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Proteomics Exploration Reveals That Actin Is a Signaling Target of the Kinase Akt*

Franck Vandermoere‡§, Ikram El Yazidi-Belkoura‡, Yohann Demont‡, Christian Slomianny¶, Johann Antol‡, Jérôme Lemoine||**, and Hubert Hondermarck‡ ††

The serine/threonine kinase Akt is a key mediator of cell survival and cell growth that is activated by most growth factors, but its downstream signaling largely remains to be elucidated. To identify signaling partners of Akt, we analyzed proteins co-immunoprecipitated with Akt in MCF-7 breast cancer cells. Mass spectrometry analysis (MALDI-TOF and MS-MS) of SDS-PAGE-separated Akt co-immunoprecipitates allowed the identification of 10 proteins: α -actinin, valosin-containing protein, inhibitor κ B kinase, mortalin, tubulin β , cytokeratin 8, actin, 14-3-3 σ , proliferating cell nuclear antigen, and heat shock protein HSP27. The identification of these putative Akt binding partners were validated with specific antibodies. Interestingly, the major protein band observed in Akt co-immunoprecipitates was found to be the cytoskeleton protein actin for which a 14-fold increase was observed in Akt-activated compared with non-activated conditions. The interaction between Akt and actin was further confirmed by reverse immunoprecipitation, and confocal microscopy demonstrated a co-localization specifically induced under growth factor stimulation. The use of wortmannin indicated a dependence on the phosphatidylinositol 3-kinase pathway. Using a phospho-Akt substrate antibody, the phosphorylation of actin on an Akt consensus site was detected upon growth factor stimulation, both *in cellulo* and *in vitro*, suggesting that actin is a substrate of Akt kinase activity. Interestingly, cortical remodeling of actin associated with cell migration was reversed by small interfering RNA directed against Akt, indicating the involvement of Akt in the dynamic reorganization of actin cytoskeleton germane to breast cancer cell migration. Together these data identify actin as a new functional target of Akt signaling. *Molecular & Cellular Proteomics* 6:114–124, 2007.

The serine/threonine kinase Akt/PKB¹ belongs to the cAMP-dependent protein kinase A/protein kinase G/protein kinase C

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¹ The abbreviations used are: PKB, protein kinase B; FGF-2, fibro-

(AGC) superfamily of protein kinases that share structural homology within their catalytic domain and have similar mechanisms of activation (1). Akt/PKB was initially identified by three independent groups based on its homology to protein kinases A and C and to the cellular homolog of the retroviral oncogene Akt (v-Akt). In mammals, three Akt/PKB genes have been identified, termed PKB α /Akt1, PKB β /Akt2, and PKB γ /Akt3, located at chromosomes 14q32, 19q13, and 1q44, respectively.

The original discovery of Akt kinase as the cellular homolog of a viral oncogene already implied its pivotal role in cell growth and survival that, since then, has been verified in various cell systems, and Akt is increasingly regarded as a crucial player in cancer cell survival and growth (2). Akt overexpression is observed in various human cancers, and the Akt signaling pathways are now considered as realistic targets for cancer therapy (2). In breast tumor biopsies, Akt1 is frequently overexpressed in a high percentage of cells, whereas Akt2 expression is mostly sparse; in contrast, most normal breast epithelial cells do not overexpress Akt kinases (3). Interestingly it has been suggested that activation of Akt1 by Her2/ phosphatidylinositol 3-kinase (PI3K) plays an important role in mediating multidrug resistance in human breast cancer cells, and Akt may therefore be a novel molecular target for therapies against breast cancer (2, 4). Akt protein modulates the function of several downstream substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth. The list of proteins known to interact with Akt includes Bad, cAMP-response element-binding protein, the forkhead family, inhibitor κ B kinase, procaspase-9, apoptosis signal-regulating kinase 1 (ASK1), murine double minute 2 (Mdm2), glycogen synthase kinase 3, p21^{Cip=Waf1}, a mammalian target of rapamycin (mTOR), and a tuberous sclerosis complex (TSC2) (5). The PI3K pathway that is stimulated by a wide range of tyrosine kinase growth factor receptors is the major activator of Akt signaling via stimulation of PDK1 that phosphorylates and activates Akt. The PI3K inhibitors LY294002 and wortmannin, both inhibiting the catalytic site of the p110 subunit, have routinely been used as research tools and are

blast growth factor-2; PI3K, phosphatidylinositol 3-kinase; MEM, minimum essential medium; PDK1, phosphoinositide-dependent protein kinase 1; PAS, phospho-Akt substrate; siRNA, small interfering RNA; PH, pleckstrin homology; PCNA, proliferating cell nuclear antigen.

