The Akt isoforms are present at distinct subcellular locations

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Santi SA, Lee H. The Akt isoforms are present at distinct subcellular locations. Am J Physiol Cell Physiol 298: C580–C591, 2010. First published December 16, 2009; doi:10.1152/ajpcell.00375.2009.—Akt is involved in the regulation of diverse cellular functions such as cell proliferation, energy metabolism, and apoptosis. Although three Akt isoforms are known, the function of each isoform is poorly understood. To gain a better understanding of each Akt isoform, we examined the subcellular localization and expression of each isoform in transformed and nontransformed cells. Akt1 was localized in the cytoplasm, which is in agreement with the currently accepted model that cytoplasmic Akt is translocated and activated at the inner leaflet of the plasma membrane. Interestingly, HEK-293 and HEK-293T cells contained Akt1 in the nucleus and cytoplasm, respectively, suggesting that SV40 T-antigen plays a crucial role in the cytoplasmic localization and activation of Akt1 in HEK-293T. Akt2 was colocalized with the mitochondria, while Akt3 was localized in both the nucleus and nuclear membrane. The subcellular localization of the Akt isoforms was not substantially altered in response to ionizing radiation or EGF. Furthermore, the ablation of one Akt isoform by small interfering RNA (siRNA) did not alter the subcellular localization of the remaining isoforms, suggesting that the major function of one isoform is not compensated for by other isoforms. Together, our data support the notion that Akt2 and Akt3 are regulated at the mitochondrial and nuclear membranes, respectively. The mitochondrial localization of Akt2 raises the possibility that this isoform may be involved in both glucose-based energy metabolism and suppression of apoptosis, two Akt functions previously identified with anti-pan-Akt antibodies.

phosphatidylinositol 3-kinase; signal transduction; subcellular localization; mitochondria; radiation

AKT/PKB (protein kinase B) intricately regulates many cellular functions such as cell growth and proliferation, cell survival, apoptosis, energy metabolism, and resistance to anticancer therapeutics (14, 17, 19). Akt consists of three isoforms, Akt1, Akt2, and Akt3 (or PKBα, β, and γ). Although these three isoforms are encoded by separate genes, they share a common NH2-terminal plekstrin homology (PH) domain, a catalytic domain in the middle, and a COOH terminus (12, 19). The identity of the overall amino acid sequence of the three isoforms is very high (~80%); however, the COOH terminus and the PH-linker region are more diverse (12).

Despite the growing amount of research demonstrating the existence of isoform-specific regulation, many papers still draw generalized conclusions about Akt function without considering the unique function of each Akt isoform. There are reasons as to why this is still the case. First, given the high degree of sequence similarity at both nucleotide and amino acid levels, many studies have concluded that the regulation of Akt1, 2, and 3 are functionally redundant. Lending further credence to this hypothesis is the fact that all three kinases contain similar phosphorylation/activation sites: threonine 308 (Akt1), 309 (Akt2), and 305 (Akt3) and serine 473 (Akt1), 474 (Akt2), and 472 (Akt3). Phosphorylated or activated Akt regulates many of its cellular functions through the phosphorylation of a series of downstream players. These substrates, which may be inhibited or activated by Akt-mediated phosphorylation, often contain the phospho-Akt-substrate (PAS) motif (R-X-R-X-X-S/T) (17). Thus all three Akt isoforms may be similarly phosphorylated and activate downstream substrates through the PAS motif. Second, effective tools for studying Akt in an isoform-specific manner have been lacking. For example, the fact that the phosphorylation sites of all three Akt isoforms differ by only one amino acid residue has made the examination of isoform-specific function difficult.

Because of the difficulty of studying isoform-specific functions, many studies have simply examined Akt function with anti-“pan”-Akt antibodies, which can detect all three Akt isoforms. On the basis of data obtained mainly from these works, the following Akt functional model has been suggested: On the activation of phosphatidylinositol 3-kinase (PI3K), Akt in the cytoplasm translocates to the inner leaflet of the plasma membrane, where it is phosphorylated/activated. This widely accepted model assumes that all three isoforms are present in the cytoplasm. However, accumulating lines of evidence suggest that this assumption may not be correct. Furthermore, data obtained from experiments carried out with small interfering RNA (siRNA) appear to suggest that the regulation of Akt isoforms may not be redundant, and may be more intricately networked than previously thought.

The purpose of this study was to lay down groundwork to eventually gain a better understanding about how each Akt isoform differentially regulates cellular functions. In this study, we took advantage that many anti-Akt “isoform-specific” antibodies are now available through several commercial sources. We identified suitable isoform-specific antibodies through rigorous and systematic characterization. Equipped with this tool, we then examined the subcellular localization and the levels of mRNA and protein of each Akt isoform in several different cancer and noncancer cell lines. Our data showed that each Akt isoform is present, in most cases, at a unique subcellular location: Akt1 in the cytoplasm, Akt2 at the mitochondria, and Akt3 in the nucleus. We found that the subcellular localization of each Akt isoform was not substantially altered in the presence or absence of other Akt isoforms, ionizing radiation, or epidermal growth factor (EGF).
MATERIALS AND METHODS

