Akt/Protein Kinase B Isoforms Are Differentially Regulated by Epidermal Growth Factor Stimulation* 

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Overexpression of epidermal growth factor receptor (EGFR) in certain cancers is well established. There is growing evidence that epidermal growth factor (EGF) activates Akt/protein kinase B (PKB) in a phosphoinositide 3-OH kinase (PI3K)-dependent manner, but it is not yet clear which Akt isoforms are involved in this signal transduction pathway. We investigated the functional regulation of three Akt isoforms, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, in esophageal cancer cells where EGFR is frequently overexpressed. Upon EGF stimulation, phosphorylation of Akt1 at the Ser-473 residue was remarkably induced. This result was corroborated by in vitro Akt kinase assays using glycogen synthase kinase 3β as the substrate. PI3K inhibitors, wortmannin or LY294002, significantly blocked the Akt kinase activity induced by EGF. Akt2 activity was evaluated by electrophoretic mobility shift assays. Robust activation of Akt2 by EGF was observed in some cell lines in a PI3K-dependent manner. EGF-induced Akt3 activation was demonstrated by Ser-472 phosphorylation of Akt3 but in a restrictive fashion. In aggregate, EGF-mediated activation of Akt isoforms is overlapping and distinctive. The mechanism by which EGFR recruits the PI3K/Akt pathway was in part differentially regulated at the level of Ras but independent of heterodimerization of EGFR with either ErbB2 or ErbB3 based upon functional dissection of pathways in esophageal cancer cell lines.

The epidermal growth factor receptor (EGFR) family is a tyrosine kinase receptor, which is composed of EGFR, ErbB2, ErbB3, and ErbB4. Overexpression of EGFR has been reported in a number of human cancers, including esophageal squamous cell carcinoma (1, 2). By contrast, ErbB2 overexpression has been noted in approximately 10-25% of human esophageal carcinomas (2–4), but this does not extend to ErbB3 and ErbB4 (5). EGFR overexpression is often associated with increased production of ligands, which bind EGFR, thereby creating an autocrine loop, which facilitates tumor growth (6). The mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K) signaling pathways are two major downstream pathways initiated by the activation of EGFR (7).

Akt (also named protein kinase B (PKB) and RAC protein kinase) is a serine/threonine protein kinase with homology to protein kinases A and C (8–10). Three mammalian isoforms (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ) have been cloned thus far. Akt2 and Akt3 have 81 and 83% homology in amino acid sequences with Akt1, respectively (11). The Thr-308 residue in the kinase domain and Ser-473 residue in the tail domain of Akt1 need to be phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and 3-phosphoinositide-dependent protein kinase-2 (PDK2), respectively, for its maximal activation. The corresponding phosphorylation sites in Akt2 (Thr-309 and Ser-474) and Akt3 (Thr-305 and Ser-472) have been identified, and Akt2 and Akt3 appear also to be regulated by PDK1 and PDK2 (12). A limited number of studies of the expression pattern of the three Akt isoforms reveal Akt1 and Akt2 are ubiquitous, whereas Akt3 is expressed predominantly in brain, heart, and kidney (13–15).

Akt has been shown to have a pivotal role in cell cycle progression (16–19), differentiation of smooth muscle cells (20), stimulation of glucose uptake and GLUT4 translocation (21), angiogenesis (22), inhibition of apoptosis (23–28), and cell growth (29). The mechanisms underlying how Akt exerts its anti-apoptotic effect in cells have attracted much attention. Targets of Akt related to apoptosis include BAD (30, 31), glycogen synthase kinase 3β (GSK3β) (32), forkhead transcriptional factors (FKHR, FKHR1, and AFX) (33–36), NF-xB (26, 37), glycogen synthase kinase 3β (GSK3β) (38), and CREB (39). In addition, gene amplification and overexpression of Akt2 in human ovarian, breast, and pancreatic carcinomas have been reported (40–42), and a recent report suggests a contribution of Akt3 to the aggressive phenotype of human breast cancer (43). Thus, Akt seems to confer a growth advantage to tumor cells.

