AKT2 up-regulation by Src phosphorylation

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Abstract

Understanding the regulation of Akt2 has been of major interest for elucidating the control of normal cellular physiology as well as malignant transformation. The paradigm for activation of Akt2 involves phosphatidylinositol 3-kinase-phosphorylation of dependent membrane localization followed by activating Thr-309 and Ser-474. Many of the activating signals for Akt2 involve the stimulation of receptor and non-receptor tyrosine kinases. In this study we show that activation of Akt2 by src phosphorylation is associated with tyrosine phosphorylation of Akt2. In addition, in MCF7 Breast cancer cells that exhibit high basal levels of Akt2 activity, Akt2 was tyrosine-phosphorylated in the basal state, and this phosphorylation was further enhanced by Src phosphorylation.

Key word: Akt2 , up-regulation, Src, Breast cancer.

ان فهم عملية السيطرة على

Akt2

هو الان موضع اندفاع كبير لدى الكثير من الباحثين من اجل فهم السيطرة على فسلجة الخلايا الطبيعية وكذالك التحول القاتل لهذه الخلايا.من المعلوم ان تفعيل PI3K يتضمن Akt ومن ثم يتبع بالتفعيل من خلال الفسفرة لكل من الثريونين 309 و السيرين 474 . هنالك اشارات تفعيل متعددة ل Akt2 و تتضمن التحفيز من خلال التايروسين كاينيز ذات المستقبلات والتي لاتحتوي مستقبلات. في هذه الدراسة اوضحنا ان تفعيل ال Src Akt2 بواسطة الفسفرة من خلال انه مرتبط بالفسفرة لتايروسين بالاضافة الى ذالك في خلايا (MCF7 خلايا لسرطان الثدي) والتي تظهر مستوى عال من فعالية Src والتي بصورة عامة تظهر فسفرة في A التايروسين عززت بواسطة Akt2

INTRODUCTION

The protein kinase Akt plays key regulatory roles in a range of physiological processes including glucose metabolism [1,2], cell

survival [3–5], proliferation [6,7], migration [8,9], and angiogenesis [10–12]. Furthermore, the kinase is inappropriately regulated in a number of tumors [13–16].

Understanding the regulation of Akt has thus been of major interest for elucidating the control of normal cellular physiology as well as malignant transformation. The paradigm for activation of Akt involves phosphatidylinositol 3-kinase (PI3K)1-dependent membrane localization followed by activating phosphorylation of Thr-308 in the activation loop of the kinase and Ser-473 at the COOH terminus [4, 5, 17]. Binding of the PH domain of Akt to PI3K-generated phosphatidylinositol 3,4,5-trisphosphate releases the auto-inhibitory function of this domain allowing phosphorylation of Thr-308 by PDK1, while the mechanism of Ser-473 phosphorylation remains to be determined definitively. Many of the activating signals for Akt involve the stimulation of receptor and non-receptor tyrosine kinases, and the most potent activator known is the tyrosine phosphatase inhibitor pervanadate, highlighting a possible role for tyrosine phosphorylation in the regulation of the enzyme. Recent reports show that the tyrosine kinases Syk and Btk are required for B cell receptor induced activation of Akt [18,19]. Akt (PKB) kinases are evolutionarily conserved in eukaryotes ranging from *C.elegans* to human. The amino acid identity between mouse, rat and human is more than 95%, whereas between *C.elegans* and human, it is only 60% [20].

Three isoforms of Akt encoded by three separate genes have been found in mammalian cells. Akt1 is ubiquitously expressed in mammalian cells and tissues. Akt2 is expressed at lower level than Akt1, except in insulin-responsive tissues: skeletal muscle, heart,

liver and kidney [21,22]. Akt 3 is expressed at the lowest level in most tissues, except for testes and brain [23].

