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Direct tyrosine phosphorylation of Akt/PKB by epidermal growth factor receptor

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Akt/PKB plays pivotal roles in many physiological responses such as proliferation, differentiation, apoptosis, and angiogenesis. Here we show that tyrosine phosphorylation of Akt/PKB is essential for the subsequent phosphorylation at Thr^{308}. Tyrosine phosphorylation of Akt/PKB was induced by stimulation of COS-7 cells with epidermal growth factor receptor (EGF) and its phosphorylation was significantly enhanced by constitutive targeting of Akt/PKB to the plasma membrane by myristoylation. Interestingly, incubation of affinity purified Myc-tagged Akt/PKB with purified EGF receptor resulted in tyrosine phosphorylation as well as Ser^{473} phosphorylation of Akt/PKB. In addition, tyrosine-phosphorylated Akt/PKB could directly associate with activated EGF receptor in vitro. Finally, alanine mutation at putative tyrosine phosphorylation site (Tyr^{326}) abolished EGF induced Thr^{308} phosphorylation of wild type as well as constitutively active form of Akt/PKB. Given these results we suggest here that direct tyrosine phosphorylation of Akt/PKB by EGF receptor could be another mechanism of EGF-induced control of many physiological responses.

Key words – Akt/PKB, Epidermal growth factor receptor, tyrosine phosphorylation, survival, growth

Introduction

Akt/PKB originally identified as a cellular oncogene, and plays pivotal roles in many biological responses as a downstream effectors of phosphatidylinositol-3′ kinase (PI3K) [6]. From the studies of last few decades, it has been reported that Atk/PKB acts as critical intracellular signaling mediator, and controls cellular growth/proliferation, cell cycle, anti-apoptotic function by inactivation of pro- apoptotic proteins, fate determination by differentiation, oncogenesis by transformation and metastasis, glucose uptake by GLUT4 translocation, and migration by cytoskeletal rearrangement [29]. Likewise, activation of Akt/PKB is caused by various extracellular stimuli such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), and nerve growth factor (NGF) [29]. To be activated by extracellular stimuli, translocation to the plasma membrane through the interaction with PIP_3 generated by PI3K is prerequisite process and subsequent serine/threonine phosphorylation [1,7]. Two phosphorylation sites, Thr^{308} and Ser^{473}, have been identified to be critical for activation of Akt/PKB induced by various growth factor stimuli. Phosphorylation of Thr^{308} in the activation loop of Akt/PKB by PDK-1 has been known to be important for the initiation of activation process, and the phosphorylation of Ser^{473} at the C-terminal hydrophobic tail either by mTOR or by autophosphorylation is required for the maximum activation of kinase activity [11,13,25]. Once activated, Akt/PKB affects downstream signaling by phosphorylation at RXRXXS/T motif in target proteins. Mutational analysis showed that either alanine replacement at Thr^{308} or Ser^{473} abolished kinase activity of Akt/PKB and subsequent loss of target phosphorylation [1]. Therefore, serine/threonine phosphorylation of Akt/PKB is critical step for the activation process.

Though serine/threonine phosphorylation of Akt/PKB seems to be important for activation process, it has been reported that tyrosine kinase activities in B lymphocytes are required for the activation of Akt/PKB. For example, Syk and Btk are required for B cell receptor-mediated activation of Akt/PKB [9,17]. In addition, cells lacking c-Src show impaired Akt/PKB activity in osteoclasts [26,27], and

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Akt/PKB activity is up-regulated by over-expression of v-Src in both Sf9 cells and mammalian cells [10,21]. However, it is still not clear whether requirement of tyrosine kinase activity is still dependent on PI3K, and is associated with the changes in serine/threonine phosphorylation of Akt/PKB. In other AGC kinases such as PKCs, it has been reported that tyrosine phosphorylation is important for its activation. For example, tyrosine phosphorylation of PKCδ in response to H_2O_2 stimulation is important for activation [16,18]. Interestingly, the tyrosine phosphorylation sites (Tyr^{512} and Tyr^{523}) of PKCδ are conserved among AGC kinases including Akt/PKB. Therefore, it is possible that direct phosphorylation of Akt/PKB is also involved in the activation process by analogy.