There is mounting evidence that insulin and many growth factors activate Akt through a PI3K signaling pathway-dependent manner (23, 38, 44–47), although PI3K-independent mechanisms have been reported (48–51). EGF has also been shown to activate the PI3K/Akt signaling pathway in several EGFR-overexpressing cell lines such as prostate cancer cells (44), epidermoid cancer cells (45), and ovarian cancer cells (52).

Since most studies focus on the Akt1 isoform or have not discriminated among the differences between the three Akt isoforms, it is still unclear which Akt isoform(s) can be activated upon ligand stimulation under physiological conditions. Therefore, we sought to investigate the regulation of the three
mammalian Akt isoforms, Akt1, Akt2, and Akt3, in cells derived from human esophageal squamous cell carcinoma where EGFR can be overexpressed. Furthermore, we sought to elucidate whether EGFR-mediated activation of the Akt isoforms requires heterodimerization of members of the ErbB receptors or, alternatively, recruits the Ras pathway. We have found that human esophageal cancer cells can be categorized into three groups as follows: cells in which EGF stimulation activates all three Akt isoforms; cells in which EGF stimulation activates only Akt1. Functionally, EGFR activates the PI3K/Akt signaling pathway differentially in a Ras-dependent manner but ErbB2- and ErbB3-independent fashion in the cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Tissue Culture**—TE-1, TE-2, TE-3, TE-5, TE-6, TE-8, TE-9, TE-10, TE-11, TE-12, TE-15, T.T, H-4, and H-7 cells are human esophageal squamous cancer cell lines; HaCaT cells are immortalized skin keratinocytes; MCF-7 cells are human breast cancer cells; A431 cells are epidermoid cancer cells; HepG2 is a liver cancer cell line; and Panc-1 cells are derived from human pancreatic adenocarcinomas. These cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (Sigma), 100 mg/ml streptomycin (Sigma), 100 units/ml penicillin (Sigma), and 1-glutamine (Life Technologies, Inc.) at 37 °C in a 5% CO₂ incubator. Cell lysates of NIH3T3 cells treated or untreated with PDGF were purchased from New England Biolabs (Beverly, MA).

**Reverse Transcription-PCR (RT-PCR) and PCR Southern Analyses**—Total RNA was prepared as described previously (53). Ten μg of total RNA was incubated with 5 units of DNase I (Life Technologies, Inc.) at 20 °C for 15 min in 50 μl of a reaction consisting of 20 μl Tris, pH 8.4, 2 mM MgCl₂, and 50 μl KCl. The reaction was heat-inactivated at 65 °C for 15 min in the presence of 2.5 mM EDTA. Ten μl of the reaction was mixed with 0.5 μg of oligo(dT)₁₄₋₂₀ primer (Life Technologies, Inc.) and incubated at 70 °C for 10 min and on ice for 5 min. Following annealing at 42 °C for 5 min, 200 units of SuperScript II RNAase H Reverse Transcriptase (Life Technologies, Inc.) was added and further incubated at 42 °C for 50 min in 20 μl of reaction consisting of 10 μM Tris, pH 8.3, 50 μM KCl, 2.5 mM MgCl₂, 0.5 mM dNTPs, and 10 mM dithiothreitol. The reaction was terminated by incubating at 70 °C for 15 min. The RNase H treated samples were subconfluent, they were switched to serum-free medium (Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (Sigma), 100 μg/ml streptomycin, 100 μg/ml penicillin, and L-glutamine (Life Technologies, Inc.) and incubated at 37 °C for 20 min.

For polymerase chain reaction (PCR), the following oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA) and used as primers: 5'-GGCTGGACAGATGCTGGA-3' (Akt1 sense), 5'-GGTAATGCAGATGCTGGA-3' (Akt1 antisense); 5'-GGCCCGCTCG- CATCGGGAAC-3' (Akt2 sense), 5'-GGCCCGCTCGAATCGGGAAC-3' (Akt2 antisense); 5'-AACTCATATAGCGAACGAAATG-3' (Akt3 sense), 5'-AACTCATATAGCGAACGAAATG-3' (Akt3 antisense); 5'-AGAAAGTGAGTCGAGCAGCA-3' (c-ErbB3 sense); 5'-TTGAGGTTGTCATTCTGCA-3' (c-ErbB3 antisense). PCR was carried out for 35 cycles (denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min) in a 50 μl reaction mixture consisting of 10 μM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, 200 μM primers, 2.5 units of Taq DNA polymerase (Fisher), and 2 μl of the cDNA. The PCR products were electrophoresed on a 1.5% agarose gel.