The activation of Akt has been shown to be a multi- step process and several proteins responsible for each step were identified. The first rate-limiting step for Akt activation is the binding of PIP3 (phophatidylinositol-(3,4,5)triphosphate) or PtdIns (3,4,5) P3) to the pleckstrin homology (pH) domain of Akt and the translocation of Akt to the plasma membrane [20, 24, 25]. Moreover, some data have shown that AKT2 is activated in many breast cancers, and its activation is induced by estrogen receptor a (ERa) via an interaction between ERa and PI3K [26]. And cells with activated AKT2 were found to be estrogen independent and resistant to the anti-hormonal therapeutic agent Tamoxifen [27].

Src is ubiquitously expressed in mammalian cells and is, in addition to platelet integrin signaling, involved in other distinct pathways and cellular targets (G-protein-coupled receptors, cytokine and immune recognition receptors, and ion channels) [28].

Src is the first discovered and best studied proto-oncogene. It`s role in focal adhesions is associated with cell detachment, migration, and invasion that is important in the spreading of cancer [29].

Increased Src activity has been demonstrated in a number of human malignancies, and control of Src by Csk was associated with the progression of cancer [29]. Higher Src activity or low levels of Csk weakened focal adhesions and supported cell detachment.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK293T, MCF7 cells (from ATCC) were maintained in DMEM with 10% fetal bovine serum. Purified c-Src and AKT2 were purchased from Upstate Biotechnology Inc. Akt *in vitro* kinase kit, Was purchased from Cell Signaling Technology, Inc. anti-AKT2 (D-17, Santa Cruz). Anti-HA antibody was from Babco. monoclonal antibodies directed against phosphorylated Tyr (pY) (Invitrogen), p-Akt2 (p-Ser474), polyclonal (BioScience), LY294002 (Calbiochem) stocks were prepared in D.D H2O and then diluted to final concentrations in bath solutions before use.

Plasmids and Transfections—pCMV6-HA-Akt2, pCMV6-HA-Src, pcDNA3 and myr-Akt2 Plasmids were kindly provided by prof G. VIGILIATTO. Transfections were performed by using Fugene 6 (Roche Molecular Biochemicals) or LipofactAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's instructions.

Immunoprecipitation, Western Blot, and in Vitro Kinase Assay—The transfected cells were lysed in the buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 Mm glycerol phosphate, 1 mM Na3VO4, 1 µg/ml aprotinin, 1 µg/ml leupetin, and 1 mM phenylmethylsulfonly fluoride). Insoluble material was removed by centrifugation, and antibodies were added to lysates for 1 h at 4 °C. Antibodies were collected with protein A- and protein G (1:1)-Sepharose beads, and protein complexes were washed three times at 4 °C with the lysis buffer. Immunoblotting was performed as described previously [30]. Akt *in vitro* kinase assays were performed as reported by Franke *et al.*[31].For measurement of

endogenous Akt activity, equivalent amounts of protein (determined by Bradford assay) were immunoprecipitated with anti-PKB/Akt antibody prebound to protein A-Sepharose beads, and kinase assays were carried out according to the instruction manual of the Src Kinase Assay Kit (Cell Signaling Technology, Inc.). Akt2 protein was used as a substrate for Src. The phosphorylated Akt2 was detected by Western blot using Anti Py (total phosphotyrosin) antibody. Src *in vitro* kinase assays were performed according to manufacturer's instructions (Upstate Biotechnology Inc.).

RESULTS

To demonstrate that Src kinases may directly regulate Akt2 activity, we tested whether Src is able to induce tyrosine phosphorylation of Akt2 in a cotransfection experiment in 293Tcells. As shown in Fig. 1 HA-tagged Akt2 is highly tyrosine-phosphorylated in the presence of Src.