Here we provide convincing evidences that Akt/PKB is directly tyrosine phosphorylated by EGF receptor, and tyrosine phosphorylation is prerequisite process for the Thr^{308} phosphorylation. Also, tyrosine phosphorylation at Tyr^{326} is sufficient to induce autophosphorylation of Ser^{473} *in vitro*. These results indicate that tyrosine phosphorylation of Tyr^{326} is one of alternative regulation mechanisms of Akt/PKB by growth factor receptor.

Materials and Methods

Materials

COS-7 and human epidermoid carcinoma A431 cells were purchased from ATCC (Rockville, MD USA). Tissue culture supplies were purchased from Corning (Corning, NY, USA), and sera were from Hyclone (Logan, UT, USA). Enhanced chemiluminescence detection system was purchased from Amersham (Aylesbury, UK). Monoclonal anti-EGFR antibody that specifically recognizes the C-terminal region (amino acids 996-1022) of human EGFR was obtained from Transduction Laboratories (Lexington, KY, USA). Monoclonal anti-phosphotyrosine antibody (4G10), monoclonal anti-EGFR antibody that specifically recognizes the extracellular domain of human EGFR, Myc-tagged Akt1 and Myr-Akt1 were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MA, USA). Wheat germ lectin agarose and other chemicals were obtained from Sigma (St. Louis, MO, USA).

Cell culture

COS-7 and A431 cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum and were maintained at 5% CO2/95% air at 37°C. Medium was replaced every 2 days. Cells were maintained in 70% confluence.

Plasmids and transfections

Site-directed mutagenesis corresponding Tyr^{326} was performed by splice-overlap extension (SOE) method as described previously [4]. Mutated version of Akt/PKB was fully sequenced in order to ensure that no other mutation was introduced inadvertently. COS-7 cells were plated on 10-Cm dishes at 70% confluence. Transfection of plasmids was performed by using LipofeactAMINE 2000 (Life Technology, Inc.) reagent accordingly to the manufacturer's instructions. At 24 hrs post-transfection, cells were serum-starved for 24 hrs followed by stimulation with EGF as indicated in figure legends.

Purification of PLC-γ1 and EGF receptor

Purification of EGF receptor was performed as described previously [3]. Briefly, A431 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EGTA/EDTA, 1 mM Na_3VO_4, 2 mM sodium pyrophosphate, 10% glycerol and 1% Triton X-100). Cell lysates were applied to wheat germ lectin agarose. Unbound proteins were washed out with lysis buffer. EGFR was eluted with lysis buffer containing 0.3 M of N-acetyl-D-glucosamine. Purification of PLC-γ1 was essentially performed as described previously [4]. Briefly, Sf9 cells expressing PLC-γ1 were harvested and washed with PBS and then lysed with lysis buffer. PLC-γ1 was purified using sequential DEAE-5PW, Phenyl-5PW, Heparine-5PW column chromatography. The eluted EGF receptor and purified PLC-γ1 were applied to a gel filtration column to exchange buffer into kinase assay buffer (20 mM HEPES-OH pH 7.4, 25 mM $MgCl_2$, 4 mM $MnCl_2$, 0.1 mM Na_3VO_4).

In vitro phosphorylation of Akt/PKB by EGF receptor

COS-7 cells were transfected with pUSEamp-Myc-Akt1/PKBα. After 24 hrs, cells were serum starved for 18 hrs to reduce endogenous phosphorylation of Akt/PKB. Cells were lysed with lysis buffer and Myc-tagged Akt/PKB was immunoprecipitated by immobilized Myc antibody as described below. Isolated Akt/PKB was further incubated with purified EGF receptor in the presence or absence of

ATP for 30 min. PLC-γ1 was also used as positive control of EGF-induced tyrosine phosphorylation. Phosphorylation of EGF receptor, PLC-γ1, Akt/PKB was detected by western blotting with respective antibodies indicated in figure legends. To dissect interaction of Akt/PKB with EGF receptor, reaction mixtures further washed three times with lysis buffer and western blotting was performed as described below.