For PCR Southern blot analysis, the Akt3 RT-PCR products were electrophoresed on a 1.5% agarose gel. The gel was denatured with 1.5 mM sodium chloride, 0.5 mM sodium hydroxide, neutralized with 1.5 mM sodium chloride, 0.5 mM Tris, pH 7.4, and 1 mM EDTA, and transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech). Hybridization was carried out using Rapid-blot buffer (Amersham Pharmacia Biotech) at 42 °C for 1 h. Subsequent washes were done once at room temperature with 2× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and twice at 42 °C with 0.5× SSC, 0.1% SDS. Autoradiography was performed using Kodak X-Omat AR film (Eastman Kodak Co.).

**Transient Transfection**—One μg of either a dominant negative ras plasmid (RasA77V) (gift from T. Wang) or an empty vector was transiently transfected into 1 × 10⁶/ml cells by a liposome-mediated method using 4 μl of LipofectAMINE and 6 μl of PLUS reagent (Life Technologies, Inc.). After overnight serum starvation, cells were stimulated with 10 ng/ml recombinant mouse EGF (Roche Molecular Biochemicals) for 30 min; total cell lysates were obtained, and Western blotting was carried out as described under “Immunodetection of Phospho-Akt and Phospho-GSK3β.”

**Antibodies**—Antibodies against EGFR, ErbB2, ErbB3, and ErbB4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or NeoMarkers (Union City, CA). An anti-phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories (Lexington, KY). Sheep anti-Akt1 and -Akt3 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-Akt antibodies were generated against the carboxyl-terminal sequences of human Akt1 and Akt3. Polyclonal rabbit anti-ErbB2 antibodies were purchased from Amersham Pharmacia Biotech, and an anti-sheep horseradish peroxidase antibody was purchased from Amersham Pharmacia Biotech.

**Immunodetection of Phospho-Akt and Phospho-GSK3β**—When cells were subconfluent, they were switched to serum-free medium (Dulbecco’s modified Eagle’s medium plus 0.1% fetal bovine serum) overnight (16–18 h) and were stimulated with 10 ng/ml EGF for 30 min at 37 °C. For the time course experiment, the cells were stimulated with 10 ng/ml EGF for 5, 15, 30, 60, and 120 min. To block the PI3K signaling pathway, cells were pretreated with 100 nM wortmannin (Upstate Biotechnology) or 100 μM LY294002 (Sigma) or vehicle (0.1% Me₂SO) for 30 min prior to stimulation with EGF. After treatment, cells were lysed in Akt lysis buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, and a protease inhibitor mixture tablet (Roche Molecular Biochemicals)). Protein concentration was determined by the BCA protein assay (Pierce). Total protein samples (10 μg) were separated on a 6% SDS-polyacrylamide gel (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked in 5% non-fat milk (Bio-Rad) in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, and 0.1% Tween 20) overnight at 4 °C. Membranes were probed with anti-ErbB antibodies specific for each of the four ErbB receptors for 1 h at room temperature, washed three times in TBST, incubated with anti-mouse or anti-rabbit horseradish peroxidase antibody diluted 1:3,000 in TBST for 1 h at room temperature, and then washed three times in TBST. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus, Amersham Pharmacia Biotech) and was exposed to Kodak X-OMAT LS film (Kodak).
fluoride (PMSF), and a protease inhibitor tablet). The total cell lysates were separated on a 10% SDS-PAGE for the detection of Akt phosphorylated at Ser-473 (100 μg/lane) and GSK3β phosphorylated at Ser-9 (10 μg/lane). The transferred proteins on PVDF membranes were probed with the phospho-Akt or phospho-GSK3β antibody (1:1,000) followed by incubation with the anti-rabbit horseradish peroxidase antibody (1:3,000) and then immunodetected as described above. The membranes were stripped by exposing them to either 62.5 mM Tris, pH 6.8, and 2% SDS for 30 min at 50 °C or reblotting solution (Re-Blot Western blot Recycling Kit, Chemicon International Inc., Temecula, CA) for 10 min at room temperature and reprobed with the anti-Akt1 antibody or anti-GSK3β antibody for confirmation of equal protein loading.