Antibodies, chemicals, and other reagents. Isoform-specific blocking peptides and the following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-Akt1 (D-17, sc-7126), anti-Akt2 (F-7, sc-5270), anti-Akt3 (M-14 and C-14, sc-11521), antilamin B1, anti-calnexin, anti-protein disulffide isomerase (PDI), anti-trans-Golgi network 38 (TGN38), and an voltage-dependent anion-selective channel 1 (VDAC1) antibodies, as well as donkey anti-goat IgG FITC, goat anti-mouse IgG FITC, donkey anti-rabbit IgG-rhodamine, and rhodamine-conjugated donkey anti-goat IgG. The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-Akt1 (2H10) (no. 2967), anti-Akt2 (no. 2962), and anti-Akt3 (no. 4059), anti-phosphorylated Akt (p-Akt S473), anti-pan-Akt, and anti-phosphatase and tensin homolog (PTEN) antibodies. The following reagents were from Upstate Biotechnology (Lake Placid, NY): anti-Akt1 (07-416), anti-Akt2 (07-372), and anti-Akt3 (05-780 and 07-383) antibodies and recombinant proteins specific for each of the Akt isoforms. Anti-α-tubulin and anti-γ-tubulin antibodies were from Sigma. MitoTracker Red CMXRos Mitochondrial Probe was from Cambrex Bioscience Walkersville (Charles City, IA). Secondary peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat antibodies were purchased from Sigma, Pierce, and Calbiochem, respectively. Fluorescein (FITC)-conjugated AffiniPure donkey anti-mouse IgG, AffiniPure Fab fragment rabbit anti-goat (IgG) (H + L), and Cy5-conjugated AffiniPure donkey anti-rabbit IgG were purchased from Jackson Immunoresearch (West Grove, PA). DNase I, Moloney murine leukemia virus (MMLV), Lipofectamine 2000 transfection reagent, and Opti-MEM I Reduced Serum medium utilized in the siRNA transfections were purchased from Invitrogen.

Cells and cell culture. The following cell lines used in these studies were purchased from American Type Culture Collection (ATCC) (Manassas, VA): three breast cancer cell lines (MDA-MB-231, MDA-MB-468, and MCF-7), the HeLa cervical carcinoma cell line, the LNCAP prostate cancer cell line, the HepG2 liver cancer cell line, HEK-293 (human embryonic kidney cell line), and HEK-293T (through A. Parissenti at the Northeastern Ontario Cancer Centre, Sudbury, ON, Canada). MDA-MB-231, MDA-MB-468, and LNCAP cells were maintained in RPMI 1640 growth medium, and MCF-7, HepG2, HEK-293, and HEK-293T cells were cultured in DMEM (both from Hyclone). RPMI 1640 and DMEM were supplemented with 10% (vol/vol) fetal bovine serum (FBS) and a penicillin-streptomycin antibiotic solution (100 U/ml penicillin, 100 µg/ml streptomycin) (Hyclone). MCF-7 and HEK-293 cells were maintained in DMEM supplemented with 10% (vol/vol) equine serum (Hyclone), 20 ng/ml recombinant human EGF (Calbiochem, Gibbstown, NJ), 0.5 µg/ml hydrocortisone (Sigma), 10 µg/ml insulin (Sigma), 100 ng/ml cholera toxin (GIBCO), and a penicillin-streptomycin antibiotic solution (100 U/ml penicillin, 100 µg/ml streptomycin) (Hyclone).

Immunocytochemistry. All cells were plated on 1.8-cm (0.13–0.17 mm thick) sterile glass coverslips (Fisher) placed in the well of a 6-well clustered dish (Sarstedt) and allowed to adhere overnight. Cells on coverslips were removed from the well, washed in DMEM [50 mM Tris·HCl (pH 7.4), 150 mM NaCl], and fixed in 4% (wt/vol) paraformaldehyde for 10 min before permeabilization. This process was repeated for the second and third probes. Fluorescent antibodies for these probes were either rhodamine (1:100) or Cy5 (1:500). Because two of the isoform-specific antibodies used for immunofluorescence were raised in the same species (i.e., Akt1 and Akt3 were both raised in goat), an alternative staining method had to be developed for triple isoform staining and Akt1-Akt3 double staining, as follows. After fixation and permeabilization, the cells were blocked for 1 h at room temperature and washed once in TBS. Anti-Akt1 antibody was diluted in TBS, incubated for 45 min, and washed off. An AffiniPure Fab Fragment rabbit anti-goat antibody (100 µg/ml diluted in PBS) was added to the coverslip (cells to sterically flood the goat immunoglobulins, thereby allowing the first goat antibody to be “zeroconverted” to rabbit. This step lasted for 30 min, and the coverslip was washed in TBS, followed by treatment with a secondary antibody for 30 min. Staining with Akt2 and Akt3 (triple condition) or Akt3 (double condition) followed next, as outlined above. To ensure the high efficiency of the Fab fragment incubation, a control was also stained with a donkey anti-goat fluorescent secondary antibody after the Fab incubation.

To examine the localization of Akt2 at the mitochondria, 50 nM of MitoTracker Red CMXRos was added to the medium of each coverslip in a six-well clustered dish and incubated for 45 min at 37°C and 5% CO2. The cells on the coverslip were removed from the medium and washed in TBS, followed by fixation and permeabilization before immunostaining with an anti-Akt2 antibody.

All immunocytochemistry experiments were visualized by confocal microscopy with a Zeiss 510 Meta laser scanning microscope (Carl Zeiss) equipped with a ×63 objective lens. Three laser filer settings were utilized for excitation with the following band-pass filter settings: argon 488 nm (band pass 505–530), HeNe 543 nm (long pass 560) and 633 nm (long pass 650). All images were captured and analyzed (including colocalization statistics and analyses) with LSM 510 software equipped with the microscope (LSM Image Examiner, Carl Zeiss).

