

## **Effects of Antiproliferative Protein (APP) on Modulation of Cytosolic Protein Phosphorylation of Prostatic Carcinoma Cell Line LNCaP**

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### **ABSTRACT**

**Antiproliferative protein (APP) isolated from conditioned media of two androgen-independent prostatic carcinoma cell lines, PC3 and Du-145 was shown to inhibit selectively cell proliferation of androgen-dependent prostate cancer cell line LNCaP in a dose dependent manner. This protein was further purified with HPLC using hydrophobic interaction column (phenyl 5PW) and was used to study the modulation of protein phosphorylation of LNCaP cells. The data indicated that antiproliferative protein could partially change the cytosolic protein phosphorylation. When compared with control LNCaP cells, in APP-treated cells 4 new proteins (88, 46, 30 and 18 kDa) were phosphorylated, while a 72 kDa phosphoprotein was de-phosphorylated. The same results were obtained when two types of protein kinase activators were used. Protein kinase activators showed that the above changes of protein phosphorylation are not due to the protein kinase C or DNA protein kinase activity. These data may indicate the inhibition of LNCaP cell's proliferation by APP may be is due to the modulation of protein kinases and as a result due to interference on second messenger pathway. *Iran. Biomed. J 2: 9-13, 1998.***

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*Keywords:* Antiproliferative protein, protein phosphorylation, LNCaP cells, Prostatic carcinoma

### **INTRODUCTION**

Prostate cancer is currently the most common malignancy and is the second leading cause of cancer death among males in the United States. For more than 50 years, hormonal therapy has been the major therapeutic procedure for metastatic prostate cancer [1]. After initial response, patients inevitably develop a tumor that is hormonally unresponsive and resistant to the current therapeutic procedures. Other available treatments such as radiation, surgery and chemotherapy have not prolonged survival of patients with metastatic carcinoma. Recently, promising results were obtained in cancer treatment by immunotherapy using cytokines in experimental animal models [2-4].

Previous studies indicated that tumor cell lines produce and release into their condition media (CM) several growth regulatory factors [5-8]. Some of these factors have been purified and some remained to be characterized.

Recently, we have identified a new antiproliferative protein (APP) from serum-free conditioned

medium of two androgen-independent prostatic carcinoma cell lines (PC3 and DU-145)[9]. This protein selectively inhibits cell proliferation of an androgen-dependent prostatic carcinoma cell line (LNCaP). No antiproliferative activity was observed against mouse fibroblast 3T3, normal human lymphocytes, human leukemic cells including promyelocyte HL-60 or T cell HUT-78, or human adenocarcinoma cell lines including prostatic cells JCA-1, ovary NIH:OVCAR-3, cervix C-33A or breast MDA-MB-231. APP did not induce apoptosis, but it prevented the LNCaP cells from entering into the S phase of the cell cycle. The antiproliferative activity was reversible and was not affected by neutralizing antibodies against the well known cytokines such as TGF<sub>1,2,3</sub>, TNF- $\alpha$ , PDGF, EGF, IL-1, IL-2, IL-3, IL-4, and IL-6. APP was partially purified by gel filtration, ion-exchange chromatography and SDS-PAGE with an apparent molecular weight of 50 kDa. Biochemical studies revealed that APP is sensitive to high temperature and protease digestion but not to reducing agent, pH 2.0, DNase or RNase digestion.

In this report, attempts were made to determine the mechanism of action of APP at the cellular level. This protein was purified by HPLC and could modulate cytosolic protein phosphorylation of the APP-treated LNCaP cells.

## MATERIALS AND METHODS

**Cell lines and conditioned media.** All cell lines were maintained in complete medium (RPMI-1640 containing 10% FBS). PC3 and DU-145 cell lines were also grown in serum-free medium (ITS<sup>+</sup>, Collaborative Research, Inc.). Mycoplasma-free cells were cultured for 72 h at 37°C and the culture supernatants were collected, centrifuged and filtered through a 0.22 µm membrane and kept frozen until use.

**Cell proliferation assay.** Cell proliferation was performed as described previously [9]. LNCaP cells were plated in 96-well plates at  $4.5 \times 10^4$  cells per ml and APP preparations were added to a final volume of 200 µl. Cells were incubated at 37°C for 48 h and during the last 4 h they were pulse labeled with 2.0 µCi/ml [<sup>3</sup>H]-thymidine. LNCaP cells were detached with trypsin, harvested on glass filters, and the incorporated <sup>3</sup>H-thymidine was measured.