**Akt2 Mobility Shift Assays**—Total protein samples (40 μg/lane) prepared in Akt lysis buffer as described above were separated on a 7.5% SDS-PAGE using a tall gel apparatus (Hoefer SE410 Sturdier Vertical Unit, Amersham Pharmacia Biotech) for adequate separation between phosphorylated and unphosphorylated Akt2 bands and transferred to a PVDF membrane. The anti-rabbit Akt2 antibody was used at 1:5,000 dilution as a primary antibody for the immunodetection of Akt2.

**Akt Kinase Assays**—After overnight serum starvation, subconfluent TE-2, TE-5, TE-8, TE-9, TE-10, and TE-12 cells were stimulated with 10 ng/ml EGF for 30 min and followed by Akt kinase assays. To inhibit the P13K signaling pathway, subconfluent TE-2, TE-3, TE-8, and TE-12 cells were pretreated with 100 μM wortmannin, 100 μM LY294002, or vehicle alone for 30 min prior to stimulation with EGF. The final concentration of Me_SO was less than 0.1%. We employed a non-radioactive kinase assay system of Akt (Akt kinase assay kit, New England Biolabs) following the company’s instructions. Briefly, cells were washed once with ice-cold phosphate-buffered saline, lysed in 1 ml of cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM β-glycerol phosphate, 1 mM FMSF, and 1 μg/ml leupeptin), and incubated on ice for 10 min. The cell lysates were centrifuged for 10 min at 14,000 rpm at 4 °C. Supernatants were adjusted so that each sample contained an equal amount of protein (150 μg) and were then incubated with cross-linked Akt antibody for 3 h at 4 °C. The immunoprecipitates were pelleted and washed twice in cell lysis buffer and twice in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM MgCl₂). The pellets were suspended in kinase buffer containing 200 μM ATP and 1 μg of a GSK3 fusion protein (CIPK/GPGRRGRRTSSFAEG) as the substrate and then incubated for 30 min at 30 °C. The reaction was terminated by addition of 3× SDS sample buffer (187.5 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 150 mM dithiothreitol, and 0.03% bromphenol blue). The samples were heated at 95 °C for 5 min, separated on a 12% SDS-PAGE, and the proteins were transferred on a PVDF membrane. Immunoblotting was done with the phospho-GSK3β antibody raised against Ser-9. The kinase assays were repeated three times in each cell line. The signals were quantified using the NIH Image 1.62 program, and statistical analysis was performed by Scheffe’s test, and p < 0.05 was considered significant.

**Immunoprecipitation/Western Blot Analysis**—TE-8 cells were serum-starved overnight and then stimulated with 10 ng/ml EGF for 30 min at 37 °C, washed three times with ice-cold phosphate-buffered saline, lysed in Akt lysis buffer as described above, and centrifuged for 15 min at 4 °C. TE-8 cells cultured in medium supplemented with 10% serum served as a positive control. The supernatants were incubated with 2 μg of the anti-EGFR, ErbB2, ErbB3, or p85 antibody or 4 μg of the anti-Akt3 antibody for 1 h at 4 °C followed by incubation with 20 μl of protein A/G Plus agrose beads (Santa Cruz Biotechnology) for 5 h at 4 °C. The beads were subsequently washed three times with Akt lysis buffer and probed with an anti-tubulin antibody as a loading control (bottom panel).
buffer, solubilized in SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromphenol blue) containing 5% β-Mercaptoethanol, boiled at 95 °C for 5 min, and subjected to SDS-PAGE followed by Western blotting under the conditions described above.

