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理事長　　中島　　章　殿

Ⅰ．研究者氏名　庚海燕

研究機関 東京大学 医科学研究所 細胞生物化学 研究指導者 竹縄忠臣 教授 職名 教授

所在地 〒108 東京都 港区 白金台 4-6-1　電話 5449-5508 内線

Ⅱ．過去の研究歴

1988.8～1992.1 中国深圳市人民医院 内科 医者

1993.1～1994.3 日本 東京大学 医科学研究所 研究生

1994.4～現在 日本 東京大学 医学部 博士課程在学中

Ⅲ．過去の研究実績

1994. 第67回日本生化学会大会. <<New PIP_2 binding protein – Histone H1 and H3>>. 1995. 第68回日本生化学会大会 <<PIP_2 bound to histone H1 decreased when histone H1 was phosphorylated by PKC>>.

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

第69回日本生化学会大学. 第19回日本分子生物学会年会合同年会. 3-P-1078. <<Interaction of PIP_2 and histone H1 reversed the transcription repression by histone H1>>.

(2) 学会誌等に発表した論文　　無 ・ 有　　（雑誌名・論文名）

<<A New Phosphalipase C δ_4 is induced at S-phase of the cell cycle and appears in the nucleus>> J. Biol. chem. 271. 355-360.

<<Phosphatidylinositol 4,5-bisphosphate reverses the inhibition of RNA transcription caused by histone H1>>. J. Biol. chem. 投稿中.

Ⅴ．今後の研究計画及び希望

1. 細胞骨骼系において phophalipase $c\gamma_1$ の機能解析. 論文書く中.

2. phosphalipase $c\delta_1$ の機能解析. 実験中.

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 2,000字程度で記載して下さい。）(別紙)

Ⅶ. 指導教官の意見

庾海燕氏は骨格筋由来の細胞膜でのインスリン脂質代謝に取り組まれ，核内の脂質代謝に注目したことは，非常に独創的な点です。また，糖尿病や細胞増殖に関わることが示唆されながら，解析の遅れているオスカルパーゼ08に着目している点は，興味深いことであると思います。また，庾氏は物理学的観点に優れた知識を持つと共に，大変勤勉で，種々の実験に積極的に取り組みます。今後，大きな成果が出ると確信しています。

Yu. 97.1.23.

Phosphatidylinositol 4,5-bisphosphate reverses the inhibition of RNA transcription caused by histone H1

Haiyan Yu*, Kiyoko Fukami*, Yutaka Watanabe**, Choichiro Ozaki**, and Tadaomi Takenawa* †

*Department of Biochemistry, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan, **Faculty of Technology, University of Ehime, Matsuyama-shi, Ehime 790, Japan

Running title: PIP2-binding protein, histone H1

† Correspondence should be addressed to T. Takenawa
(Tel:81-3-5449-5510, Fax:81-3-5449-5417)

The abbreviations used are: PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; IP3, inositol 1,4,5-trisphosphate; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; CL, cardiolipin; PG, phosphatidylglycerol; PLC, phospholipase C; PKC, protein kinase C; PKA, protein kinase A; PH domain, pleckstrin homology domain; ARF, ADP-ribosylation factor; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulphonyl fluoride; DIFP, diisopropyl fluorophosphate; Hepes,4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes,4-morpholineethanesulfonic acid; TFA, trifluoroacetic acid

Introduction

PIP2 is recognized as the source of the bioactive second messenger inositol trisphosphate(IP3) for intracellular Ca^{2+} mobilization and diacylglycerol(DG) for PKC activation. In addition to its role as a signal-generating lipid, PIP2 has been shown to modulate the functions of various proteins such as PKC(1,2), μ-calpain(3), ADP-ribosylation factor(ARF) 1(4), and phospholipase D(5). PIP2 also binds to actin-regulating proteins such as profilin(6), cofilin(7), gelsolin(8), gCap(9), and α-actinin(10) and regulates the functions of these proteins. It has been reported that a decrease in the amount of PIP2 bound to α-actinin and vinculin correlates with the depolymerization of actin *in vivo*(11). Recently, it has been also demonstrated that the interaction of PIP2 with PH domains(12) and the PTB domain(13) is important for anchoring proteins at the membrane surface. All these data suggest that PIP2 not only generates second messengers, but also modulates the function of PIP2-binding proteins.