**Protein purification by HPLC.** APP was further purified by HPLC using hydrophobic interaction column, Phenyl-5PW (8 × 75 mm), (Waters). PC3 positive fractions from S-200 column (120 µg/100 µl) was injected into the HPLC column. A linear gradient from 100% A [1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 0.1 M phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub>), pH 7] to 100% B (0.1 M phosphate buffer, pH 7) was used during 60 min at flow rate of 0.5 ml/min. Fractions were concentrated, dialyzed against RPMI-1640 and after filtering through a 0.22 µm membrane were used for cell proliferation assay.

**Cytosolic protein phosphorylation.** LNCaP cells were treated with HPLC-purified APP and cultured for 2 days. Cells were subjected to *in vitro* phosphorylation assay using [<sup>32</sup>P] ATP. Cells were lysed and after centrifugation, cytosolic proteins were separated with 10% SDS-PAGE and phosphoproteins were analyzed by autoradiography. Also, two types of protein kinase activators were used: poly(dA)-poly(dT) for DNA-PK (double-stranded DNA-dependent protein kinase) and Ca<sup>+2</sup>

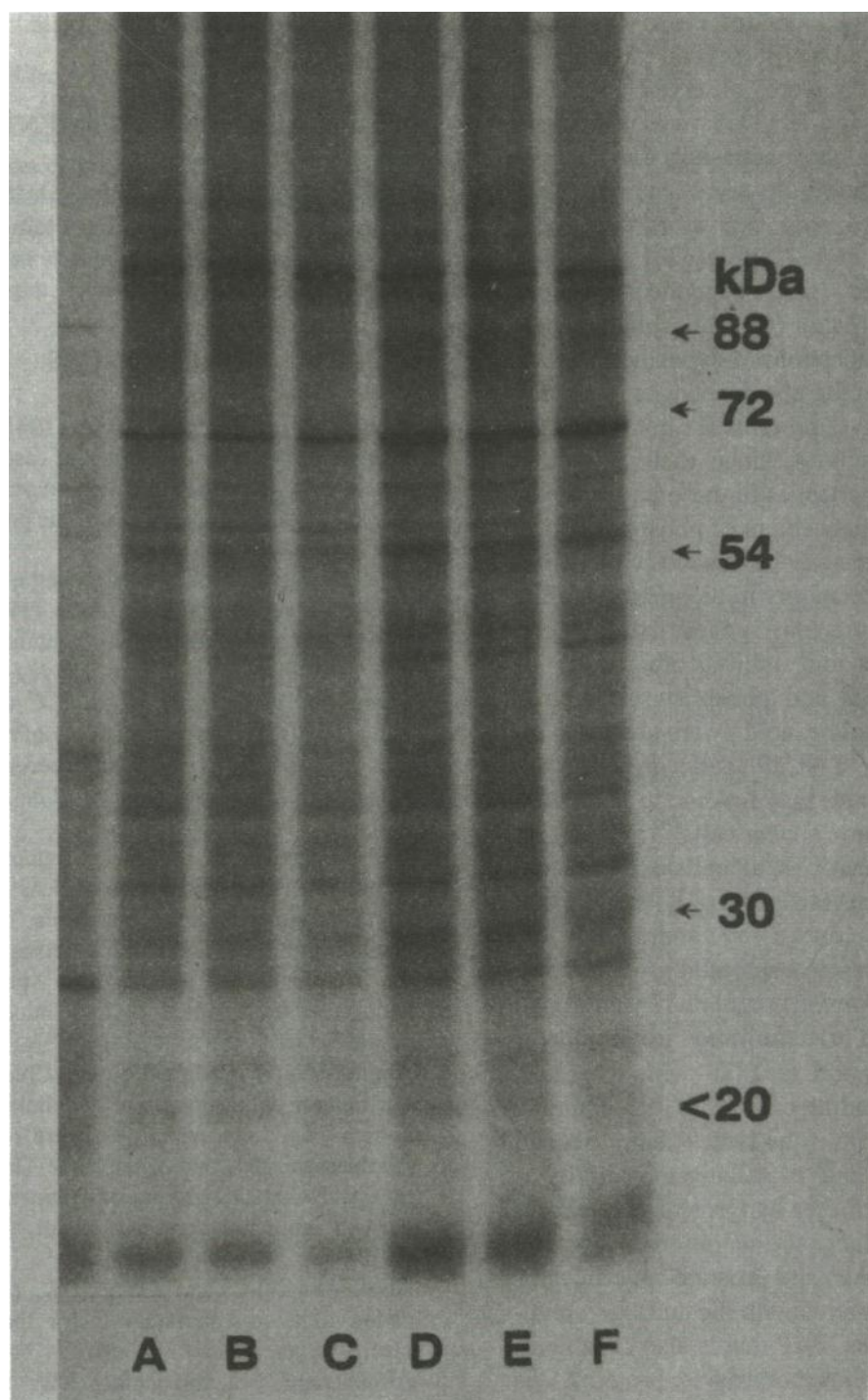
phosphatidyl serine for PKC (Ca<sup>+2</sup>/phospholipid-dependent protein kinase).