RESULTS

Expression of ErbB Family Members in Human Esophageal Cancer Cell Lines—We sought initially to determine the expression of the four ErbB family members in human esophageal cancer cells by Western blot analysis using specific antibodies for each receptor. This was done to assess the levels of each receptor as a basis for the determination of whether there was heterodimerization between receptors that could eventually be involved in the activation of Akt. EGFR was expressed in TE-2, TE-5, TE-8, TE-9, TE-10, TE-12, and HaCaT cells, and the expression level of EGFR in TE-8 and TE-12 cells was comparable to A431 cells that are well established EGFR over-expressing cells (Fig. 1). ErbB2 was expressed in TE-2 and TE-5 cells and barely expressed in TE-10 and HaCaT cells (Fig. 1). Although we performed Western blots with an anti-ErbB3 antibody, we were able to detect signals from esophageal cancer cell lines only when immunoprecipitation was employed prior to the immunoblot. The expression level of ErbB3 was much weaker than in A431 cells which are well known ErbB3 expressing cells (data not shown), suggesting that the expression level of ErbB3 in esophageal cancer cells is relatively low. The presence of ErbB3 mRNA in all cell lines was confirmed by RT-PCR analysis using ErbB3-specific primers (data not shown). We could not detect expression of ErbB4 in the esophageal cancer cell lines tested even when immunoprecipitation with an ErbB4 antibody was conducted prior to the immunoblot (data not shown).

Akt1 and Akt2 Are Ubiquitously Expressed but Akt3 Has Cell Type-limited Expression—Akt/PKB is one of the major downstream molecules of EGFR transduced via the PI3K signaling pathway. Since we found EGFR is highly expressed in human esophageal cancer cells, we next examined the expression of the three Akt isoforms in those cell lines. The expression of Akt1 and Akt2 was assessed by RT-PCR and Western blot analysis utilizing primers and specific antibodies for each isoform, respectively, and the expression of Akt3 was analyzed by RT-PCR alone since an anti-Akt3-specific antibody useful for Western blot has yet to be generated to our knowledge. As shown in Fig. 2, both Akt1 and Akt2 proteins were expressed in the human esophageal cancer cell lines tested. Akt2 expression was ubiquitous, whereas Akt1 expression was variable and even low as in TE-1, HCE-4, and HCE-7 cells. The expression levels of both isoforms did not reveal any obvious correlation with the expression level of EGFR.

RT-PCR analysis was consistent with that of the Western blot for Akt1 and Akt2. The Akt1 and Akt2 transcripts were ubiquitous and detected in various cancer cell lines including MCF-7, HepG2, and Panc-1 cells (Fig. 3A). By contrast, Akt3 mRNA showed cell type-specific expression with negative and positive expression in HepG2 and Panc-1, respectively. Interestingly, Akt3 mRNA revealed specific expression among esophageal cancer cells with high expression in HCE-4, HCE-7, TE-1, TE-8, TE-9, and TE-10 cells and relatively low or no expression in other cells (Fig. 3A).

In order to assess the presence or absence of Akt3 mRNA, PCR Southern blot analysis was performed using a [γ-32P]ATP-labeled Akt3-specific probe. PCR Southern blot analysis revealed that all cell lines and normal human esophagus express Akt3 mRNA. Signals were detected in RT-PCR-negative cells like TE-12 and TE-15 cells, raising the sensitivity of detection.

![Fig. 4. Western blot with the anti-phospho-Akt1 (Ser-473) antibody upon EGF treatment in various human esophageal cancer cell lines.](image)

**Fig. 4.** Western blot with the anti-phospho-Akt1 (Ser-473) antibody upon EGF treatment in various human esophageal cancer cell lines. Cells were treated with EGF (10 ng/ml for 30 min) after overnight serum starvation, and cell lysates were prepared as described under “Experimental Procedures.” Western blotting with the anti-phospho-Akt1 antibody, which recognizes only phosphorylated Ser-473 residue, was performed in human esophageal cancer cell lines (TE-2, TE-5, TE-8, TE-9, TE-10, and TE-12 cells) (top panel). PDGF-treated NIH3T3 cells served as a positive control. The phospho-Akt antibody demonstrated two bands at 60 kDa. The phosphorylation of Akt was enhanced by EGF stimulation in all the esophageal cancer cell lines examined, although TE-5 cells showed high basal phosphorylation of Akt. The intensity of upper bands was stronger than that of lower bands in TE-2, TE-5, TE-9, and TE-12 cells (lanes 2, 4, 8, and 12), and the intensity of both bands was equivalent in TE-8 and TE-10 cells (lanes 6 and 10). The same membrane was reprobed with the anti-Akt1 antibody, which recognizes Akt1 regardless of its phosphorylation status, and served as a loading control (bottom panel).
and confirming the sequence specificity of the PCR products. Although these experiments are not quantitative, given the equivalent level of β-actin RT-PCR products in all samples tested, Akt3 transcription is likely to be regulated differentially.