Increasing evidence indicates that PIP2 is present in the nuclear membrane and nuclear matrix as well as in the plasma membrane and cytoplasmic cytoskeleton. Likewise, the isolated membrane-free nuclei from Friend erythroleukemia cells have enzymes that produce both PIP and PIP2(14), and a decrease in nuclear PIP and PIP2 was observed in Swiss 3T3 cells in the early stages of the response to insulin like growth factor(IGF-1)(15) and in Hela cells during S-phase(16). In addition, PIP2 hydrolysis activity has been demonstrated in nuclei. Martelli *et al.* have reported that the purified nuclei of 3T3 cells contain PLCβ and that IGF-1 treatment stimulates the activity of nuclear PLC(17). Furthermore, a PLC isoform confined to the nuclei has been

Materials and Methods

Materials---Histone H1, H3, staphylococcus aureus V8(V8 protease), and PKA were purchased from Boehringer Mannheim. PKCs purified from rat brain were a kind gift from Dr. Inagaki(Aichi Cancer Center, Japan), and Cdc2 kinase was from Upstate Biotechnology Incorporated. [^{3}H]PIP2(7.6 Ci/mmol) and [γ-^{32}P]ATP were purchased from Du Pont-New England Nuclear and Amersham, respectively. *Drosophila* Embryo Nuclear Extract *in vitro* Transcription System was from Promega. The peptide gel filtration column(Superdex Peptide HR 10/30), protein gel filtration column(Superose 6) and reverse phase C18 column(TSKgel ODS-80TMCTR) were from Pharmacia Biotech and TOSOH(Japan), respectively. DEAE-cellulose(Whatman), cellulose phosphate(Whatman), and Hi-Trap heparin column(Pharmacia) were purchased as indicated. PIP2 and PIP was prepared from bovine spinal cords by the method previously reported(27), PI, phosphatidylcholine(PC), phosphatidylserine(PS), and CL were purchased from Sedary, PG was from Sigma. Diparmitoyl PIP3 was chemically synthesized.

Preparation of antibodies---Polyclonal antibodies to histone H1 and H3 were prepared by injecting purified histone H1 or H3 into New Zealand white rabbits. Thereafter, booster injections were administered every two weeks for two months. The antibody was purified by 20-38 % ammonium sulfate fraction. Monoclonal antibody to PIP2 was prepared as described before(24,25).

with 0.1 N HCl. A part of the treated histone H1 was subjected to SDS-PAGE, transferred to nitrocellulose filters, and then immunostained with anti-PIP2 antibody. To show that the association of PIP2 affect the apparent molecular weight of histone H1, the other parts of the treated histone H1 were applied to gel filtration column(Superose 6) previously equilibrated with the buffer containing 20 mM Tris-HCl(pH, 7.5) and 0.1 M NaCl, and eluted with the same buffer. For dot-blot analysis, peptides and proteins were spotted on a nitrocellulose filter and stained with anti-PIP2 antibody.

Extraction of PIP2 from histone H1 and histone H3---200 μg of purified histones H1 and H3 were extracted with 2 ml chloroform/methanol(2/1) and 0.5 ml 1N HCl. The chloroform layer was evaporated under a N2 stream and the residue was spotted onto a thin-layer chromatography(TLC) plate. The plate was developed in chloroform/methanol/ammonia/H2O(14/20/3/5, v/v) and immunostained with anti-PIP2 antibody.

Preparation and analysis of peptides from histone H1---50 μg Histone H1 was digested overnight with 50 μg/ml V8 protease in 50 mM phosphate buffer, pH 7.8. The peptides from the digest were separated by high performance liquid chromatography(HPLC) on a C18 column with a linear gradient of 0-60 % acetonitrile in 0.091% trifluoroacetic acid(TFA) as described before(28). Each eluted peptide was lyophilized and then solubilized in water. The molecular mass of the peptide was determined by HPLC on a peptide gel filtration column equilibrated and eluted with 10% acetonitril in 0.1% TFA. The amino acid sequencing was performed by a protein sequencer(Shimadzu, Japan).

plasmid(32), and the supercoiled pKr plasmid was used for *in vitro* transcription. RNA synthesis was performed as described in the Technical Bulletin(Promega) using a *Drosophila* embryo nuclear extract in the presence or absence of histone H1 or phospholipid vesicles. Phospholipid/histone H1 vesicles were prepared as follows. Lipids in organic solution were dried under a N2 stream, suspended in histone H1-containing solution by sonication. The quantity of cDNA obtained from a specific primer extension reaction(reverse transcription) shows the amount of the specific mRNA. The primer for reverse transcription is 5'TATTACTCGCGGTTGTGTGTGGCACAAC, which hybridizes to the *Kruppel* mRNA from +45 to +73. The primer extension reaction was performed at 37 ^{o}C for 60 min in the buffer described in the Technical Bulletin. The predominant products, 72 and 68 bases in length, were separated on a denaturing gel containing 8 % acrylamide, 7 M urea, and TBEx1 buffer. The gel was subjected to autoradiography.

of PIP2 with histone H1 and H3 did not occur during preparation of histones.