## RESULTS AND DISCUSSION

The phosphorylation of proteins by protein kinases is an important mediator of cell metabolism. Protein kinase activity has been shown to be involved in regulation of intercellular communication, intracellular signal transduction, cell division, protein activity and several other cellular systems [10]. Most of the serine/threonine kinases studied to date are soluble, cytosolic proteins. This group includes protein kinases C (PKC), cAMP-dependent protein kinase (PKA), and cGMP-dependent protein kinase (PKG). In contrast, tyrosine kinase activities are often found in the cytosolic regions of membrane-associated receptors. In many cases, such as the EGF receptor and the insulin receptor, ligand binding to the receptor's extracellular domain leads to activation of the tyrosine kinase activity [11]. This results in phosphorylation of the receptor as well as other intracellular targets, initiating a cascade of signal transduction.

In this report, attempts were made to determine whether APP plays a regulatory role in protein phosphorylation of treated LNCaP cells. This question may be addressed using protein kinase activators, inhibitors or, alternatively by using purified kinases. For this reason, APP that was previously semi-purified by column chromatography [9], was further purified by HPLC using hydrophobic interaction column (Phenyl-5PW). This column separates macromolecules based on their hydrophobicity in the presence of high salt condition. Fourteen fractions were eluted from the column. Cell proliferation assay showed that only the last fraction out of the column had antiproliferative activity on LNCaP cells. This result indicates that APP is very hydrophobic and could be purified in an active form by HPLC. About 0.3 µg of purified protein was used for assay and 56.8% suppression of proliferation was obtained. This indicates purification factor of about 600 × by comparing to the original CM.

To study cytosolic protein phosphorylation, LNCaP cells were treated with purified APP for 2 days and then the cells were subjected to *in vitro* phosphorylation assay. Fig. 1 shows autoradiography data of LNCaP cell cytosolic protein phosphorylation and de-phosphorylation in the presence or absence of APP. When control cells



**Fig. 1.** Autoradiogram of LNCaP cells cytosolic phosphorylated proteins separated by SDS-PAGE. Lanes A, B and C are untreated LNCaP cells as controls and Lanes D, E, and F are APP-treated cells. DNA-PK activator was added to lanes B and E and PKC activator to lanes C and F.

were compared with APP-treated cells, several changes in protein phosphorylation were observed. Four new proteins (88, 46, 30 and 18 kDa) were phosphorylated, while a 72 kDa phosphoprotein was dephosphorylated. The same results were obtained when two types of protein kinase activators were used: DNA-PK activator [poly(dA)-poly(dT)] for DNAPK (double-stranded DNA-dependent protein kinase) and PKC activator ( $\text{Ca}^{+2}$  phosphatidyl serine) for PKC ( $\text{Ca}^{+2}$ /phospholipid-dependent protein kinase). It seems that the above changes in protein phosphorylation of five proteins are not due to the PKC or DNA-PK activity, since their activators failed to induce any changes in these phosphoprotein. In contrast, enhancement of phosphorylation of 54 kDa protein seems to be due to the DNA-PK activity, since it was seen only in the presence of its activator. These results may suggest that APP can activate some kinases and inhibits others. Inhibitors of Ser/Thr-kinases and phosphatases such as calphostin C and okadaic acid were shown to inhibit effectively the LNCaP cell growth [12]. Therefore, these enzymes may involve in regulation of the growth of prostatic cancer cells.

Recent data show that LNCaP cells express large amounts of androgen receptors (AR) mRNA that are under negative control by androgen [13]. LNCaP cells also possess epidermal growth factor (EGF) receptors that are up-regulated by androgen to secrete EGF and transforming growth factor (TGF- ) and to respond to EGF with increased growth [14]. The binding of EGF to its membrane tyrosine kinase receptor activates many second messenger pathways and modulates the activity of several intracellular kinases by tyrosine phosphorylation [15]. EGF reduces the secretion of prostatic acid phosphatase (PAP) and prostate-specific antigens (PSA) and interferes with the androgen regulation of these proteins. PAP that is also capable of dephosphorylating phosphotyrosyl proteins is down-regulated by androgens. PSA also can dephosphorylate phosphotyrosine proteins, and was shown to dephosphorylate EGF receptor [16].

It may be possible that APP interferes with androgen receptor or EGF receptor so that EGF or TGF- cannot be secreted. In this case, APP may interfere with second messenger pathway and the cell growth can be inhibited. While the biochemistry and molecular biology of protein

kinases have progressed, it has been much more difficult to understand their function in intact cells.

## ACKNOWLEDGMENTS

Author would like to thank Dr. Sanski Konno for his assistance in cytosolic protein phosphorylation.

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