SDS-PAGE and Western blotting. Preparation of cell extracts and subsequent protein analysis by SDS-PAGE and Western blotting were as suggested by Cell Signaling Technology with some modifications. Briefly, proteins were harvested in Cell Lysis Buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100] supplemented with 1 mM PMSF, 1 µM protease inhibitor cocktail tablets (Roche), 2 mM sodium orthovanadate (Na3VO4), and 10 mM sodium fluoride (NaF) and quantified with a bicinchoninic acid (BCA) assay (Pierce). For Western blotting, 30–35 µg of protein was separated by gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) with a semi-dry transfer apparatus (Bio-Rad). After the transfer, the membrane was incubated in blocking buffer for 1 h at room temperature in 0.1% TBST [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 0.1% (vol/vol) Tween 20] with 5% Carnation nonfat skim milk. The membrane was then washed and incubated with a secondary antibody in 0.1% TBST with 5% nonfat milk for 1 h at room temperature. The blot was then washed again before visualization with enhanced chemiluminescence (ECL) (GE Healthcare). The intensity of protein bands of scanned images was determined by densitometry with AlphaEase Fluorochrom (FC) 8900 version 4.0.1 software, which was equipped with the AlphaImmoblot scanner (Fisher Scientific).

Detection of Akt antibody specificity. For the peptide blocking experiments, antigen binding was prevented by preincubating the
antibody with a fivefold excess (wt/vol) of blocking peptides. After the incubation, the antibody-peptide solution was used in place of the primary antibody incubation in the immunocytochemistry procedure, and the resultant data were captured by confocal microscopy. Isoform-specific recombinant proteins for Akt1, Akt2, and Akt3 (20 ng) were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane, followed by Western blot analysis with each Akt isoform-specific antibody. Every lot of each isoform-specific antibody was examined in this manner to ensure that no cross-reactivity was present between the different lots of antibodies, even when they were purchased from the same vendor with the same catalog number.

For immunoprecipitation, 200 μg of protein from MDA-MB231 cells was mixed with 0.5–2.0 μg of isoform-specific primary antibodies against Akt1 (Cell Signaling Technology), Akt2 (Santa Cruz Biotechnology), or Akt3 (Upstate Biotechnology). The protein-antibody mixture was then incubated overnight at 4°C on a rotating shaker. Protein A/G Sepharose beads (Santa Cruz Biotechnology) were added to the protein-antibody mixture the following day and then incubated for an additional 2 h at 4°C, with constant rotation, before collection by centrifugation. After washing, the beads were collected, resuspended in 3× sample buffer [0.1 M Tris·HCl (pH 6.8), 10% (wt/vol) SDS, 40% (vol/vol) glycerol, 2% (wt/vol) DT, 10% (wt/vol) bromophenol blue], and boiled for 5 min. The immunoprecipitate was collected by centrifugation. If the immunoprecipitate subjected to electrophoresis on a 10% polyacrylamide gel, along with a whole cell extract control (30–35 μg) and supematant.

Quantitative PCR. Total RNA was extracted with an RNeasy Kit (Qiagen) according to the manufacturer’s instructions and quantified with a spectrophotometer (UV 1101 Biotech Photometer) taking standard OD260/280 absorbance measurements. RNA integrity was analyzed with a RNA 6000 Nano Assay LabChip Kit and analyzed with an Agilent 2100 Bioanalyzer. The RNA samples were treated with DNase I, and then 1 μg of total RNA was reverse transcribed with MMLV, 10 mM dNTPs (ID Labs) and T20-VN primers (20 ng) before collection by centrifugation. The resultant data were captured by confocal microscopy.

To verify the localization of Akt2, MDA-MB231 cells were transfected with a recombinant construct. Akt2 cDNA in a pCR 2.1-TOPO vector (a kind gift from J. M. Woodgett, Ontario Cancer Institute, Toronto, ON, Canada) was digested with SpeI and EcoRV, and a DNA fragment containing the Akt2 open reading frame was cloned into a PECPF-N1 vector at the BamHI and KpnI site. To transiently express the recombinant Akt2 in MDA-MB231, the transfected cells were plated onto a 1.8-cm sterile glass coverslip placed in a six-well clustered dish and allowed to adhere overnight. Four micrograms of plasmid DNA was transfected with Lipofectamine 2000 according to the manufacturer’s protocol.

Small interfering RNA transfections. MDA-MB231 cells were plated onto a sterile glass coverslip 24 h before transfection with siRNA. Cells were transfected with Opti-MEM I Reduced Serum Medium (GIBCO) (which is actually serum free) and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The plates were incubated overnight, and the transfection medium was replaced with complete RPMI medium 24 h after transfection initiation. The cells were returned to the incubator, and samples were taken at 48 h after transfection initiation. A scrambled control (10 nM) and/or a reagent control (mock-transfected with Lipofectamine 2000 only) were included for all siRNA transfection experiments. All siRNA oligonucleotides, including the scrambled controls, were purchased from Ambion. The siRNA sequences and final concentrations used in this study are as follows: Akt1 (10 nM) 5′-GGCUCCCCU-CAACAACUC-3′; Akt2 (10 nM) 5′-GGAGAAGAGAGCGGCACA-3′; Akt3 (20 nM) 5′-GGACGACAGCUUUCUAU-3′. The treatment with radiation and epidermal growth factor. MDA-MB231 cells were plated onto a sterile glass coverslip placed in a six-well clustered dish and allowed to adhere overnight [RPMI, 10% (vol/vol) FBS]. The next day, the medium was removed and the cells were serum starved overnight [RPMI, 0.1% (vol/vol) FBS] before treatment with either radiation or EGF. Cells were radiated at 5 Gy (RS320 Irradiation System, Gilmay Medical) or treated with 100 ng/ml EGF (Santa Cruz Biotechnology).