To clarify the specificity of PIP2-binding to histone H1 further, histone H1 was incubated with various amounts of PIP2 and western blot was carried out. As shown in Fig. 1d, exogenously added PIP2 was detected on histone H1 in dose dependent manner, suggesting that this binding is specific and very strong. **To confirm that anti-PIP2 antibody does not crossreact with histone H1, PIP2 bound to histone H1 was removed by the incubation with 0.1 N NaOH. Though histone H1 protein was stable under this treatment, it was no more stained with anti-PIP2 antibody(Fig. 1e), suggesting that anti-PIP2 antibody did not react with histone H1 protein by itself. Furthermore, gel filtration study showed that 0.1 N NaOH-treated histone H1 eluted more slowly than non-treated histone H1(Fig. 1f), showing that apparent molecular weight of histone H1 was reduced by removal of bound PIP2.**

It appears to be important to understand the interaction between PIP2 and histone H1. Therefore, we next determined the PIP2-binding site in histone H1. When histone H1 was digested by V8 protease and subjected to C18 reverse column chromatography, seven peptides were obtained(No 1-7 in Fig. 2a). Among these peptides, peptide 1 was identified as containing the PIP2-binding site by dot-blot analysis with anti-PIP2 antibody in addition to original histone H1(No. 0)(Fig. 2b). PIP2 bound to peptide 1 was still remained after HPLC as seen in α-actinin(28). The molecular mass of peptide 1 was

of PKC(34), PKA(35), and cdc2 kinase(36) in rat H1d, human H1b, and bovine H1, we recognized that all the phosphorylation sites are nearly conserved among the subtypes and that the PKC phosphorylation site is close to the PIP2-binding site. Thus, we assume that the phosphorylation of serine-104 by PKC induces the decrease in PIP2 binding to histone H1.

Since histones have been known to suppress the transcription by RNA polymerase II, we were interested in the effect of inositolphospholipids on the transcriptional inhibition mediated by histone H1 in eukaryotic cells. We next examined whether PIP2 can counteract the repression of RNA polymerase II transcription by histone H1 using a *drosophila* embryo transcription system. Transcription by RNA polymerase II was inhibited 90-95% by 1 μM H1 and completely inhibited by 4 μM H1 in the absence of phospholipid(second lanes from left in Fig. 4a, b, and c). The addition of PIP2 to the reaction mixture in the ratio of 10 mol PIP2 to 1 mol histone H1(1 μM) reversed the transcriptional inhibition by histone H1 to 62 % of the basal transcription level(lane 4 compared to lane 1 in Fig. 4a). Antirepression caused by PIP3 or PIP was also found, but these effects were much weaker than that of PIP2. The degree of transcriptional activity was about 12 % in the presence of PIP3(10 μM) and 6% in the presence of PIP(10 μM). And a slightly effect of CL and PG at 10 μM were observed as Hirai *et al*(23) have reported(Fig. 4b). On the other hand, PI, PS, and PC had no effect at all at 40 μM(Fig. 4c). In addition, we found that IP3 does not affect the transcriptional inhibition by histone H1(data not shown). All these data suggest that inositolphospholipids, especially PIP2, interact with histone H1 and are involved in regulation of the RNA transcription.

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non-treated histone H1 and the lower one shows that of the NaOH-treated histone H1.

Fig. 2. Identification of the PIP2 binding site of histone H1.

(a)Peptides from histone H1 digested with V8 protease were applied to a C18 reverse-phase column and eluted with a linear gradient of 0-60% acetonitrile in 0.091 % TFA. (b)The eluted peptides (peptides 1-7) in (a) were lyophilized and spotted onto a nitrocellulose membrane along with authentic histone H1(0), and then immunostained with anti-PIP2 antibody. (c)The molecular mass of the PIP2 binding peptide was identified by peptide gel filtration column chromatography using standard proteins including cytochrome C(12.5 kDa), aprotinin(6.5 kDa), insulin B-chain(3.5 kDa), substance P(1.3 kDa). (d)The first N-terminal 29 amino acid residues were determined by protein sequencing.