Akt1 and Akt3 Are Activated by EGF Stimulation through the PI3K Signaling Pathway in Human Esophageal Cancer Cell Lines—Since it has been reported that EGF stimulates the PI3K/Akt signaling pathway, we tested this notion in human esophageal cancer cell lines. EGF (10 ng/ml) induced a time-dependent increase in the tyrosine phosphorylation of high molecular mass (~170 kDa) proteins that peaked at 30 min in TE-2 and TE-12 cells (data not shown). Activation of Akt by EGF stimulation was analyzed by two independent experimental approaches with a phospho-specific antibody, which recognizes Akt1 only when phosphorylated at the Ser-473 residue, and by in vitro kinase assays. Akt was not phosphorylated in serum-free basal conditions in most cells except for TE-5 cells. Two bands of ~60 kDa were recognized when the phospho-Akt antibody was employed, and the slower migrating band was totally abrogated by wortmannin and LY294002 treatment, which was consistently observed in each kinase assay. Me2SO (0.1%) treatment alone did not affect the kinase activity (data not shown). WO, wortmannin; LY, LY294002. C, Akt kinase activity activated by EGF is significantly abolished by PI3K inhibitors in esophageal cancer cell lines. The signals of the Akt kinase assays in B were quantified using the NIH Image 1.62 program, and data are presented as mean ± S.E. Analysis of variance followed by Scheffe’s test was performed, and p < 0.05 was considered significant. The PI3K inhibitors significantly abrogated the EGF-induced Akt kinase activity in all the cell lines examined.
Regulation of Akt Isoforms by EGF

cells started to appear at 5 min, peaked at 1 h, and decreased to 2 h. In TE-5 cells, phosphorylation started at 15 min and was sustained to 2 h. These observations imply that unknown mechanisms, perhaps through the actions of tyrosine and lipid phosphatases (54), attenuate the activation of Akt by EGF.

As a next step, we sought to clarify the signaling pathway responsible for the phosphorylation of Akt upon EGF stimulation in these cell lines. We employed well established PI3K inhibitors, wortmannin and LY294002, to blunt the PI3K signaling pathway. The inhibitors themselves did not affect cell viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (data not shown). PDGF-treated and untreated NIH3T3 cells served as a positive and negative control for the Akt2 mobility shift, respectively (lanes 13 and 14). WO, wortmannin; LY, LY294002; WB, Western blot.

Since a previous report indicated that the phospho-Ser-473 Akt antibody could recognize the Akt3 isoform as Ser-473 is conserved in the human Akt3 sequence, and the migration of expressed Akt3 is faster than that of Akt1 (13, 15), we postulated that the fast-migrating bands noted in Fig. 4 might represent phosphorylated Akt3. In order to test this, we performed immunoprecipitation with the Akt3-specific antibody in TE-8 cells under EGF-unstimulated or EGF-stimulated conditions and then immunoblotted with the phospho-Ser-473 Akt antibody. As shown in Fig. 7, when immunoprecipitation was performed with the anti-Akt3 antibody, only the fast-migrating band could be detected upon EGF treatment, verifying that the slow migrating and the fast migrating bands correspond to Akt1 and Akt3, respectively. Therefore, EGF induced the phosphorylation of only Akt1 in TE-2, TE-5, TE-9, and TE-12 cells and both Akt1 and Akt3 isoforms in TE-8 and TE-10 cells. The phosphorylation of both Akt1 (slow migrating bands) and Akt3 (fast migrating bands) was inhibited by pretreatment with a PI3K inhibitor, wortmannin or LY294002 (Fig. 7), demonstrating that the phosphorylation of Akt3 is also mediated through the PI3K signaling pathway.

