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PDF issue: 2025-12-05

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(Citation)
Life Sciences,78(14):1624-1629

(Issue Date)
2006-02

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000304

Casein kinase Iε down-regulates phospho-Akt via PTEN, following genotoxic stress-induced apoptosis in hematopoietic cells

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Key Words: casein kinase Iε, Akt, PTEN, cell survival, leukomogenesis

Abstract

Here, we show a functional role of casein kinase I (CKI) ε in hematopoietic cell survival through the modification of phosphatidylinositol 3-kinase (PI3K)/Akt signaling. Introduction of wild-type (WT)-CKIε into interleukin-3 (IL-3)-dependent 32D cells increased the sensitivity to genotoxic stresses, such as γ-irradiation, etoposide, and IL-3 deprivation, whereas kinase-negative (KN)-CKIε suppressed it. Contrary to KN-CKIε, WT-CKIε attenuated the IL-3-induced activation of Akt with the increase of PTEN activity. Similarly, the increase of Akt activation, as well as PTEN inactivation, was accompanied both by a decrease of CKIε expression induced by *all-trans* retinoic acid and by the addition of a specific inhibitor for CKIε in HL-60 cells. CKIε seems to activate PTEN by physical interaction. These results suggest that the CKIε-induced down-regulation of PI3K/Akt signaling through PTEN lead to amplified sensitivity to apoptosis. Thus, the suppression of CKIε in many human leukemia cell lines may play a role in the cell immortalization.

Introduction

Members of the casein kinase I (CKI) family of monomeric serine/threonine kinases are highly conserved from yeast to human, and are ubiquitously expressed in different cell types (Gross and Anderson, 1998; Vielhaber and Virshup, 2001). In mammals, seven isoforms (α, β, γ1-3, δ, and ε) have been identified. By regulating the stability of potential substrates in vitro and transport-dependent cellular processes, CKI is likely to regulate DNA and RNA metabolism, cellular morphology, vesicular trafficking, DNA repair, and the activity of various transmembrane receptors. In addition, recent genetic analyses in diverse fields have demonstrated that CKIε plays an essential role in regulating several critical in vivo processes, such as circadian rhythm (Lowrey et al., 2000), and embryogenesis and morphogenesis via Wnt signaling in various species (Peters et al., 1999). In the CKI family, CKIδ and CKIε, which were first cloned while screening for a budding yeast mutant, *hrr25*, are known to have similar functions because of their structural similarity (Hoekstra et al., 1991; Fish et al., 1995).

Recently, we have reported the specific biological function of CKIε using recombinant CKIε cDNA-introduced murine myeloid progenitor 32D systems (Okamura et al., 2004). CKIε is down-regulated along with hematopoietic granulocytic differentiation. Then, it self-inhibits cytokine-induced granulocytic differentiation by stabilizing the suppressor of cytokine signaling 3 (SOCS3) and β-catenin. The 32D cells overexpressing CKIε grew as well as wild-type (WT) 32D cells in the standard culture condition. However, the 32D cells overexpressing CKIε

seemed to be more apoptotic in the poor culture condition. Therefore, we speculated that CKIε might be involved in the regulatory mechanism of the cell survival. This study is focused on the sensitivity of various genotoxic stresses.

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which can be activated by many growth factors and cytokines, was shown to be involved in a variety of cellular functions including inhibition of apoptosis (Nicholson and Anderson, 2002). The serine/threonine kinase Akt, a novel downstream target of this signaling, stimulates several pathways and synergistically promotes cell survival by phosphorylating suitable substrates such as Bad, caspase-9, IKKα, the Forkhead transcription factors, Mdm2, YAP, and the tumor suppressor TSC proteins (Nicholson and Anderson, 2002; McCormick, 2004). Recent findings indicate that Akt is constitutively activated and promotes cellular resistance to chemotherapy, ionizing radiation, and TRAIL in many tumors (Martelli et al., 2003; Pommier et al., 2004). Specifically, a branch of its pathways involving the kinase mTOR seems relevant for cancer cell survival (Bjornsti and Houghton, 2004). The activation of Akt is likely to be achieved by two main mechanisms (Pommier et al., 2004). First is due to direct *Akt* amplification. Second, Akt can be activated indirectly by PTEN inactivation.