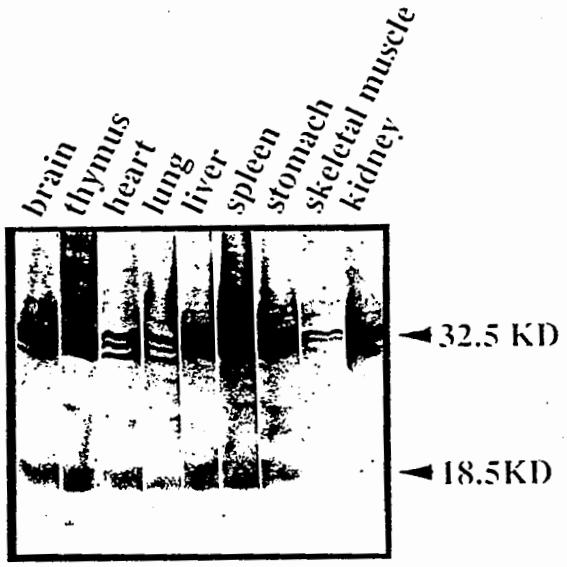
Fig. 3. Effect of the phosphorylation of histone H1 on PIP2-binding.

(a)0.1 μg of Histone H1 phosphorylated for 60 min by PKC, PKA, or cdc2 kinase was subjected to 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was immunostained with anti-PIP2 antibody and autoradiographed. (b)The time course for the change in PIP2 level during the phosphorylation of histone H1 by PKC is shown. Histone H1 phosphorylated by PKC for 0, 5, 15, 30, and 60 min was subjected to 10% SDS-PAGE, transferred to a nitrocellulose filter, immunostained with anti-PIP2 antibody,

Table 1. The PIP2 binding ability of the peptides was analyzed by the inhibition of PLCδ1 activity.

The activities of PLCδ1 were measured in the absence of peptide, or in the presence of 500 μM full length histone H1, or 500 μM histone H1 peptides 1-7 from V8 protease digestion.

a



b

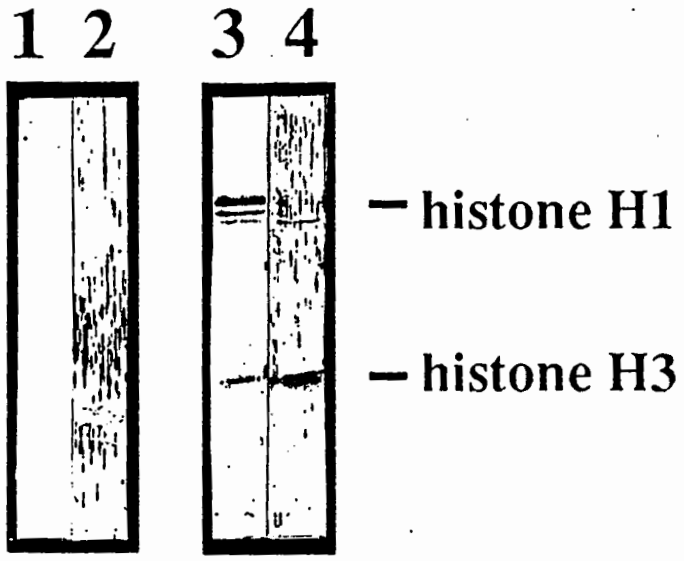
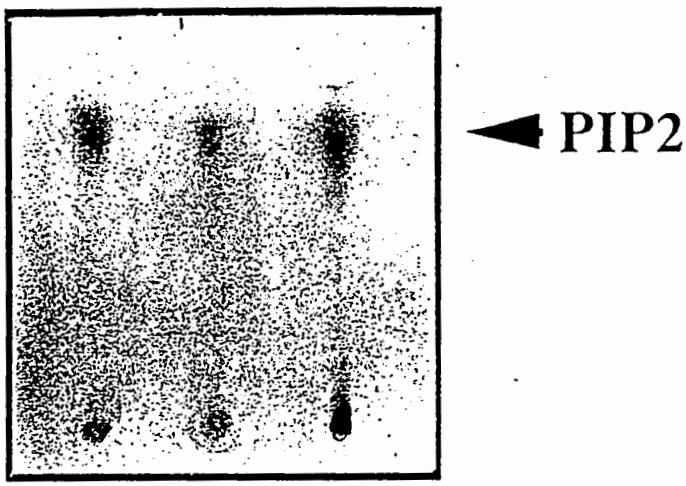