In order to confirm the result obtained from the phospho-Akt analysis, Akt kinase assays were performed using a GSK3 fusion protein as the substrate. Of note, the antibody used for immunoprecipitating Akt1 (New England Biolabs) does not cross-react with Akt2, but possible cross-reactivity with Akt3 is as yet unknown. As shown in Fig. 8A, kinase activity of Akt1 was enhanced upon EGF stimulation in all the esophageal cancer cell lines examined except for TE-10 cells, and the Akt kinase activity activated by EGF was significantly suppressed by the PI3K inhibitors, wortmannin and LY294002, in TE-2, TE-3, TE-8, and TE-12 cells (Fig. 8, B and C). These results are consistent with those observed in the phospho-Akt analysis and substantiate the observation that EGF activates Akt kinase activity through the PI3K signaling pathway in human esophageal cancer cells.

Akt2 Is Activated upon EGF Stimulation through the PI3K Signaling Pathway in Some Human Esophageal Cancer Cell Lines—We next set out to explore the regulation of Akt2 by EGF in esophageal cancer cell lines. We employed mobility shift assays with an Akt2-specific antibody to determine the phosphorylation status of Akt2 since an antibody specifically recognizing phosphorylated Akt2 is not available. The Akt2-specific antibody detected a marked electrophoretic mobility shift in TE-2, TE-8, and TE-10 cells in response to EGF stimulation, and there was no obvious mobility shift in TE-5, TE-9, and TE-12 cells (Fig. 9A). The PI3K inhibitor wortmannin or LY294002 inhibited the Akt2 mobility shift in TE-2, TE-8, and TE-10 cells (Fig. 9B), confirming that EGF induced the phos-
phosphorylation of Akt2 through a PI3K-dependent signaling pathway in these cell lines.

In summary, EGF promotes the phosphorylation of all three isoforms of Akt in TE-8 and TE-10 cells, Akt1 and Akt2 in TE-2 cells, and only the Akt1 isoform in TE-5, TE-9, and TE-12 cells but consistently in a PI3K-dependent manner. This implies variable regulation of the different Akt isoforms upon EGF stimulation even within cancer cells of the same origin.

**EGFR Recruits the PI3K/Akt Signaling Pathway in a Partially Ras-dependent Manner**—We further explored the molecular mechanisms underlying PI3K recruitment by EGF stimulation in esophageal cancer cells. Since previous reports have shown that ErbB3 can be an adaptor protein bridging between EGFR and PI3K in A431 and breast cancer cells (55, 56), we carried out immunoprecipitation in TE-8 cells with the antibody against EGFR, ErbB2, or ErbB3 followed by immunoblotting with the anti-phosphotyrosine antibody or antibodies against each ErbB receptor. As expected, EGFR was highly phosphorylated upon EGF stimulation, whereas ErbB2 and ErbB3 were not phosphorylated (Fig. 10A). When TE-8 cells were cultured in medium supplemented with 10% serum, protein–protein interactions between EGFR, ErbB2, and ErbB3 and between ErbB3 and p85, the regulatory subunit of PI3K, were clearly demonstrated (Fig. 10B). On the contrary, when cells were stimulated by 10 ng/ml EGF for 30 min in serum-free medium, protein–protein interactions among the ErbB family members or between p85 and the ErbB family members were not observed (Fig. 10A and data not shown). Therefore, we conclude that neither ErbB2 nor ErbB3 is likely to be responsible for the EGF-activated EGFR/PI3K/Akt cascade in esophageal cancer cells.

Next, we explored the possibility that Ras could serve as the bridge between EGFR and PI3K/Akt since it has been shown that catalytic subunit of Ras, p110, is a direct target of Ras (57). We suppressed Ras activity by transfecting a dominant negative ras vector (RasAsn-17) in esophageal cancer cells, and we examined its effect on the phosphorylation of Akt stimulated by EGF (10 ng/ml for 30 min.) by Western blotting with the phospho-Akt antibody. We found that the phosphorylation of Akt induced by EGF was totally abolished in TE-5 cells and partially blunted in TE-8 and T.T cells by the dominant negative ras vector (Fig. 11A). Our results suggest that EGFR recruits the PI3K/Akt pathway via a Ras-dependent manner, although the degree to which Ras is involved appears to be variable.