Immunoprecipitation and western blotting

Cells were treated with EGF (100 ng/ml) for 10 min, washed twice with PBS, and lysed in the lysis buffer. After sonication, the cell homogenates were centrifuged at 10,000 *g* for 10 min. Fifty micrograms of cell lysate or in vitro kinase reaction mixture was electrophoresed on 8% polyacrylamide gels for immunoblotting. For immunoprecipitation, 400 μg of cell lysates were immuoprecipitated with either anti-Myc antibody-conjugated protein A agarose. The immunoprecipitates were subjected to SDS-PAGE and immunoprobed with indicated antibodies. The blots were developed with ECL reagents and the signal was detected by autoradiography.

Results and Discussion

Phosphorylation at serine, threonine, and tyrosine residues is essential nature of biological modulation of intracellular signaling molecules. Phosphorylation at amino acid residues confers negative charge to whole macromolecules and induce conformational changes to become active or inactive status. Likewise, Akt/PKB is also phosphorylated on Thr^{308} and Ser^{473} residues along with recruitment to plasma membrane upon activation of receptor tyrosine kinases [1]. The important roles of recruitment to the plasma membrane can be emphasized by the fact that association with PIP_3 via PH domain is critical initiation step for activation of Akt/PKB [2,5]. Furthermore, phosphorylation of Thr^{308} is mediated by PDK-1 which is a plasma membrane localized upstream kinase [1]. In this report, we have provided evidences that targeting to plasma membrane results in the tyrosine phosphorylation in addition to serine/threonine phosphorylation of Akt/PKB. Targeting to the plasma membrane of Akt/PKB has been reported to be important biological process for the activation of Akt/PKB upon stimulation of growth factors [2]. Furthermore, engineered Akt/PKB tagged by myristoylation site results in permanent localization to the plasma membrane, and maintains the activation status even in the absence of extracellular growth factor stimulation [15]. In line with this, tyrosine phosphorylation of Akt/PKB was induced by EGF stimulation, and addition of myristoylation site to the Akt/PKB showed dramatic increase in tyrosine phosphorylation along with Thr^{308} and Ser^{473} phosphorylation in EGF-dependent manner (Fig. 1). These results demonstrate that EGF-dependent tyrosine phosphorylation of Akt/PKB requires translocation to the plasma membrane. In previous report, it had been suggested that Src kinase was involved in the tyrosine phosphorylation Akt/PKB [8]. However, our results showed that over-expression of Src in 3Y1 fibroblast cells did not affect tyrosine phosphorylation of Akt/PKB (data not shown). It is possible that the discrepancy upon the effect of Src kinase may be due to the different context of cells. Nevertheless, it is notable that significant enhancement of tyrosine phosphorylation in myristoylated Akt/PKB by EGF-dependent manner. These findings lead us to dissect the role of EGF receptor itself in the tyrosine phosphorylation of Akt/PKB.

EGF receptor consists of immunoglobulin homology domain at extracellular side followed by membrane panning region and intracellular tyrosine kinase domain [20]. Like other growth factor receptors, the activation of EGF receptor is initiated by agonist binding and trans-phosphorylation at tyrosine residues of intracellular domain. Activated EGF receptor subsequently induces tyrosine phosphorylation of phospholipase C-γ (PLC-γ) and p85