Fig. 1 Yu et al

c



d

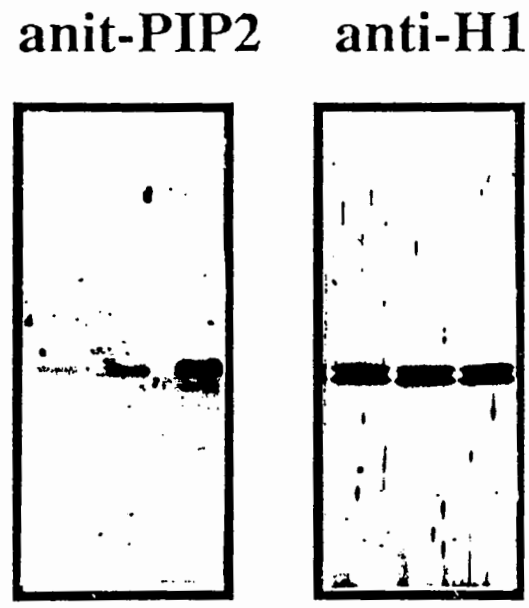
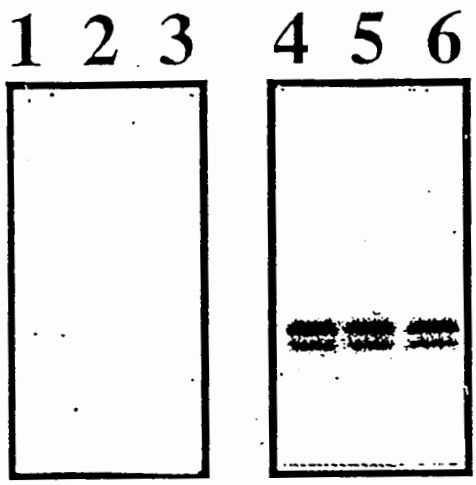


Fig. 1 Yu et al

e