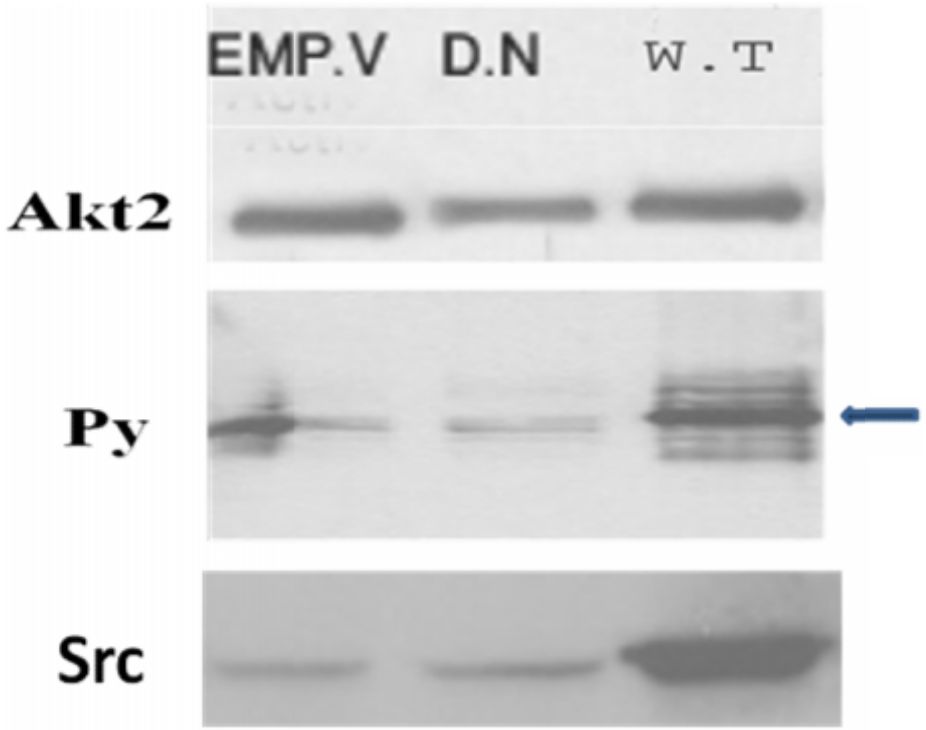

Figure 1, 293T cells were cotransfected with Src empty vectore (EMP.V) , dominant negative (D.N) and W.T. Then I.P anti HA, and W.Blot anti Akt2, Total Tyr.Py, and anti Src. W.blot result shown tyrosin phosphorylation of Akt2 by Src in vivo .

Tyrosine phosphorylation of Akt2 was enhanced by *in vitro* kinase assays that shown direct interaction and phosphorylation of Akt2 by Src (Fig. 2).

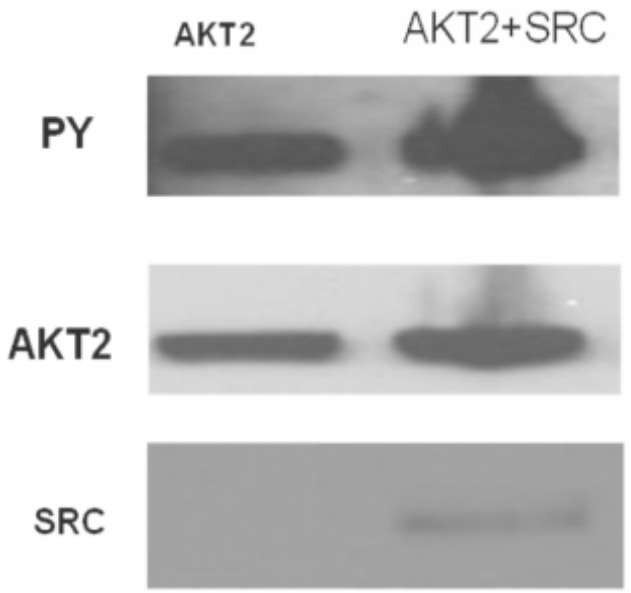

Figure 2. Invetro Src kinase assay by used Purified c-Src and Akt2, shown the possibility of direct interaction of Src Kinase with Akt2 kinase.