RESULTS

Examination of antibody specificity. To characterize the specific interaction and unique function of each Akt isoform, we rigorously examined many isoform-specific anti-Akt antibodies currently available through commercial sources. We employed the following four different examination methods: 1) a competition assay with antigen-specific peptides; 2) isoform-specific antigen recognition by anti-Akt antibodies with purified recombinant Akt proteins; 3) immunoprecipitation of Akt proteins with an isoform-specific antibody, followed by Western blot analysis; and 4) knockdown of each Akt isoform with isoform-specific siRNA.

To examine the specificity of antibodies generated against Akt1, 2, or 3, an anti-Akt “isoform-specific” antibody was incubated with its own targeting peptide. As shown in Fig. 1, incubation of anti-Akt1 antibody (Santa Cruz Biotechnology) with an Akt1-targeting peptide abolished the interaction between Akt1 protein and the anti-Akt1 antibody (compare Fig. 1, i and ii). In contrast, incubation of the anti-Akt1 antibody with an Akt 2- or Akt3-targeting peptide did not affect the interaction between Akt1 protein and the anti-Akt1 antibody (Fig. 1, iii and iv). It should be noted that incubation with the secondary antibody alone did not generate any (nonspecific) signal (Fig. 1v). Together, the anti-Akt1 antibody from Santa Cruz Biotechnology (catalog no. sc-7126) specifically recognized Akt1. Similarly, anti-Akt2 and anti-Akt3 antibodies used.
were specific to Akt2 and 3 proteins, respectively (Fig. 1, vi–xv) (catalog nos. sc-5270 and sc-11521, respectively).

We also analyzed the specificity of Akt isoform-specific antibodies, using purified recombinant proteins (Supplemental Fig. S1). We found that two antibodies among nine commercially available Akt “isoform-specific” antibodies that we examined were cross-reactive with other isoforms; thus these two were excluded in the subsequent experiments (S. Santi, unpublished data). We also found that different lots of antibodies sometimes showed different reactivity, even though they had the same catalog number (S. Santi, unpublished data). Therefore, we had to rigorously examine every lot of antibodies before use. As shown in Supplemental Fig. S1A, the anti-Akt1 antibody lot G0804 (Santa Cruz Biotechnology, catalog no. sc-7126) was slightly cross-reactive with Akt3 in the recombinant protein-based analysis. On the basis of the data shown in Supplemental Fig. S1, A and B, the following isoform-specific antibodies were selected for use in our subsequent experiments: Akt1 from Cell Signaling Technology (catalog no. 2967), Akt2 from Santa Cruz Biotechnology (catalog no. sc-5270), and Akt3 from Upstate Biotechnology (catalog nos. 05-780). As shown in Supplemental Fig. S1C, the anti-Akt1 antibody (Cell Signaling Technology, catalog no. 2967), the anti-Akt2 antibody (Santa Cruz Biotechnology, catalog no. sc-5270), and the anti-Akt3 antibody (Upstate Biotechnology, catalog no. 05-780) that we used effectively and specifically pulled down each corresponding Akt isoform. Thus subcellular localization of each Akt isoform could be studied with two sets of isoform-specific antibodies based on the data shown in Fig. 1 and Supplemental Fig. S1, with the exception of anti-Akt2, where only one antibody was found suitable (Santa Cruz Biotechnology, sc-5270).

Each Akt isoform showed a distinct subcellular localization. The currently accepted Akt functional model may be applicable for all three isoforms only if all of them localize in the cytoplasm. To prove this, we examined the subcellular localization of the Akt isoforms by immunostaining MDA-MB231 cells with the isoform-specific antibodies described above. As expected, Akt1 was mainly localized in the cytoplasm (Fig. 2, i, v, and ix). Akt2 was also localized in the cytoplasm. Unexpectedly, however, Akt2 showed a punctuate staining pattern clustered around the perinuclear region (Fig. 2, ii, vi, and x). Akt3 localized mainly to the nucleus and nuclear membrane (Fig. 2, iii and xi). The merged images of double or triple staining were consistent with this interpretation (Fig. 2, iv, vii, and xii). Thus our data demonstrated that Akt1, 2, and 3 in MDA-MB231 cells distinctly localize in the cytoplasm, a discrete region in the cytoplasm, and in the nucleus and nuclear membrane, respectively.

To determine whether the distinct subcellular localization of the Akt isoforms observed in MDA-MB231 cells is a common phenomenon, we carried out a similar experiment with two additional breast cancer cell lines (MDA-MB468 and MCF-7), one noncancer breast epithelial cell line (MCF-10A), one noncancer breast epithelial cell line (MCF-10A), one cervical cancer cell line (HeLa), one prostate cancer cell line (LNCaP), one liver cancer cell line (HepG2), and two human embryonic kidney cell lines (HEK-293 and HEK-293T). Data obtained from indirect immunostaining experiments showed that the subcellular localization of the Akt isoforms in MDA-MB468 and MCF-7 breast cancer cells was largely consistent with that for MDA-MB231 (Fig. 3). The subcellular localization pattern of the Akt isoforms in MCF-10A cells (nontrans-
formed) was also largely consistent with that of three breast cancer cell lines (MDA-MB231, MDA-MB468, and MCF-7) (Figs. 2 and 3). The pattern of Akt subcellular localization in HeLa, LNCaP, and HepG2 was also largely consistent with that of human breast cancer cell lines (Fig. 3).