Here, we showed the biological modulation of PTEN by CKIε for hematopoietic cell survival. CKIε increased the sensitivity to genotoxic stress-induced apoptosis by down-regulating PI3K/Akt signaling through stimulating PTEN.

Materials and methods

Cells and culture

The murine interleukin-3 (IL-3)-dependent myeloid progenitor cell line, 32D, was maintained in RPMI 1640 containing 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) and 15% WEHI-conditioned medium (WEHI-CM). Wild-type (WT)- or kinase-negative (KN)-CKIε cDNA (accession number AB091043) fragment was introduced into 32D cells by the retroviral vector as described previously (Okamura et al., 2004). KN-CKIε interferes in the biological activity in a dominant-negative manner. As a control, 32D cells expressing only *Neo*-gene were employed. The human promyelocytic leukemia cell line, HL-60, was maintained in RPMI 1640 containing 10% FCS. Other human leukemia cell lines used in this study are the following; ML-1, THP-1, and U-937 derived from myeloid series, Mo7E from megakaryocytes, CCRF-CEM, HPB-ALL, HPB-MLT, Jurkat, MOLT-14, and MOLT-16 from T cell series, and Nalm-6 and Nalm-26 from B cell series. CD34-positive ($CD34^+$) hematopoietic cells were isolated from mobilized peripheral blood cells by Isolex50 (Baxter, Deerfield, IL) (Matsuoka et al., 1997).

To investigate PI3K/Akt signaling, gene-infected 32D cells were starved in only RPMI 1640 for 60 min, and then stimulated with mouse recombinant IL-3 (Genzyme, Cambridge, MA) in a medium containing 10% FCS. Similarly, starved HL-60 were stimulated with *all-trans* retinoic acid (ATRA) (Sigma Chemical Co. St. Louis, MO) or cultured in the presence of CKIε-specific inhibitor, CKI-7 (Seikagaku Corp. Tokyo, Japan).

Antibodies

CKIε-specific polyclonal anti-sera were obtained after immunization of healthy rabbits with a peptide, IPASQTSVPFDHLGK, coupled to keyhole limpet hemocyanin by utilizing glutaraldehyde (Okamura et al., 2004). Antibodies to the total and serine 473-phosphorylated Akt (P-Akt), serine 380/threonine 382/383-phosphorylated PTEN (P-PTEN) and Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from New England Biolabs (Beverly, MA). An antibody to PTEN (FL-403) was kindly provided by Santa Cruze Biotech (Santa Cruze, CA).

Apoptosis study

For induction of apoptosis, 1×10^5 cells/ml exponentially growing gene-infected 32D cells were irradiated at room temperature using a ^{60}Co γ-ray source with a dose of 7.0 Gy or treated with 0.2 μM etoposide (Sigma) in complete medium, and/or incubated without WEHI-CM as IL-3 deprivation. Apoptotic cells were assessed daily by flow cytometry (FACScan; Becton Dickinson San Jose, CA) using fluorescence-conjugated anticoagulant (Annexin-V-FLUOS; Roche, Germany).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Okamura et al., 2004). Briefly, cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1.0 μg/ml leupeptin, 1.0 μg/ml aprotinin, 1.0 μg/ml pestatin, and 1 mM phenylmethylsulfonyl fluoride. For immunoprecipitation, the total

cell lysate was incubated with specific antibodies, followed by the addition of protein G-Sepharose (Amersham Pharmacia Biotech) on a rotating shaker for 4 hours at 4^{0}C. The immunoprecipitates were washed three times with lysis buffer, and three times with phosphate-buffered saline. The cell lysate was separated on 12-15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany). The membranes were probed with specific antibodies. Immune-complexes were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL).

RNA blot analysis

Total RNAs of human leukemia cell lines and CD34^{+} cells were isolated by the acid guanidinium thiocyanate-phenol chloroform method (Nagata et al., 1996). RNA blots were hybridized with ^{32}P-labeled human CKIε-specific cDNA probe, which was prepared from the C-terminal-coding (nucleotide 920-1302) fragment.

Statistical analysis

Data are presented as the means ± SEM of two or three clones of each gene-infected 32D cell line from three independent experiments. Student *t*-test was applied in analyzing the results of the apoptosis study. $P < 0.05$ was considered significant.