Akt2 activity induced by Src is inhibited by the PI3-kinase inhibitor LY294002, although the tyrosine phosphorylation (PY) of Akt2 remains unchanged (fig. 3,4).

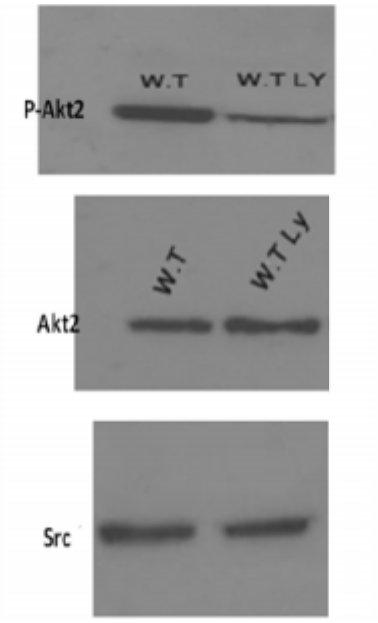

Figure 3. 293T cells were cotrasfected with Src W.T and HA-Akt2, then stimulate by the PI3-kinase inhibitor LY294002, (the figure explain before and 5 min after exposure to 10 μmol/l LY294002 (LY)), protein extract were prepared and W.Bloted anti p-Akt2, Akt2 and Src.

To address whether tyrosine phosphorylation of Akt2 is required for its biological function, in MCF7 cells, Src can still significantly enhance the kinase activity of a membrane-bound myristoylated Akt2 (myr-Akt2) (Fig. 4).

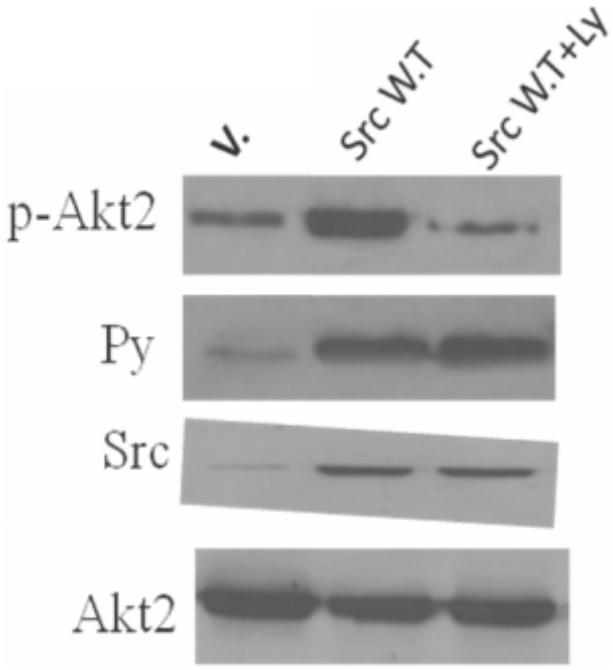

Figure 4. Tyrosine phosphorylation of Akt2 is required for its biological functions. Mcf7 breast cancers cell lines were cotransfected with Src W.T, myr-Akt2, then stimulate by the PI3-kinase inhibitor LY294002, (the figure explain before and 5 min after exposure to 10 µmol/l LY294002 (LY)), protein extract were prepared and W.Bloted anti p-Akt2, Akt2, Tyr (PY) and Src.

To define the Akt2 protein stability we transfected MCF7cells with Src and stimulated with cyclohexamide (an inhibitor of protein synthesis) figure 5. The present of Src increase Akt2 half life from less than 6 hours to more than 12 hours.