In contrast, HEK-293 cells contained Akt1 throughout the entire cell and Akt2 mainly in the nucleus (Fig. 3). The subcellular localization of Akt3 in HEK-293 was also in the nucleus, which is similar to other cell lines examined. In a stark contrast to HEK-293, HEK-293T cells contained Akt1 exclusively in the cytoplasm (like many other transformed cells). Unlike Akt1, the subcellular localization of Akt2 in human embryonic kidney cells was not altered by the presence of T-antigen (T-ag) (Fig. 3).

**Messenger RNA and protein levels of Akt isoforms.** Our data shown in Figs. 1–3 appeared to suggest that the subcellular localization of the Akt isoforms is not a critical factor for tumor development and progression in the cancer cell lines. It was shown previously that Akt overexpression is often correlated with an aggressive progress of certain cancers. Therefore, we examined the levels of Akt mRNA and proteins in several cancer and noncancer cell lines. The MDA-MB231, MDA-MB468 breast cancer cell lines and MCF-10A immortalized breast epithelial cell lines expressed all three Akt isoforms to a similar extent (Fig. 4A). In contrast, the MCF-7 breast cancer cell line expressed very little Akt3 (Fig. 4A, lane 3). However, this cell line showed the highest level of Akt1 protein among the nine cell lines examined (Fig. 4, A and B). LNCaP and HepG2 expressed “normal” levels of Akt1 and Akt2 but a very low level of Akt3 protein (Fig. 4A, lanes 8 and 9), while HeLa cells expressed a normal level of Akt2 protein but very little Akt1 or Akt3 protein (Fig. 4A, lane 5, and B). Both HEK-293 and HEK-293T showed high levels of Akt3, although they contained normal levels of Akt 1 and 2 proteins. Taken together, the levels of Akt 1 and 2 proteins were similar in all cell lines examined. In contrast, very low levels of Akt3 were observed in the MCF-7, HeLa, LNCaP, and HepG2 cell lines. Overall, our data do not support the notion that levels of Akt isoforms are directly correlated with tumor development or tumor progression.

Mutations in PTEN, the phosphatase that counteracts PI3K activity, can cause high levels of Akt activation, which is manifested by phosphorylation of the serine 473 residue on Akt. As expected, data from Western blotting showed that Akt was activated (i.e., phosphorylated) in MDA-MB468 and LNCaP, where PTEN is deleted (Fig. 4A, lanes 2 and 8). This finding is consistent with two previous reports that PTEN is deleted in these cell lines (11, 21). Unexpectedly, both the levels of PTEN and Akt phosphorylation were very low in the HepG2 cell line, suggesting that additional mutation(s) may exist in this cell line. Despite containing a normal (or a near normal) level of PTEN, HEK-293 and HEK-293T cells showed high levels of Akt activation (Fig. 4A, lanes 6 and 7). The level of Akt activation was particularly high in the HEK-293T cells, although the overall level of pan-Akt protein level in this cell line was relatively low. This finding suggests that SV40 T-ag directly or indirectly contributed to the Akt phosphorylation in HEK-293T cells. Combining these data with those shown in Figs. 2 and 3, the subcellular localization of the Akt isoforms is likely independent of the PTEN status, or phosphorylation of Akt (p-Akt).

As shown in Fig. 4C, the relative mRNA level of each Akt isoform was similar to that of the corresponding protein. The extremely low level of both Akt3 mRNA and protein in some of the cell lines (Fig. 4) was disconcerting, given that indirect immunostaining showed that these cells apparently contained Akt3 proteins (e.g., Fig. 3, HeLa, MCF-7, LNCaP, and HepG2). In this regard, it may be important to note that there are somewhat conflicting reports as to whether Akt3 is present in these cell lines. For example, some studies demonstrated that neither MCF-7 nor LNCaP cells expressed Akt3, while others found that Akt3 was present in these cells (8, 20). The discrep-
ancy shown in these reports may be, at least in part, due to the different anti-Akt3 antibodies used. One potential source of discrepancy shown in our data could also have resulted from the two different Akt3 antibodies used in these experiments. The anti-Akt3 antibodies used for immunostaining and Western blot analyses were generated against the COOH terminus and the NH2 terminus, respectively. Therefore, we carried out Western blotting as well as an immunostaining assay using both the COOH terminus- and NH2 terminus-specific anti-Akt3 antibodies, to further verify the results of the indirect immunostaining of Akt3 observed in Fig. 3.

The NH2 terminus-specific anti-Akt3 antibody (Upstate, 05-780) did not detect Akt3 protein in the MCF7, HeLa, LNCaP and HepG2 cell lines in the Western blotting (Fig. 5A), which is consistent with data shown in Fig. 4A. In contrast, the COOH terminus-specific anti-Akt3 antibody (Santa Cruz Biotechnology, sc-11521) detected bands where Akt3 proteins were expected to be present (Fig. 5A, right). However, a closer examination suggested that these upper bands may not be Akt3. We therefore characterized the nature of the bands detected by the COOH terminus-specific anti-Akt3 antibody by Western blotting using Akt3-ablated cells. As shown in Fig. 5B, the intensity of the lower band was reduced greatly in MDA-MB231 cells treated with Akt3 siRNA, suggesting that the lower band represents Akt3 and the upper band does not. Thus our data confirm that Akt3 protein was not detectable by Western blotting in the MCF-7, HeLa, LNCap, and HepG2 cells.