Results

CKIε increases the sensitivity of genotoxic stress-induced apoptosis

To elucidate the functional significance of CKIε in hematopoietic cell survival, apoptosis induced by several genotoxic stresses, such as γ-irradiation (7.0 Gy), etoposide (0.2 μM), and IL-3 deprivation in 32D cells expressing WT- or KN-CKIε cDNA were examined. The Annexin V positive rate was measured as apoptotic cells by flow cytometry. Under normal culture conditions without any stress, there was no significant difference in cell growth or viability among the three cell lines as described previously (Okamura et al., 2004). However, WT-CKIε cells were significantly more sensitive to these apoptotic stresses than control cells (Table 1). In contrast, KN-CKIε cells were relatively less sensitive during the first few days after the treatments (Table 1). These results indicated that CKIε increased the sensitivity to genotoxic stress-induced apoptosis in hematopoietic cells.

CKIε down-regulates the phosphorylation of Akt via PTEN

In reference to this biological response, it was considered that CKIε might be involved in a cell survival signaling, PI3K/Akt. Because the pathway was one of the downstream targets of cytokine stimulation, the involvement of CKIε in IL-3-induced Akt activation of the 32D cell lines was investigated by immunoblot analysis. There were significant differences in the phosphorylation levels of Akt among the three cell lines (Fig. 1). By IL-3 stimulation, serine 473-phosphorylated Akt (P-Akt), an active form of Akt, was induced strongly within 20 min

in all cell lines. In comparison to control cells, P-Akt decreased rapidly, and was barely detectable by 60 min in WT-CKIε cells. In contrast, a high level of P-Akt was sustained for 120 min in KN-CKIε cells.

Next, the activity of PTEN, a negative regulator of PI3K/Akt signaling, was evaluated after the IL-3 treatment. PTEN is known to be inactivated by phosphorylation of serine 380/threonine 382/383 in its regulatory domain (Vazquez et al., 2000; Miller et al., 2002). In the absence of IL-3, serine 380/threonine 382/383 was already phosphorylated to some extent in all cell lines (Fig. 1). In control cells, IL-3 increased the phosphorylation gradually in a time-dependent manner. On the other hand, it decreased in WT-CKIε cells, whereas it more clearly increased in KN-CKIε cells (Fig. 1). These results suggested that CKIε up-regulated the PTEN activity following the inhibition of IL-3-induced PI3K/Akt signaling.

Physical interaction of CKIε with PTEN to down-regulate Akt signaling

Human promyelocytic leukemia HL-60 cells, which were differentiated into granulocytes by treatment with ATRA (Matsui et al., 1984), were also employed to assess the functional significance of CKIε in PI3K/Akt signaling. Immunoblot analysis was performed for the detection of CKIε and P-Akt or phosphorylated-PTEN (P-PTEN). The expression of CKIε was down-regulated along ATRA-induced granulocytic differentiation (Fig. 2A). On the contrary, the steady state of both P-Akt and P-PTEN gradually increased day by day (Fig. 2A).

To further confirm the functional interaction of CKIε with the Akt

activation via PTEN, HL-60 cells were cultured in the presence of a specific inhibitor for CKIε, CKI-7. Both the P-Akt and P-PTEN were increased by CKI-7 in a dose-dependent manner, although the expression levels of CKIε were not affected (Fig. 2B).

We also examined the physical interaction of PTEN with CKIε in HL-60 cells treated with or without ATRA. As shown in Figure 2C, PTEN was co-immunoprecipitated with CKIε-specific antibody. In addition, CKIε was co-immunoprecipitated with PTEN-specific antibody. These results suggested that CKIε physically interacted with PTEN to up-regulate its activity following the Akt inactivation.

The suppression of CKIε expression in several human leukemia cell lines

Finally, the CKIε expression of human leukemia cell lines was examined by RNA blot analysis. CKIε is strongly expressed in immature normal hematopoietic cells, and down-regulated along granulocytic differentiation as described (Okamura et al., 2004). Thus, it is of interest how the CKIε expression is regulated in leukemia cells. As shown in Figure 3, the expression level of CKIε mRNA was different in each human leukemia cell line. Compared to normal $CD34^{+}$ hematopoietic progenitors, it was suppressed in many cell lines, especially in ML-1, THP-1, Jurkat, and MOLT-16 cells, regardless of cellular origin. The suppression of CKIε might affect the biological characteristics of leukemia cells.