f

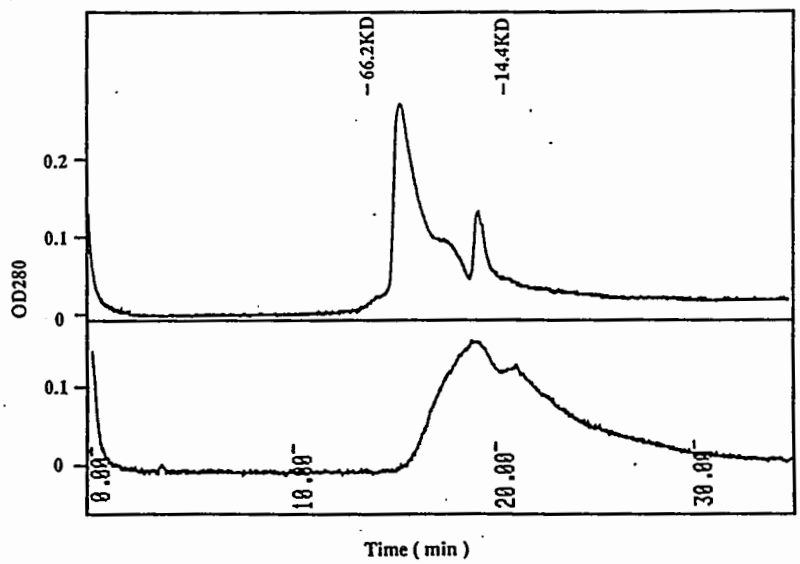
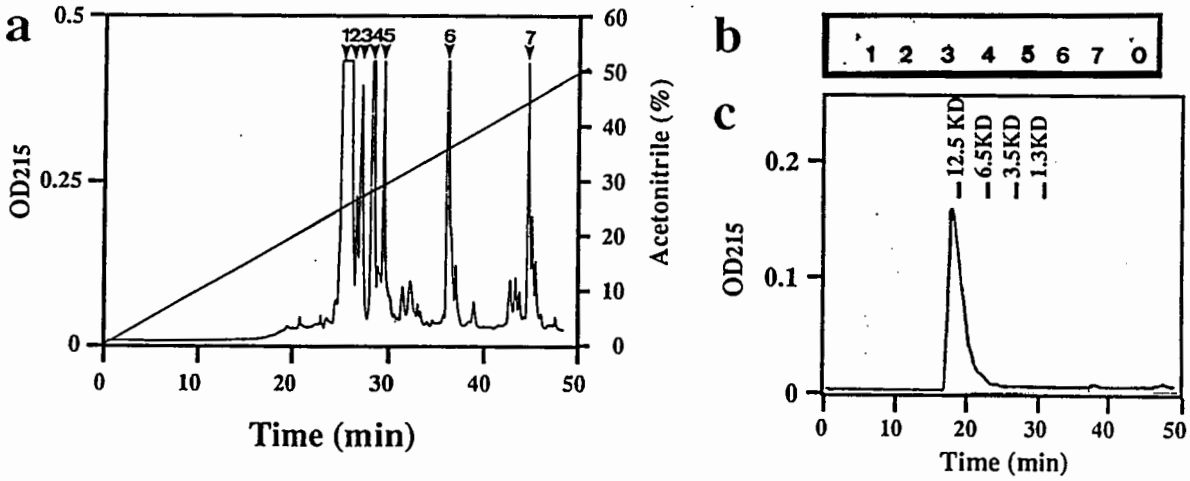
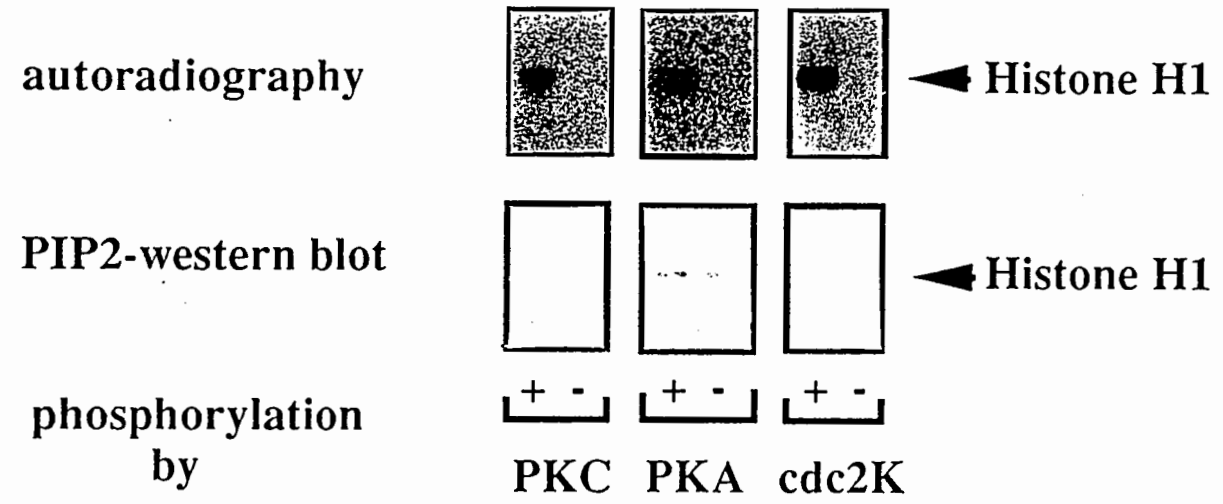