The data from Western blotting appeared to be contradictory to that shown in Fig. 3, where Akt3 was detected by immunostaining. To clarify this potential discrepancy, we carried out an immunostaining assay in MDA-MB231 (a positive control), MCF-7, HeLa, LNCap, and HepG2 cells, using antibodies against the Akt3 NH2 or COOH terminus. As shown in Fig. 5C, we could detect Akt3 proteins in the nuclei of all five of these cell lines, with either the NH2 terminus- or COOH terminus-specific anti-Akt3 antibody. This positive staining is not an artifact, since the anti-Akt3 (COOH terminus) antibody did not show a positive signal when Akt3 was ablated by siRNA (Fig. 5). Our data thus suggested that MCF-7, HeLa, LNCap, and HepG2 cells do contain Akt3, which is consistent with data presented in Fig. 3.

Akt2 is colocalized with the mitochondria. Data in Fig. 2 showed that Akt2 was clustered at discrete sites around the nuclear membrane in the cytoplasm, suggesting that Akt2 may be localized at cytoplasmic organelles. To identify the organelles “associated” with Akt2, we immunostained MDA-MB231 cells with an anti-Akt2 antibody in combination with biomarkers for different organelles. Our data showed that Akt2 was not colocalized with the endoplasmic reticulum (ER) (Fig. 6A, iv–ix). Analysis of the microscopic images with software equipped with the Carl Zeiss confocal microscope used indi-
icated that there was essentially no colocalization of Akt2 with the ER, as the Pearson $R$ correlation value between Akt2 and calnexin (smooth ER) or between Akt2 and PDI (rough ER) was $R = 0.11$ ($R^2 = 0.04$ and $R^2 = 0.03$, respectively). Unlike the ER, there was some shared localization between Akt2 and TGN38 (a marker for the Golgi apparatus) ($R = 0.30$, $R^2 = 0.10$). However, the majority of Akt2 proteins were not colocalized with the Golgi apparatus (Fig. 6A, x–xii).

Our data demonstrate that the majority of Akt2 was associated with the mitochondria in MDA-MB231 cells, as Akt2 was colocalized with MitoTracker Red staining sites (Fig. 6A, xiii–xv). This conclusion was further supported by statistical data, as the Pearson correlation showed a significant, positive association was present between Akt2 and the mitochondria: $R = 0.53$, $R^2 = 0.30$. Since the total cellular area was assessed in this statistical analysis, as opposed to regions of interest (ROIs) where both the staining intensity of Akt2 and MitoTracker would be highest, this $R$ value is most likely conservative due to the fact that the lighter background staining of Akt2 would also have been incorporated in this analysis. The association of Akt2 with the mitochondria was also apparent in MDA-MB468, MCF-7, HeLa, HepG2, and LNCaP cells (Supplemental Fig. S2). Pearson $R$ correlation values in these cell lines were $0.29–0.38$, with the highest value noted in MDA-MB468 cells ($R = 0.56$, $R^2 = 0.32$).

Next the subcellular localization of the Akt isoforms was further examined by centrifugation-based fractionation of MDA-MB231 cells and subsequent Western blot analysis. As expected, Akt1, 2, and 3 localized in the cytoplasm, the mitochondria, and the nucleus, respectively (Fig. 6B). A substantial amount of Akt2 was also found in the nuclear fraction (Fig. 2B). However, this is most likely due to cross-contamination of the mitochondria in the nuclear fraction, since a large amount of VDAC1, a mitochondrial protein, was also found in the nuclear fraction. Similarly, lamin B1 and Akt3 were also cross-contaminated in the mitochondrial fraction (Fig. 6B). Despite this cross-contamination between the mitochondrial and nuclear fractions, this set of data is consistent with data obtained from immunostaining assays. Thus Akt1, 2, and 3 are mainly localized in the cytoplasm, the mitochondria, and the nucleus, respectively.

We also examined the mitochondrial localization of Akt2 by expression of exogenous recombinant Akt2 proteins. In this experiment, we first confirmed that green fluorescent protein-tagged Akt2 was colocalized with endogenous Akt2 when the Akt2-enhanced cyan fluorescent protein (ECFP) construct was
transiently transfected into MDA-MB231 cells (Fig. 6, top). Without using any antibody, we then detected that Akt-ECFP recombinant proteins were colocalized with the mitochondria (Fig. 6, bottom). Together, data shown in Figs. 2, 3, and 6 clearly demonstrate that Akt2 is predominantly localized at the mitochondria.

Subcellular localization of Akt isoforms was not altered by ablation of other isoforms, treatment with X-ray, or in response to epidermal growth factor. One of the outstanding questions with respect to the function of Akt isoforms is whether inactivation of one isoform is compensated for by other isoform(s). We rationalized that the “compensating” Akt isoform may translocate to the subcellular location of “compensated” Akt isoform. To test this hypothesis, we ablated the Akt isoforms with siRNA to discern whether the remaining Akt isoform altered its subcellular location. We found that a single Akt isoform knockdown did not alter the localization of the remaining two Akt isoforms in MDA-MB231 cells (Supplemental Fig. S3). Similarly, the double knockdown of the Akt isoforms in different combinations did not alter the subcellular localization of the remaining isoform (Fig. 7).

To gain further insight into the function of the Akt isoforms, MDA-MB231 cells were either radiated with 5 Gy of ionizing radiation or stimulated with EGF. Our data suggested that the subcellular localization of the three Akt isoforms remained largely unchanged by serum starvation, 5 Gy, or 100 ng/ml EGF (Fig. 8).

Most of the Akt2 proteins associated with the mitochondria and Akt3 in the nucleus are likely active. We examined the activation status of Akt2 and Akt3 localized to the mitochondria and the nucleus, respectively. Akt proteins were immunoprecipitated with isoform-specific antibodies from cells stimulated with EGF, followed by Western blot analysis using an anti-phospho-Akt Ser473 antibody. As shown in Fig. 8, B and C, Akt2 proteins associated with mitochondria and Akt3 in the nucleus are phosphorylated at Ser473, suggesting that they are active forms.