Discussion

Here, we show a new biological function of CKIε for hematopoietic cell survival. CKIε functionally up-regulated the PTEN activity by physical interaction following the inhibition of PI3K/Akt signaling. It seems compatible with the fact that CKIε increased the sensitivity to various genotoxic stress-induced apoptosis in 32D cells. However, the over-expression of recombinant CKIε could not change the IL-3 dependency of the 32D cell lines by itself. The cytokine deprivation finally led most cells to apoptosis without differences among the three cell lines two days after the treatment (Table 1). These results indicate that CKIε affects hematopoietic cell survival only due to exposure to a genotoxic stress. Therefore, the functional role of CKIε seems to be different from anti-oncogenes such as p53, loss of which cause cell immortalization by itself.

PTEN, known as a tumor suppressor, negatively regulates PI3K/Akt signaling by dephosphorylating a lipid second-messenger, phosphatidylinositol-3,4,5-triphosphate, generated by activated PI3K (Cantley and Neel, 1999). The phosphatase activity of PTEN is conversely inhibited by the phosphorylation of serine 380/threonine 382/383 in its regulatory domain (Vazquez et al., 2000; Miller et al., 2002). However, it remains to be elucidated how PTEN is activated. Here, we showed that the phosphorylation of the negative regulatory sites of PTEN increased with the decreased expression of CKIε or in the presence of CKI-7. Thus, CKIε is thought to be involved in the phosphorylation of serine/threonine residue(s) other than the negative regulatory sites. Several possible recognition motifs for CKIε exist

in the regulatory domain of PTEN including PDZ domain (Wu et al., 2000; Vazquez et al., 2000; Miller et al., 2002; Das et al., 2003). Thus, it is important to elucidate whether CKIε directly phosphorylates PTEN or indirectly activates it by interacting with an unidentified novel regulator for PTEN. Further analysis is necessary to determine the molecular mechanism of PTEN activation by CKIε.

In many leukemia cell lines, the CKIε expression was suppressed, which would contribute to obtaining the characteristics of leukemia cells through the modification of several intracellular signalings. The activation of PI3K/Akt signaling by the suppression of CKIε function would be beneficial to leukemia cell growth. We previously reported that CKIε inhibited signal transducers and activators of transcription 3 (STAT3) activation by stabilizing SOCS3 (Okamura et al., 2004). Recent analysis demonstrated that STAT3-deficient mice were resistant to skin tumor development (Chang et al., 1997). Constitutive activation of STAT3 had been found in various malignancies. Furthermore, somatic mutation of the *CKIε* gene was reported in mammary ductal carcinoma (Fuja et al., 2004). These findings arise a possibility that CKIε plays a role as a tumor suppressor to prevent tumorigenesis not only by the PTEN activation, but also by the STAT3 inactivation.

Moreover, therapy-resistance of many malignancies appears to be controlled in part by constitutive activation of Akt due to the loss of PTEN function (Nicholson and Anderson, 2002; Martelli et al., 2003; McCormick, 2004; Pommier et al., 2004). In this study, we showed the CKIε-induced down-regulation of PI3K/Akt signaling through PTEN. Therefore, the suppression of CKIε seen in many leukemia cell lines

might have contributed not only to leukomogenesis, but also to the acquisition of resistance to anti-cancer therapy.

Conclusion

CKIε increases the sensitivity to genotoxic stress-induced apoptosis by down-regulating PI3K/Akt signaling through the physical interaction with PTEN in hematopoietic cells. The aberration of this CKIε-mediated suppression might be involved not only in leukomogenesis, but also in the resistance to anti-cancer therapy of hematological malignancies.

Acknowledgements

This work was supported in part by Grants-in-Aid for scientific Research from the Ministry of Health, Welfare, and Labor and from the Ministry of Education, Culture, Sports, Science and Technology in Japan .

We would like to thank Dr. Steve Tronick (Santa Cruse Biotech.) for the gifts of several antibodies.

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Figure Legends

Figure 1.

CKIε suppressed the phosphorylation of Akt and PTEN by IL-3 in 32D cells.

1×10^5 cells/ml 32D transfectants were stimulated with 5 ng/ml IL-3 for the indicated periods (min). Total cell lysate was subjected to immunoblot for analysis of the expression levels of the total and serine473-phosphorylated Akt (P-Akt) and the total and serine380/threonine382/383-phosphorylated PTEN (P-PTEN) using their specific antibodies. These data represent three independent experiments.