We further investigated the effect of Ras blockade on each Akt isoform. The effect of blunted Ras upon EGF-activated Akt2 was assessed by mobility shift assays. The mobility shifted bands corresponding to phosphorylated Akt2 were totally inhibited in TE-2 and TE-8 cells and partially in TE-10 cells (Fig. 11B).

Finally, the effect of abolishing Ras upon EGF-activated Akt1 and Akt3 was individually assessed by employing transfection of RasAsn-17 and immunoprecipitation with the anti-Akt3 antibody followed by Western blotting with the phospho-Akt antibody. As shown in Fig. 11C, RasAsn-17 revealed variable
effects on Akt1 and Akt3. In TE-8 cells, the EGF-induced phosphorylation of the slow migrating band corresponding to Akt1, but not the fast-migrating band corresponding to Akt3, was inhibited by Ras\textsuperscript{Ason-17}. This result was further confirmed by employing immunoprecipitation with the anti-Akt3-specific antibody by showing only Akt3-specific bands. By contrast, in TE-10 cells, the EGF-induced phosphorylation of Akt1 was unaffected by Ras\textsuperscript{Ason-17}, whereas the EGF-induced phosphorylation of Akt3 was totally abolished.

**DISCUSSION**

The overexpression of EGFR in certain human cancers has been well documented (1, 2, 58) and is often associated with increased production of EGFR ligands such as EGF and transforming growth factor-\(\alpha\) creating a positive autocrine loop that confers advantage for tumor cells to grow (6). In epidermoid and breast cancer cells, other EGFR family members such as ErbB2, ErbB3, and ErbB4 are also overexpressed and allow for heterodimerization among the ErbB family members (59–62).

It is still unknown which ErbB family receptors are mainly expressed in human esophageal cancer. We have demonstrated using cells derived from human esophageal squamous cell carcinomas that EGFR is the ErbB family member dominantly expressed in the cells. Our data expand upon a recent report using cells derived from human esophageal squamous cell carcinoma expressing high level of Akt2, Akt2 was not activated by EGF stimulation in TE-5, TE-9, and TE-12 cells. Lack of Akt3 activation by EGF in TE-2, TE-5, and TE-12 cells may be explained by the low level of Akt3 in these cells, but Akt3 was not activated in TE-9 cells, which do express Akt3. This raises the question of why Akt2 or Akt3 is not simultaneously activated by physiological concentrations of EGF. It is possible that different ligands may activate different Akt isoforms in cells, and even the same ligand may recruit different Akt family members depending on the potency of ligand stimulation. Interestingly, differential regulation of Akt isoforms was very recently demonstrated where Akt1 activity was serum-inducible, and Akt2 and Akt3 were constitutively activated in glioblastoma cell lines (66).

What are the mechanisms underlying activation of the different Akt members upon growth factor stimulation in cells? Although the overall mechanism of Akt activation is not completely understood, it has been described that Akt activity is regulated by its translocation from the cytosol in quiescent cells to the plasma membrane in activated cells by virtue of binding to phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate produced by PI3K (67). At the plasma membrane, PKD1 and PKD2, which may be a modified PKD1 (68), phosphorylate Akt at Thr-308 and Ser-473 residues of Akt1, respectively, and equivalent phosphorylation sites of Akt2 and Akt3 as well (12). Differential regulation of the three Akt isoforms described in our studies raises the possibility that yet undiscovered cellular player(s) other than PKD1 and PKD2 might exist and be involved in the regulatory machinery of Akt that could be differentially involved in activation of each Akt isoform. It is also conceivable that an Akt3-specific kinase exists (54). It was very recently reported that the Tcl1 oncogene was a cofactor of Akt1 but not of Akt2, which enhances Akt1 kinase activity and promotes its nuclear transport, suggesting that Tcl1 plays a pivotal role in differential regulation of Akt isoforms in certain lymphoid cells (69), although this is not applicable in epithelial cells. Identification of the cellular molecules responsible for the differential regulation of Akt family members in epithelial cells is under current investigation.

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