DISCUSSION

On activation of the receptor tyrosine kinase-PI3K cascade, Akt in the cytoplasm translocates to the inner leaflet of the plasma membrane, where it is activated by phosphorylation. This widely accepted model is based on data generated mainly with anti-pan-Akt and anti-phosphorylated Akt antibodies. Since these anti-Akt antibodies do not distinguish among the...
different Akt isoforms, the currently accepted Akt functional model may or may not accurately portray the activation mechanism of all three Akt isoforms. Therefore, it is essential to develop an appropriate tool to study the unique function of each Akt isoform. As part of developing such tools, we rigorously characterized commercially available anti-Akt antibodies that had been generated against each Akt isoform. We identified two sets of antibodies that showed specific interactions with each Akt isoform. Equipped with these isoform-specific antibodies, we examined the subcellular localization of each Akt isoform.

We found, as expected, that Akt1 localized mainly in the cytoplasm, a substantial portion of which was detected at the plasma membrane (Figs. 1–3 and 6–8). This observation is consistent with the currently accepted Akt functional model, which is not surprising since many anti-Akt antibodies interact with Akt1 very efficiently (e.g., see Fig. 4A). Thus the currently accepted model seems to accurately portray the activation mechanism of Akt1.

In contrast to Akt1, our data have unequivocally demonstrated that Akt2 localizes mainly at the mitochondria, although a minor population of the Akt2 isoform may also localize at the Golgi apparatus (Fig. 6). Bijur and Jope (2) detected a basal level of Akt in the matrix and the inner and outer membranes of the mitochondria in HEK-293 and SH-SY5 neuroblastoma cells. In their study using anti-pan-Akt antibodies, the treatment of cells with insulin-like growth factor-I (IGF-I) augmented the level of Akt phosphorylation (i.e., activation) at the mitochondria. These authors thus speculated that a selective uptake of Akt into the mitochondria might occur. Furthermore, this mitochondrial localization of Akt2 was further confirmed with ECFP-Akt2 recombinant protein (i.e., no indirect immunostaining). An Akt2-ECFP plasmid construct was transiently expressed in MDA-MB231 cells, followed by staining with an anti-Akt2 antibody (top) and MitoTracker (bottom).

Fig. 6. Further analysis of the subcellular localization of Akt2 and Akt3 in the MDA-MB231 breast cancer cell line. Cells on a glass coverslip were probed with an anti-Akt2 or -Akt3 antibody in combination with various subcellular markers to determine their subcellular localizations. A: Akt3 and Akt2 are largely colocalized with lamin B1 (i–iii) and mitochondria (xiii–xv), respectively. The following subcellular localization markers were used: calnexin, smooth endoplasmic reticulum protein; PDI, rough endoplasmic reticulum protein; trans-Golgi network 38 (TGN38), Golgi apparatus; and MitoTracker Red CMXRos, mitochondria. B: MDA-MB231 cells were fractionated into the cytosolic (Cyto), mitochondrial (Mito), and nuclear (Nuc) fractions, followed by SDS-PAGE-Western blot analysis to examine the subcellular localization of Akt1-3. Voltage-dependent anion-selective channel 1 (VDAC1) and lamin B1 were used to identify the mitochondria and nucleus, respectively. WCE, whole cell extracts. C: the mitochondrial localization of Akt2 was further confirmed with ECFP-Akt2 recombinant protein (i.e., no indirect immunostaining). An Akt2-ECFP plasmid construct was transiently expressed in MDA-MB231 cells, followed by staining with an anti-Akt2 antibody (top) and MitoTracker (bottom).
finding that Akt2, but not Akt1 and Akt3, localizes at the mitochondria, the Akt characterized by these authors is most likely the function of Akt2, not Akt1 or Akt3. Since we found that Akt2 associated with the mitochondria is phosphorylated (Fig. 8), our data are also consistent with their finding that the mitochondrially localized Akt is an active form (2).

Our finding of Akt2 being associated with the mitochondria is consistent with published data that this isoform is involved in the regulation of energy metabolism, and raises the possibility that it may also be involved in apoptosis. Calera et al. (6) and Kupriyanova and Kandror (13) previously showed that Akt2 is associated with Glut transporters. This observation may be important since the ability of Akt to promote cell survival would require both glucose and ATP (9). Akt is also known to associate with mitochondrial hexokinases (9, 15), the enzymes important for the regulation of the first step in glycolysis (18). It should be noted that glucose transporters (GLuts) deliver glucose to hexokinase II that is bound to VDAC, which is associated with the mitochondria (18). The association of Akt with hexokinases would effectively couple the role of Akt to both glucose metabolism and the regulation of apoptosis. Since a constant supply of glucose is essential for cancer cell survival, the association of Akt2 with both Glut4 and the mitochondria places Akt2 in an ideal position for the regulation of energy metabolism and cell survival in cancer cells.

It is well known that Akt enhances cell survival by repressing apoptosis through phosphorylation of downstream substrates such as NF-κB, Bad/Bcl-2, and the FoxO family. At least in the case of Bad/Bcl-2, this signaling would converge at the mitochondria. In support of this idea, a number of published reports indicate that Akt may play a role in the “functional integrity” of the mitochondria (9, 15, 16). Thus phosphorylated Akt may protect the mitochondria to prevent apoptosis, thereby maintaining the mitochondrial integrity and promoting cell survival. Since we found that Akt2 is the only Akt isoform localized at the mitochondria, our data are consistent with the notion that Akt2, but not Akt 1 and 3, is critical for the survival of cells through the regulation of energy metabolism and the suppression of apoptosis.