Figure 2

(A) ATRA-induced down-regulation of CKIε activated Akt signaling in HL-60 cells.

1×10^5 cells/ml HL-60 cells were differentiated to granulocytes by the addition of 10 μM ATRA for the indicated days. Then, total cell lysate was subjected to immunoblot analysis using anti-CKIε, anti-P-Akt, anti-Akt, anti-P-PTEN, and anti-PTEN antibodies.

(B) CKIε inhibitor activated Akt signaling with the phosphorylation of PTEN in a dose-dependent manner.

HL-60 cells were cultured in the presence of a CKIε-specific inhibitor, CKI-7, for 24 hours. Then, total cell lysate was subjected to immunoblot analysis as well.

(C) Physical interaction of CKIε with PTEN.

HL-60 cells were treated under conditions with or without 10 μM ATRA. After the indicated days, total cell lysate was immunoprecipitated

with anti-CKIε (*left-panels*) or anti-PTEN (*right-panels*) antibody. Each blot was probed using anti-PTEN (*upper-panels*) and anti-CKIε (*lower-panels*) antibodies. Black and white arrowheads indicated the position of PTEN and CKIε, respectively. These data represent three independent experiments. IB, immunoblot; IP, immunoprecipitation.

Figure 3.

Expression profile of CKIε in human leukemia cell lines.

Total RNAs of human leukemia cell lines was subjected to RNA blot analysis using ^{32}P-labeled human CKIε-specific cDNA probes. Ethidium bromide (EtBr) staining indicated the amount of RNAs loaded in each lane. M, myeloid series; Me, megakaryocyte; T, T cell series; B, B cell series; HPC, human CD34^{+} hematopoietic cells.

Table 1. Apoptotic cells induced by genotoxic stresses (%)

Treatment	Cell	Day 1	Day 2	Day 3
γ-irradiation (7.0 Gy)	control	12.80±2.91	38.81±7.31	78.76±0.92
	WT-CKIε/32D	13.28±2.68	61.41±2.88**	89.31±1.46**
	KN-CKIε/32D	7.81±0.55**	38.30±5.11	79.47±2.29
Low-dose etoposide (0.2 μM)	control	5.86±0.89	6.00±2.00	11.00±3.01
	WT-CKIε/32D	6.53±1.94	13.00±4.02**	27.50±6.59**
	KN-CKIε/32D	4.72±0.75*	5.50±2.25	10.75±2.95
IL-3 deprivation	control	6.00±0.47	90 <	ND
	WT-CKIε/32D	13.75±0.80**	90 <	ND
	KN-CKIε/32D	5.47±0.46*	90 <	ND

Data are presented as the means ± SEM of two or three clones of each gene-infected 32D cell line from three independent experiments.

*P < 0.05, **P < 0.01 for comparison with control.

ND: not determined.

Figure 1 Okamura et al.

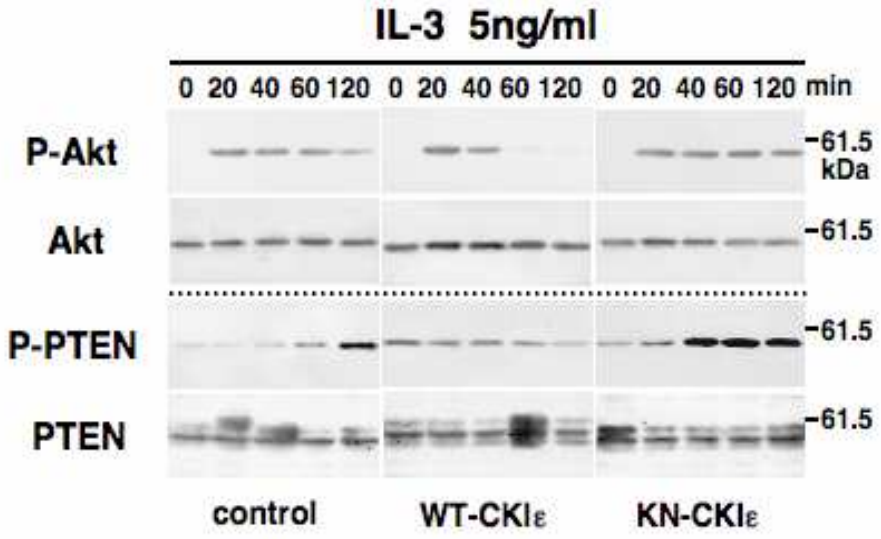

Figure 2 Okamura et al.

