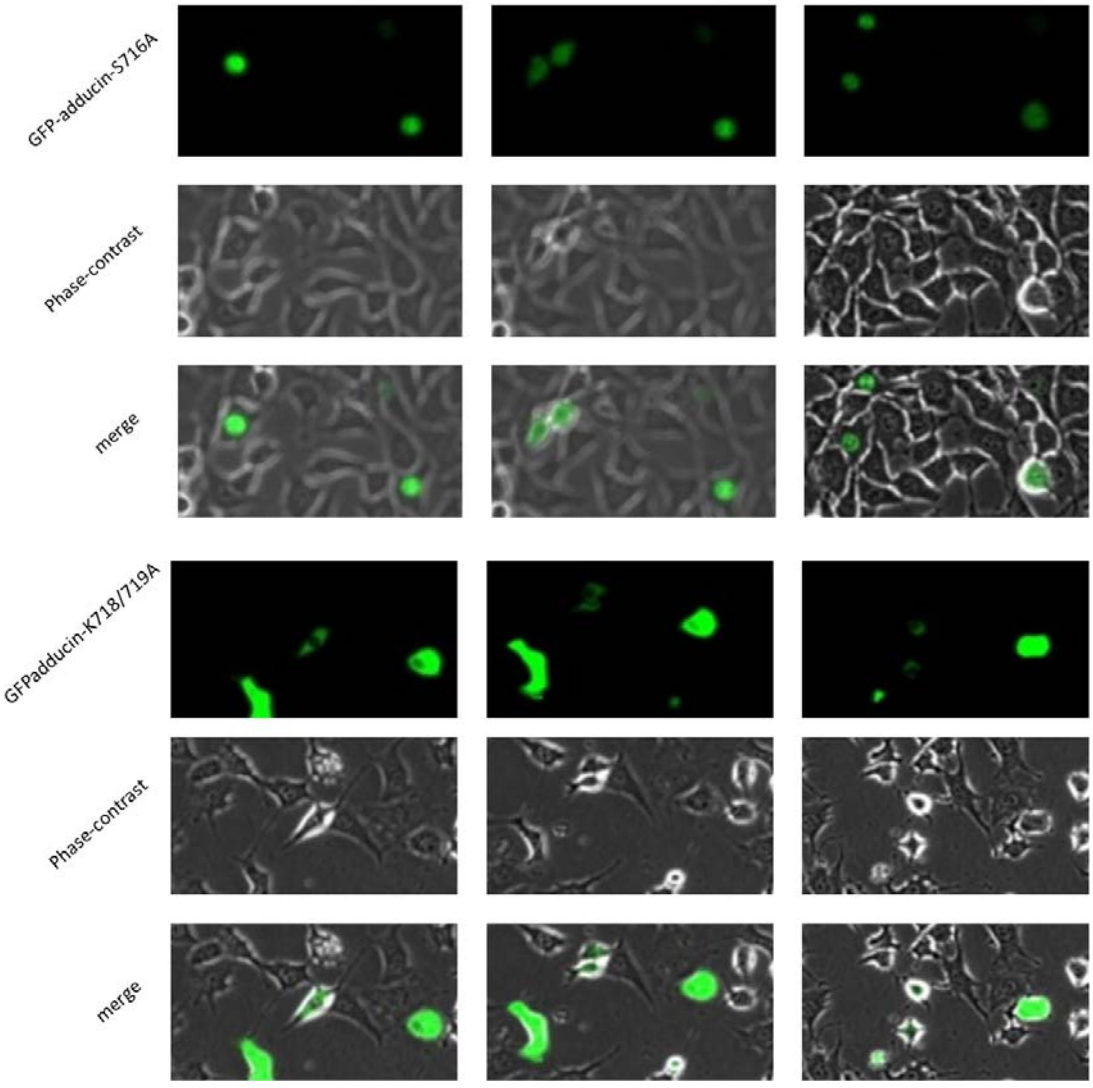


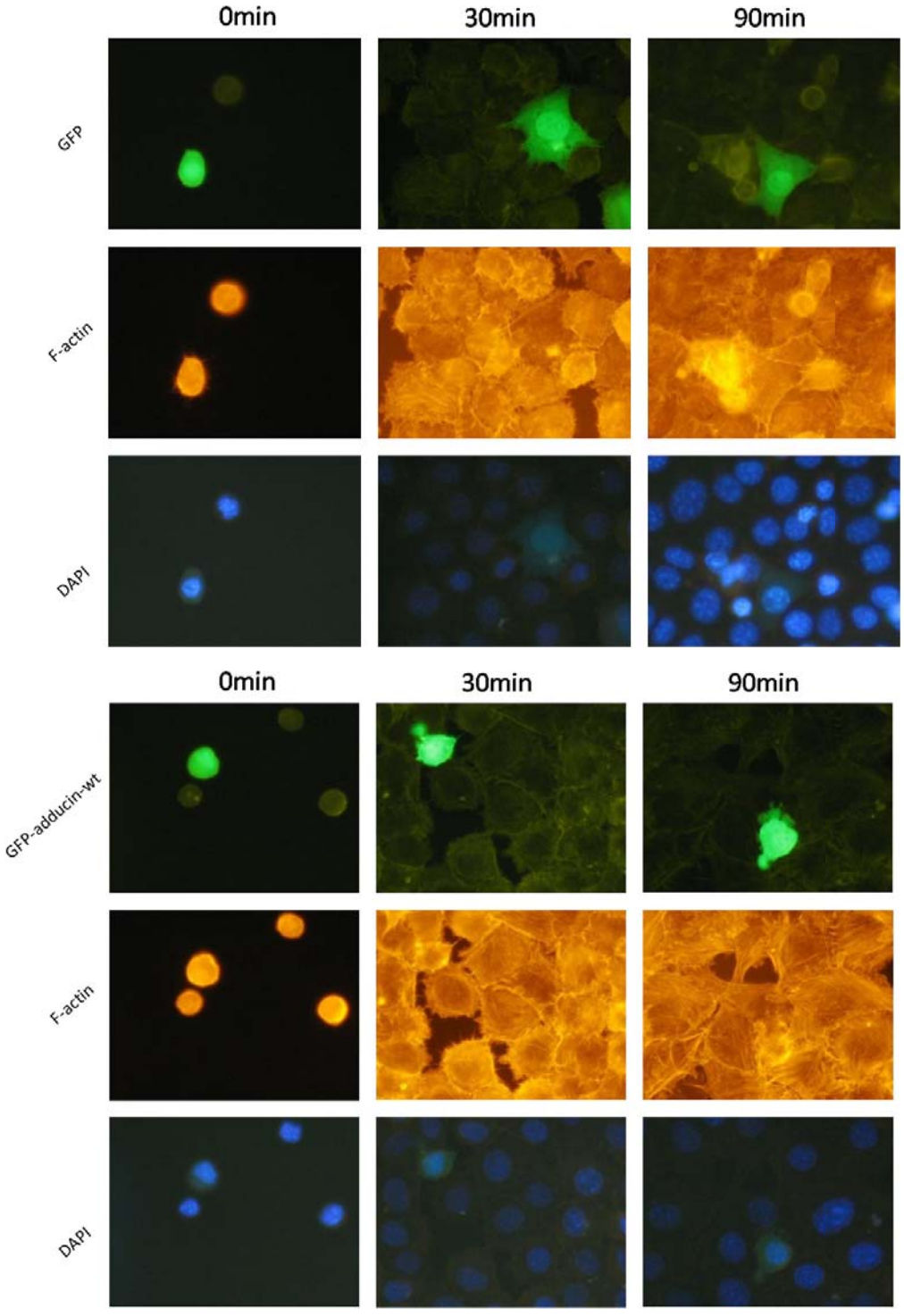
Figure 8. NIH3T3 fibroblasts transfected with adducin or its mutants can undergo cell division