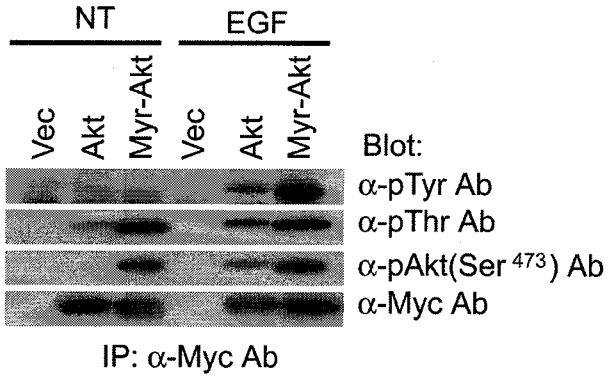

Fig. 1. Tyrosine phosphorylation of Akt/PKB by EGF stimulation. COS-7 cells were transfected with either Myc-tagged wild type or myristoylated Akt/PKB as described in Materials and Methods Cells were stimulated with EGF (100 ng/ml) for 10 min. Cell lysates were immunoprecipitated with anti-Myc antibody and blotted with indicated antibodies.

subunit of PI3K [12,22,23]. In vitro kinase assay of our experiments also demonstrated tyrosine phosphorylation of PLC-γ1 by EGF receptor (Fig. 2). At the same experimental context, EGF receptor also could induce tyrosine phosphorylation of Akt/PKB. In correlation with tyrosine phosphorylation of Akt/PKB, Ser^{473} phosphorylation was also induced by EGF receptor. It has been suggested that phosphorylation of Ser^{473} is mediated either by mTOR kinase activity or by autophosphorylation of Akt/PKB itself [24,25]. Since we have used purified EGF receptor and affinity purified Myc-tagged Akt/PKB for the *in vitro* kinase assay, the phosphorylation at Ser^{473} might be due to autophosphorylation of Akt/PKB. Likewise, we could not detect Thr^{308} phosphorylation which is mediated by PDK-1 in our experimental condition (data not shown). Given these results, it is possible that Akt/PKB is directly tyrosine phosphorylated by EGF receptor, and tyrosine phosphorylation induces Ser^{473} phosphorylation of Akt/PKB.

Upon the occupation of receptors with cognate agonists, downstream target molecules form direct molecular complexes with receptors. For example, immunoprecipitation

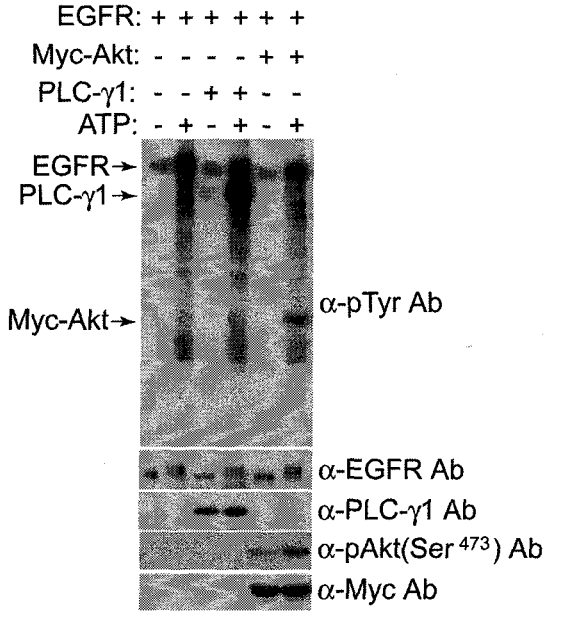

Fig. 2. Direct tyrosine phosphorylation of Akt/PKB by EGF receptor. Affinity purified Akt/PKB was incubated with affinity purified EGF receptor as described in Materials and Methods Purified PLC-γ1 was included as positive control of EGF-induced tyrosine phosphorylation. Reactions were initiated by addition of 100 mM of ATP for 30 min followed by western blotting with indicated antibodies. anti-pAkt(Thr^{308}) data was omitted since there were no signals detected.