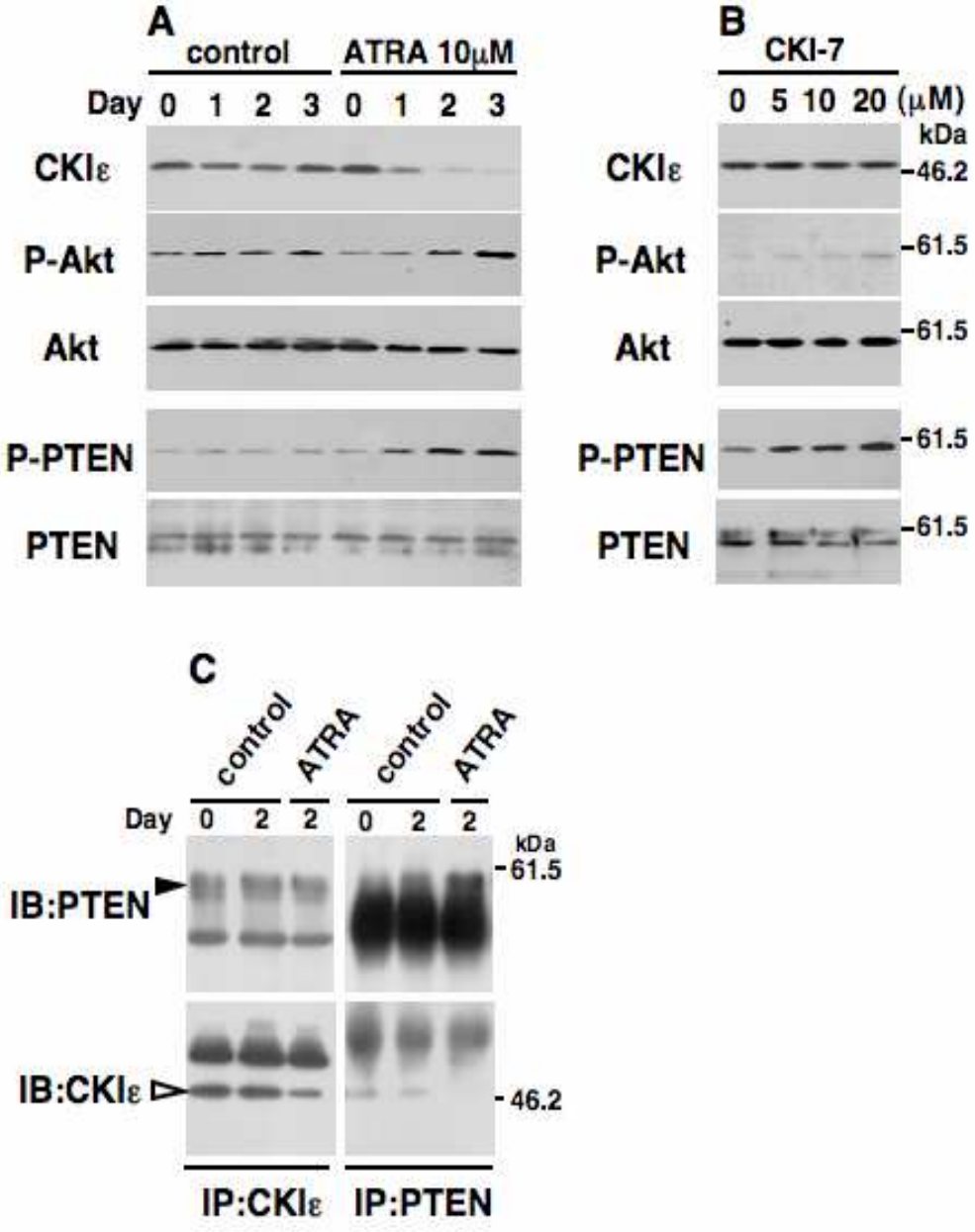

Figure 3 Okamura et al.