(6) The effect of different adducin mutants on cell spreading

a. NIH3T3 fibroblasts transfected with wild type GFP-adducin adhered at 30 minutes and spread at 90 minutes under the cell spreading assay.

b. NIH3T3 fibroblasts transfected with GFP-adducin (S716A) mutant adhered at 30 minutes and spread at 90 minutes under the cell spreading assay.

c. NIH3T3 fibroblasts transfected with GFP-adducin (K718/719A) mutant adhered at 30 minutes and spread at 90 minutes under the cell spreading assay.



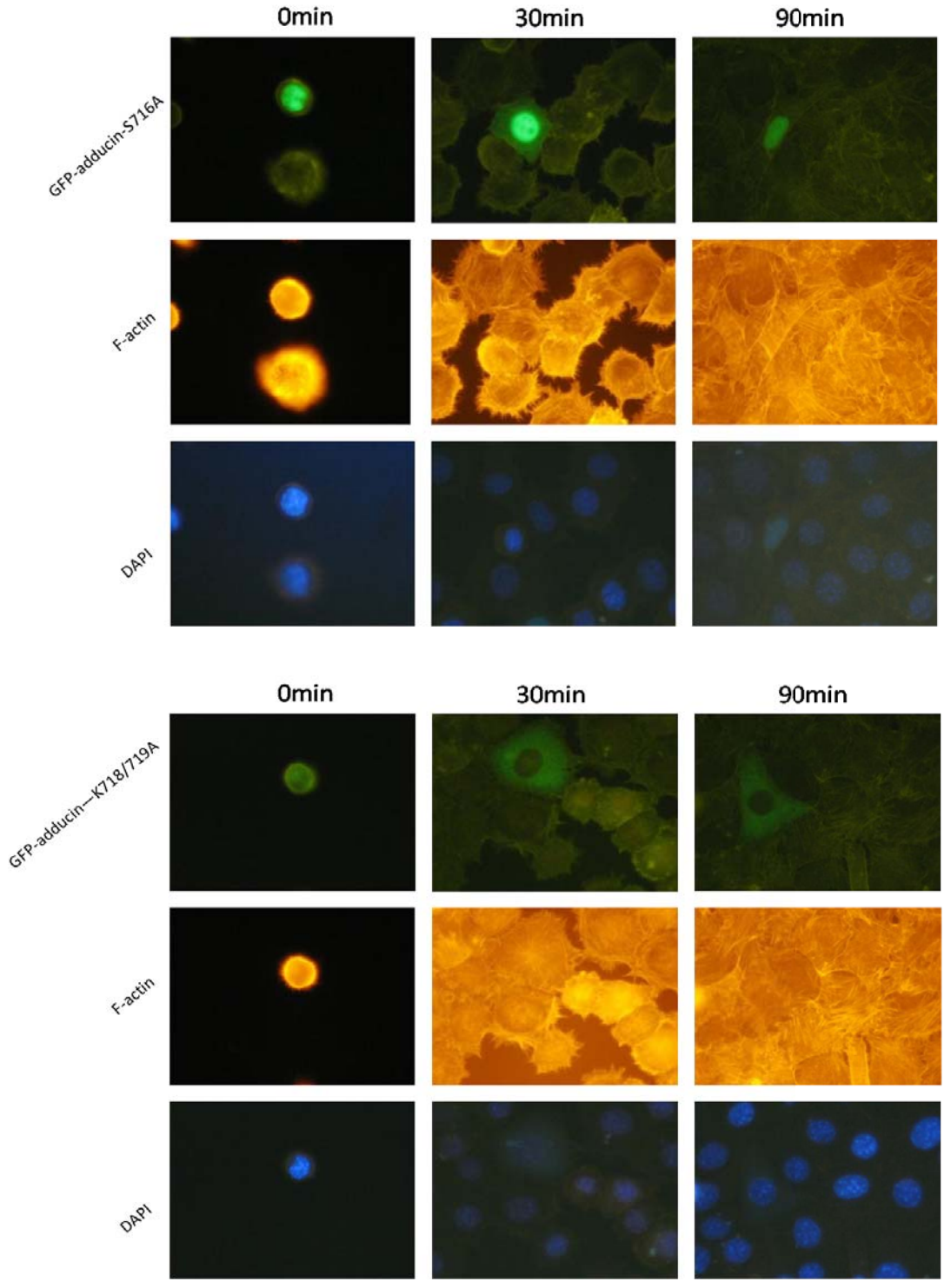


Figure 9. Expression of GFP-adducin or its mutants in NIH3T3 cells does not affect their spreading on fibronectin

5. Discussion

Adducin contains a putative nuclear localization signal (NLS) at its C-terminal domain, characterized by consecutive positive charged residues. In this study, we found that mutation of a single residue at the lysine 718 of adducin within the NLS motif renders it a tendency to express in the cytoplasm. Moreover, mutation of two lysine residues at 718 and 719 completely abolishes the nuclear localization of adducin. In addition, the tail domain of adducin ends with a highly conserved 22-residue myristoylated alanine-rich C kinase substrate (MARCKS)-related domain, which contains the major phosphorylation site for protein kinase C. Our results show that adducin with mutation at the serine 716, but not serine 726, became solely localized in the nucleus, indicating that the serine 716 of adducin is involved in its subcellular localization. Therefore, we conclude that the C-terminal domain of adducin does contain a NLS which leads adducin to the nucleus, while the phosphorylation of the serine 716 may keep it in the cytoplasm.

In this study, we used NIH3T3 fibroblasts as a model to examine the cellular function of adducin. The reason is because NIH3T3 cells do not express endogenous adducin, any changes by ectopic expression of adducin might be easier to be observed. Moreover, to distinguish the function of adducin in the nucleus *versus* the cytoplasm, adducin and two its mutants *i.e.