Fig. 1 Yu et al



d AKPKAKKAGAAKPKKAAGAAKKTKKATGA

Fig. 2 Yu et al


autoradiography
Histone H1
PIP2-western blot
Histone H1
phosphorylation by
+ -
+ -
+ -
PKC
PKA
cdc2K

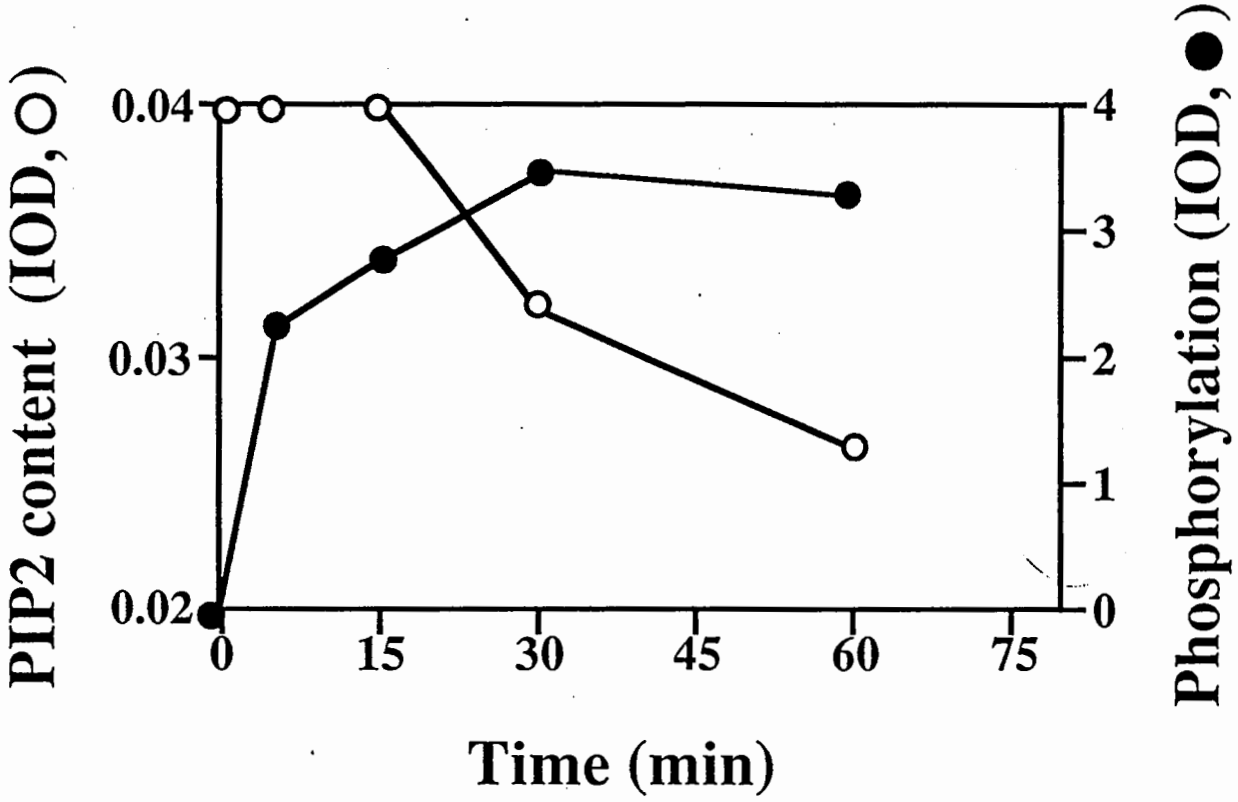
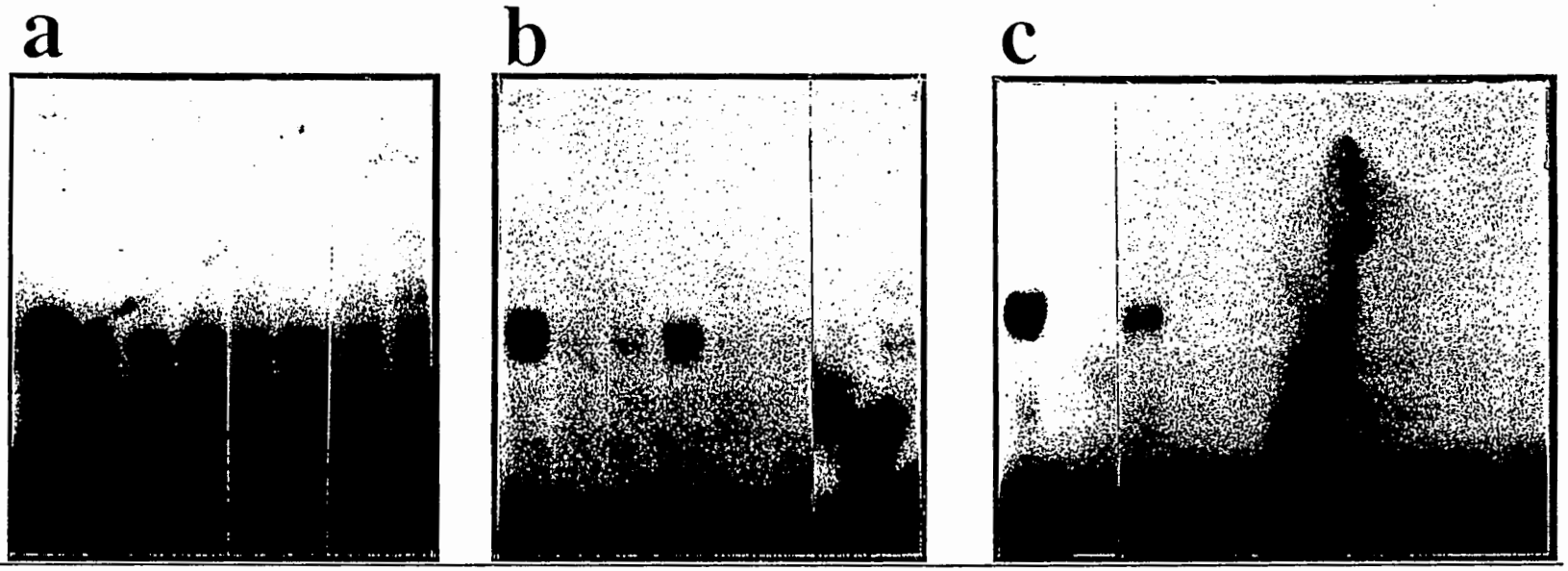

PIP2 content (IOD, ○)
0.04
0.03
0.02
0
15
30
45
60
75
Time (min)
4
3
2
1
0
Phosphorylation (IOD, ●)

Fig. 3 Yu et al



Phospholipid :Histone H1	(-)	(-)	4	10	4	10	4	10	(-)	(-)	4	10	4	10	4	10	(-)	(-)	10	1	10	1	10	1	10	1
Phospholipid			PIP2		PIP3		PIP				PIP2		CL		PG				PIP2		PI		PS		PC	
Histone H1	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
			(1μM)								(1μM)								(4μM)							
Antirepression (%)	100	0	25	62	4.8	12	0	6																		