It is interesting to note that the noncancer HEK-293 cells contained all three Akt isoforms in the nucleus, although Akt1 was also present in the cytoplasm (Fig. 3). In contrast, the HEK-293T cell line contained Akt1 exclusively in the cytoplasm. Since the only difference between these two cell lines is the presence of S40 T-ag in HEK-293T, the difference in the subcellular localization of Akt1 is likely due to the function of T-ag. It is thus possible that Akt1 is activated by T-ag indirectly through the translocation of Akt1 from the nucleus to the cytoplasm.

The term “nuclear Akt” is often used in the literature. Nuclear Akt has been suggested to be either pan-Akt that has been translocated from the cytoplasm or phosphorylated Akt (1, 3, 4, 7, 22). Given that the commercially available anti-pan-Akt antibodies and anti-phosphorylated Akt antibodies recognize all three Akt isoforms, it is difficult to definitively conclude the true identity of nuclear Akt described in these studies. Since we found that Akt3 is the only Akt isoform presented in the nucleus, it is likely that the nuclear Akt described in these studies may actually be Akt3. This possi-
bility is further strengthened by the fact that both Akt1 and Akt2 did not translocate into the nucleus on administration of ionizing radiation or EGF stimulation (Fig. 8). However, it should also be noted that all three Akt isoforms were found in the nucleus in HEK-293 cells (Fig. 3). The Akt3 isoform showed the lowest expression level among the three Akt isoforms in the cell lines examined (Figs. 4 and 5). The levels of both Akt3 protein and mRNA were below detection by Western blotting or qPCR in some cell lines (Figs. 4 and 5). This may be the reason why there have been seemingly conflicting reports on the presence of Akt3 mRNA and protein in some cells. Okano et al. (20) did not detect Akt3 mRNA in the MCF-7 and HepG2 cell lines by RT-PCR, which is consistent with our result shown in Fig. 4. However, they found by Southern blotting that both MCF-7 and HepG2 cells contained Akt3. Similarly, Brognard et al. (5) found that the levels of Akt2 and Akt3 were very low in certain non-small-cell lung cancer cell lines. However, RT-PCR analysis of the isoforms in their study showed the presence of transcripts for all three isoforms. The reports by Okano and Brognard are largely consistent with our data: We could hardly detect any Akt3 mRNA by qPCR and proteins by Western blotting in MCF-7, HeLa, LNCaP, and HepG2 (Figs. 4 and 5) but could unequivocally detect Akt3 proteins by indirect immunostaining (Fig. 5). A question remains as to why Akt3 was not detected in these cell lines by either Western blotting or qPCR. One possibility is that the levels of Akt3 mRNA and protein are too low in these cell lines to detect by qPCR and Western blotting, respectively. Alternatively, but not exclusively, Akt3 in these cell lines may contain mutation(s), which affect PCR-based amplification and Western analysis but do not affect detection by immunostaining of Akt3 in its natural (normal folding) form in the intact cell (Fig. 5).

We found that ablation of one or two Akt isoforms by siRNA did not significantly alter the subcellular localization of the remaining Akt isoform (Fig. 7), suggesting that the subcellular location of the Akt isoforms is not affected by the presence or absence of the other Akt isoforms. These data are consistent with the idea that the major function of an Akt isoform does not generally compensate for that of other Akt isoforms. This conclusion is based on the assumption that a similar mode of activation would occur for a similar function if one Akt isoform can compensate for the function of the other isoform(s). Since the subcellular localization of all three Akt isoforms is not notably altered in response to ionizing radiation and epidermal growth factor (EGF). MDA-MB231 cells were plated onto a coverslip and allowed to attach overnight. The next day, the cells were placed in the medium containing 0.1% fetal bovine serum (FBS), followed by incubation overnight (~16 h). The cells were then treated with 5-Gy X-rays (A) or 100 ng/ml EGF (B). The subcellular localization of each Akt isoform was examined by confocal microscopy 1 h after treatment. C: samples prepared as in B were analyzed by immunoprecipitation with anti-Akt isoform-specific antibodies and Western blotting with anti-phospho-Akt Ser473 antibodies. Lanes 1, 3, and 5 are samples without EGF treatment, and lanes 2, 4, and 6 are samples treated with EGF.
isoforms is different, the mode of activation is also likely different. The fact that Akt2 and 3 are localized to the mitochondria and the nucleus, respectively, raises a question of whether these two Akt isoforms are phosphorylated and activated at the inner leaflet of the plasma membrane as the currently accepted Akt activation model suggests. Since the subcellular localization of Akt2 and Akt3 are not substantially altered (e.g., they did not translocate to the plasma membrane) in response to ionizing radiation or EGF, it is unlikely that these two Akt isoforms are activated in the same manner as Akt1 is. Our data actually raise a possibility that Akt2 and Akt3 are activated at the mitochondria and the nuclear membrane, respectively, since Akt2 associated with the mitochondria and Akt3 in the nucleus are active (Fig. 8). Furthermore, our data are also consistent with the notion that all three Akt isoforms have their own unique functions that are not shared by other isoforms, since the ablation of one Akt isoform did not alter the subcellular localization of other isoforms (Fig. 7).

How may the three Akt isoforms be localized to very different subcellular compartments, although they show extensive sequence similarity? Sequence alignment shows that there are three regions with substantial sequence diversity: 1) amino acids 43–50 (numbering is for Akt1), 2) amino acids 111–145, and 3) amino acids 453–465. Therefore, these regions may be involved, directly or indirectly, in the regulation of their subcellular localization and unique function.

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