* S716A and K718/719A were employed. The S716A mutant only expressed in the nucleus, but on the other hand, the K718/719A double mutant only expressed in the cytoplasm. Unfortunately, transient expression of adducin or its mutants in NIH3T3 cells did not apparently affect their cell cycle progress or cell spreading on fibronectin. The failure for adducin to cause a biological consequence in NIH3T3 cells may be due to (i) the type of cells chosen for biological assays, (ii) the low efficiency of transfection in NIH3T3 cells, and (iii) inappropriate types of the assay used for the function of adducin. Adducin is expressed in epithelial cells, platelets, erythrocytes, and nerve cells. It is possible that the function of adducin may be less important in fibroblasts than in other types of cells or it is replaced by other closely related proteins in fibroblasts. In addition, low efficiency rate of transfection in NIH3T3 cells might mislead interpretation of the results. Finally, adducin may actually generate some subtle alterations in the function of NIH3T3 cells, which may not be detected by the assays used in this study.

Nuclear actin has emerged as a new field in cell biology, which has been implicated to exert functions in transcription, chromatin dynamics, and mitosis. Spectrin, the major binding partner of adducin, has been found to reside in the nucleus, therefore, our finding that adducin contains a NLS for its nuclear localization was not

too surprising. The question is whether adducin actually forms complexes with spectrin and actin in the nucleus. If so, does the adducin-spectin-actin complex participate in certain nuclear events? In this study, we have known more about the mechanism for adducin shuttling between the nucleus and the cytoplasm, which will be helpful for us to uncover the role of adducin both inside and outside the nucleus.

6. References

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評語

1) 本實驗方向明確，架構良好。

2) 作者本人有非常好的學業成績及求知態度。

3) 因時間不足，本作品仍有不同程度之不完整性，但已足以取得獎項。

4) 建議繼續。