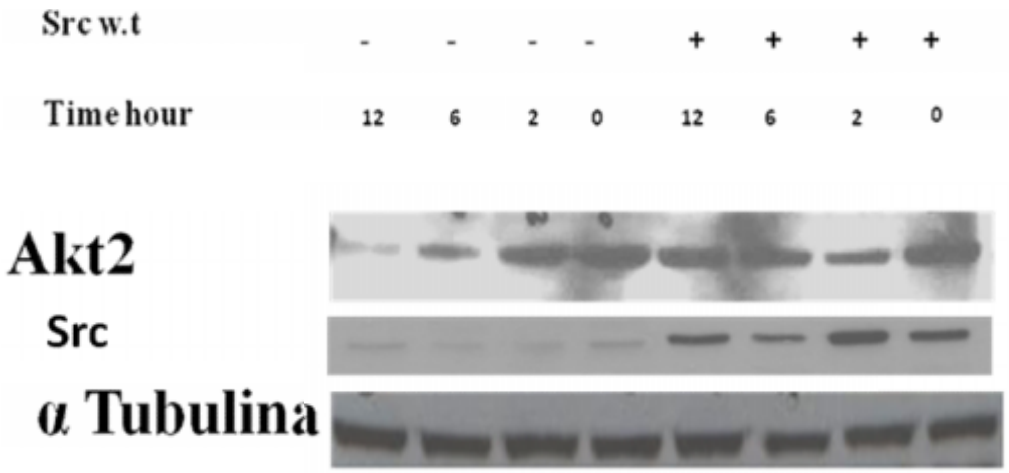

Figure 5. MCF-7 cells were transfected with Src W.T and incubated with cyclohexamide (CHX, 25 μ g/ml), an inhibitor of protein synthesis,later they were collected and extract the protein and W.blot anti Akt2, Src . αTubuline as a loading control.

DISCUSSION

In general, phosphorylation plays an essential role in regulation of the function of virtually all kinases. With no exception, Akt2 is also regulated by several phosphorylation events. In this report, we demonstrated that, in addition to phosphorylation of Thr309 and Ser474, tyrosine phosphorylation may also play an important role in regulation of Akt2 activity.

These data suggest that Src family kinases may be required for Akt2 activation in response to growth factors in these cells. This is corroborated by our observation that Src active can further enhance the kinase activity of myr-Akt, which is shown to be independent of PI3-kinase. While a constitutively active PI3-kinase has little effect on the kinase activity of myr-Akt2, which is consistent with the previous observation that the activity of myr-Akt2 is independent of PI 3-kinase [32]. These data raise the

possibility that Src may regulates Akt2 activity through additional mechanism(s) in which PI 3-kinase is not involved and most likely by directly phosphorylating Akt2. In this study, we demonstrated that the tyrosine kinase Src is able to phosphorylate Akt2 both *in vivo* and *in vitro*; therefore, Src family kinases may directly regulate Akt2 activity through a tyrosine phosphorylation-dependent manner rather than solely depends on activation of PI 3-kinase as suggested previously.

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ABBREVIATIONS

AKT **Protein kinase B**
DNA **Deoxyribonucleic acid**
EGF **Epidermal growth factor**
EGFR **Epidermal growth factor receptor**
HER2 **Human Epidermal growth factor Receptor 2**
IHC **Immunohistcheistry**
KRAS **Kirsten rat sarcoma 2 viral oncogene homolog**
MAPK **Mitogen-activated protein kinase**
mRNA **Messenger ribonucleic acid**
PI3K **phosphatidylinositol3-kinase**
PIK3CA **phosphoinositide-3-kinase, catalytic, alpha polypeptide**
PIP2 **Phosphatidylinositol (4,5)-bisphosphate**
PIP3 **phosphatidylinositol(3,4,5)-trisphosphate**
PTEN **Phosphatase and Tensin Homolog Deleted on Chromosome Ten**
RNA **Ribonucleic acid**
SDS-PAGE **Sodium dodecyl sulfate -polyacrylamide gel electrophoresis**
Src **Src (pronounced "sarc" as it is short for sarcoma) is a family of proto-oncogenic tyrosine kinases**

- **Abbreviations:**

PI3-kinase

phosphatidylinositol 3-kinase

MAP

mitogen-activated protein

DMEM

Dulbecco's modified Eagle's medium

myr-Akt

membrane-targeted, myristoylated Akt

PIP

phosphatidylinositol phosphate