of PLC-γ1 in EGF-stimulated cells results in the co-immunoprecipitation of EGF receptor or vice versa [23]. In correlation with this, our results also showed that tyrosine phosphorylated EGF receptor co-immunoprecipitated with Akt/PKB (Fig. 3A). Since we have used purified EGF receptor and affinity purified Akt/PKB from serum starved COS-7 cell lysates, molecular complex may be due to the direct association of Akt/PKB with EGF receptor. In addition, phosphorylation at Thr^{308} was not detected in this experimental condition, which eliminates possible contamination PDK-1 or p85 subunit of PI3K. More interestingly, co-immunoprecipitation of EGF receptor with Akt/PKB was not detected when the kinase reaction was not initiated by the addition of ATP (Fig. 3B) demonstrating activation of EGF receptor tyrosine kinase is necessary for the molecular interaction between EGF receptor and Akt/PKB. Given these results, we suggest that EGF receptor induce tyrosine phosphorylation of Akt/PKB by direct interaction.

It has been reported that XDXDY motif could be either a autophosphorylation site or a target phosphorylation site. For example, EDNDY of PTP-1B is tyrosine phosphorylated by EGF receptor [19]. Also, PDGF receptor contains EDNDY motif and thereby autophosphorylation at EDNDY motif provides docking site for PLC-γ recruitment [14]. Likewise, the analysis of mouse Akt/PKB sequence has revealed that Tyr^{326} matches with XDXDY motif, and is conserved among Akt/PKB isoforms (Fig. 4A), which raise the possible target site for tyrosine phosphorylation. Tyr^{326} is located at juxtaposition of Thr^{308}. Thr^{308} lies in the activation loop, and

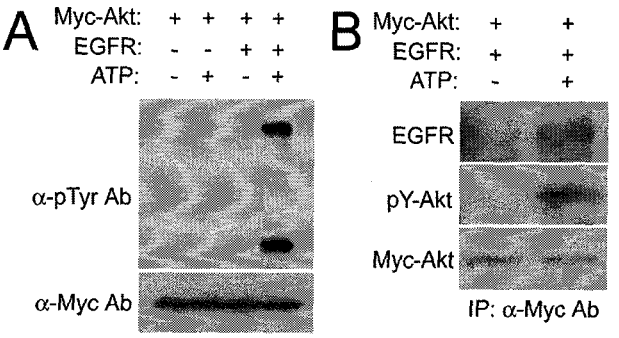

Fig. 3. Direct associated of EGF receptor with Akt/PKB. Affinity purified EGF receptor and Akt/PKB was incubated and kinase reactions were initiated by addition of ATP as described in Fig. 2. Total tyrosine phosphorylation was detected by a-pTyr antibody (A) or Akt/PKB was immunoprecipitated with a-Myc antibody and blotted with indicated antibodies.