Fig. 4 Yu et al

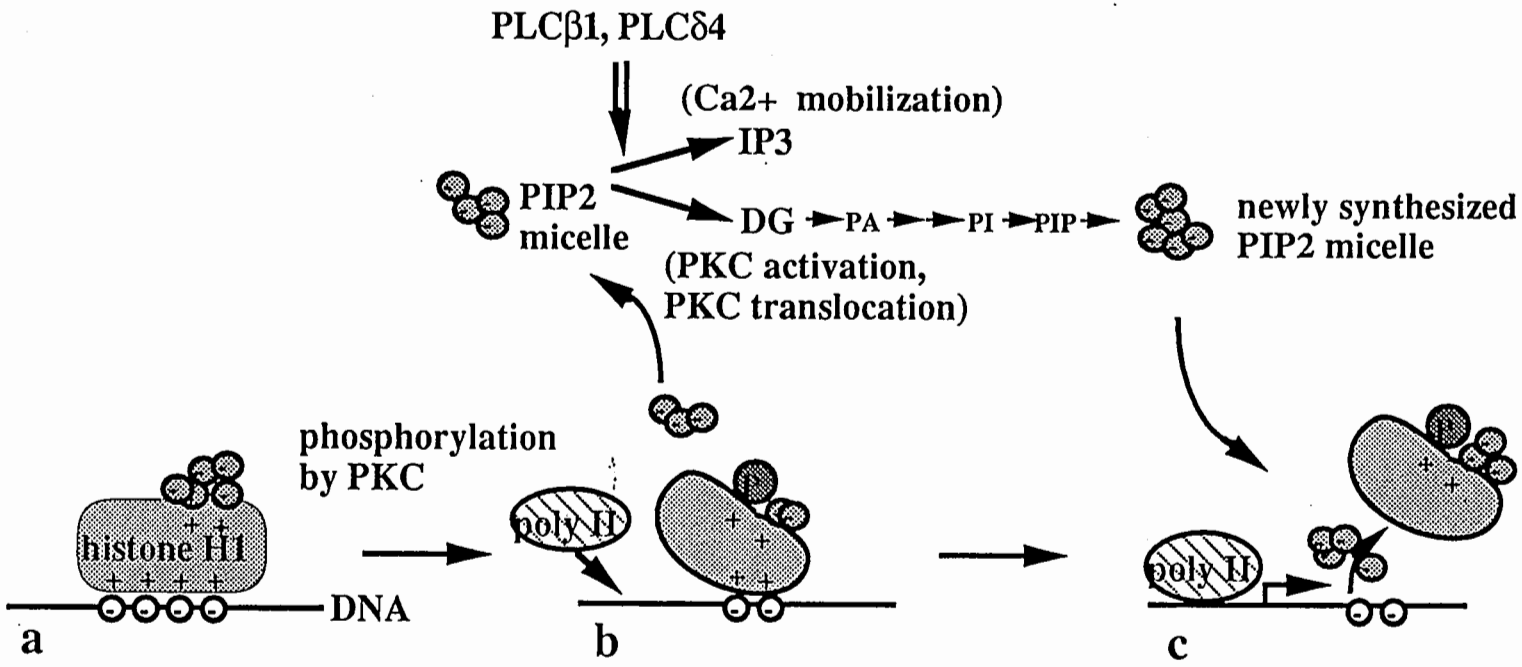

PLCβ1, PLCδ4
(Ca2+ mobilization)
IP3
PIP2
micelle
DG
PA
PI
PIP
newly synthesized
PIP2 micelle
(PKC activation,
PKC translocation)
phosphorylation
by PKC
histone H1
DNA
poly II
poly II
a
b
c

Fig. 5 Yu et al

protein(peptide)	PLC activity	
500 μM	μmol /min /mg	%
(-)	9.0 ±0.4	100.0 ±4.4
HI in full length	4.3 ±0.2	47.8 ±2.2
peptide 1	4.0 ±0.4	44.4 ±4.4
peptide 2	8.4 ±0.5	93.3 ±5.6
peptide 3	8.5 ±0.2	94.4 ±2.2
peptide 4	7.4 ±0.2	82.2 ±2.2
peptide 5	8.4 ±0.4	93.3 ±4.4
peptide 6	9.2 ±0.3	102.2 ±3.3
peptide 7	9.3 ±0.4	103.3 ±4.4

Table 1 Yu et al