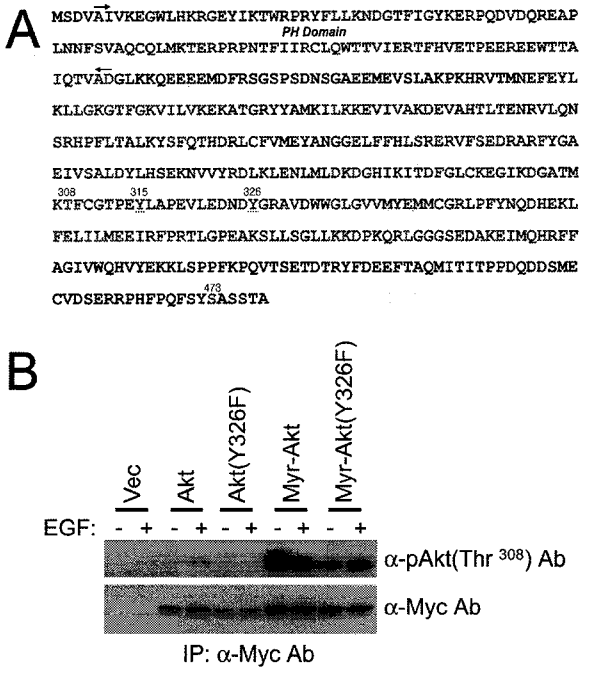

Fig. 4. Alanine mutation at Tyr[326] results in abrogation of Thr[308] phosphorylation. (A) Known Thr[308] and Ser[473] site as well as possible tyrosine phosphorylation sites were indicated. (B) Alanine mutation at Tyr[326] was introduced into either wild type or myristoylated Akt/PKB and transfected into COS-7 cells as described in Materials and Methods Transfected cells were stimulated with EGF (100 ng/ml) for 10 min and cell lysates were immunoprecipitated with anti-Myc antibody followed by western blotting with indicated antibodies.

phosphorylation of this residue opens up the activation loop, leading to conformational change [28]. By analogy, it is possible that phosphorylation at Tyr[326] induces conformational change and provides more relevant structure to be phosphorylated at Thr[308]. In correlation with this idea, mutation at Tyr[326] abolished phosphorylation at Thr[308] in both wild type and myristoylated Akt/PKB (Fig. 4). Therefore, it is likely that tyrosine phosphorylation at Tyr[326] is necessary for the subsequent phosphorylation at Thr[308] or Ser[473].

Current model for the activation of Akt/PKB only lies on the phosphorylation at Thr[308] and Ser[473]. However, our data provide strong evidences that phosphorylation at Tyr[326] is prerequisite process for the phosphorylation at Thr[308]. Hence, we hypothesized new model of Akt/PKB activation mechanism as follows: i) translocation to the plasma membrane through the activation of PI3K provides local interaction of EGF receptor with Akt/PKB, ii) EGF receptor directly phosphorylates at Tyr[326] of Akt/PKB and induces structural changes, iii) open up the activation loop by phosphorylation at Tyr[326] will give accessible Thr[308] and result in the phosphorylation, finally, iv) phosphorylation at Ser[473] by autophosphorylation or mTOR, which may provide stably active status of Akt/PKB. In conclusion, the mechanism by which extracellular stimuli-regulated Akt/PKB activity is actually far more complex than we previously thought and appear to be controlled by more than PI3K-dependent pathway though PI3K is critical factor for the final activation.

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초록 : EGF 수용체에 의한 Akt/PKB의 tyrosine 인산화에 대한 연구

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Akt/PKB는 세포의 증식, 분화, 사멸, 혈관신생 등 매우 많은 생리활성 조절에 있어 매우 중요한 역할을 수행한다. 우리는 Akt/PKB의 tyrosine 잔기의 인산화가 Thr^{308}의 인산화에 필수적임을 밝혔다. COS-7 세포주에 EGF를 처리하면 Akt/PKB의 tyrosine 잔기에 인산화가 촉진되었으며 이러한 인산화 촉진은 Akt/PKB에 myristoylation site를 이용해 세포막으로 이동시키면 더욱 더 증가하였다. 특히, 분리된 Akt/PKB와 EGF 수용체를 이용해 인산화 반응을 실시하면 tyrosine 잔기의 인산화뿐만 아니라 Ser^{473}에 대한 인산화도 증가하였다. 더욱이 tyrosine 잔기에 인산화 된 Akt/PKB는 활성화된 EGF 수용체와 직접적인 결합을 이루고 있음을 확인하였다. 마지막으로 예측되는 tyrosine 잔기인 Tyr^{326}을 Alanine으로 치환하면 정상 Akt/PKB뿐만 아니라 활성화된 Akt/PKB의 EGF에 의한 Thr^{308} 인산화가 사라짐을 확인하였다. 이러한 결과들을 바탕으로 EGF 수용체에 의한 직접적인 Akt/PKB의 tyrosine 인산화는 EGF에 의한 많은 생리활성 조절기전의 또 다른 기전이라 볼 수 있다.