known to inhibit tumor growth *in vivo* (6). As the PI3K catalytic domain is highly conserved among PI3K family members, it is not surprising that either compound discriminates among the various isoforms of PI3K. Another potentially powerful strategy for inhibiting Akt is to disrupt the binding of its PH domain to 1,4,5-triphosphate phosphoinositide, thereby preventing its membrane translocation and activation by PDK1. Novel analogs of the 1,4,5-triphosphate phosphoinositide ring have been shown to be effective Akt inhibitors in cell culture (7). Because only a subset of the cellular processes regulated by the PI3K/Akt pathway are involved in tumorigenesis, the choice of drug targets must take into account the adverse effects resulting from the inhibition of other PI3K/Akt-dependent cellular processes. Therefore it would be desirable to identify and target components of branches further downstream in the PI3K/Akt pathway.

In this work, we used a proteomics-based approach to explore Akt signaling. Akt-co-immunoprecipitated proteins were analyzed by mass spectrometry, and a series of putative signaling partners were defined. Interestingly the cytoskeleton protein actin was found to be the major partner interacting with Akt under condition of growth factor stimulation. Akt-actin interaction resulted in actin cytoskeleton cortical remodeling associated with cell migration, revealing actin as a functional target of Akt signaling.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Biowhitaker. Boyden microchambers (Transwell®) were from Costar. Recombinant human fibroblast growth factor-2 was from R&D Systems. Wortmannin (PI3K inhibitor) was from Calbiochem; G250 Coomassie Brilliant Blue, DMSO, and peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibody were from Sigma. Antibodies against Akt/PKB (rabbit immunofluorescence-purified IgG), α -actinin-4, valosin-containing protein, inhibitor κ B kinase β , mortalin, tubulin β , cytokeratin 8, heat shock protein 27, and anti-panactin were from Upstate Biotechnologies. Anti-proliferating cell nuclear antigen was from Roche Diagnostic, and anti-14-3-3 σ was from Sigma. The electrophoresis reagents were purchased from Proteomics Solution. Dynabeads (M-280 tosyl-activated) were from Dynal Biotech. siRNA against Akt was obtained from Dharmacon.

Cell Culture—Breast cancer cell line MCF-7 was obtained from the American Type Culture Collection and routinely grown as monolayers. Cells were grown as described previously (8) at 37 °C in a humidified atmosphere of 5% CO₂ in minimal essential medium (MEM)-Earle's salts supplemented with 20 mM HEPES, 2 g/liter sodium bicarbonate, 2 mM L-glutamine, 1% non-essential amino-acids, 10% FCS, 100 units/ml penicillin/streptomycin, and 50 μ g/ml gentamycin.

Co-immunoprecipitation of Akt and Interacting Proteins—The protocol used for identification of Akt binding partners is summarized in Fig. 1A. MCF-7 cells were rinsed in PBS, pH 7.5, serum-starved for 3 h in MEM-Earle's salts, and treated by 10 μ M C₂-ceramide (Sigma) in fresh minimal essential medium. Proteins were extracted from cells after 15 min of 5 ng/ml FGF-2 stimulation. To inhibit PI3K/Akt, cells were pretreated for 3 h by 100 nM wortmannin before FGF-2 stimulation. Then cells were rinsed with PBS, pH 7.5, and scratched in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, 1% Nonidet P-40, 100 μ M sodium orthovanadate). After centrifugation (10,000 \times g for 5 min), proteins were quantified using the Bradford method

(Bio-Rad). Akt and interacting proteins were co-immunoprecipitated from 15 mg of total proteins, using antibodies covalently bound on magnetic beads according to the Dynal Biotech protocol, using 50 μ g of antibody with an incubation overnight at 4 °C. Co-immunoprecipitated proteins were rinsed three times with PBS, pH 7.5, before boiling for 5-min Laemmli buffer. Co-immunoprecipitations were reproduced five independent times.

Gradient Electrophoresis—Akt and interacting proteins that were co-immunoprecipitated as described above were solubilized in SDS solution (0.3% SDS and 1% β -mercaptoethanol). After migration, the gels were fixed (50% ethanol, 0.2 M orthophosphoric acid), and proteins were detected with colloidal blue staining (1 h in a mixture of 30% methanol, 0.2 M orthophosphoric acid, 170 g/liter ammonium sulfate before incubation in the same solution containing 0.66 g/liter G250 Coomassie Brilliant Blue). Band densities were quantified using Quanti-One software (Bio-Rad).

Protein Identification by Mass Spectrometry—Protein identifications were performed by MALDI-TOF and MS-MS. The protein bands of interest were trypsin-digested as described previously (8). MALDI-TOF and MS-MS analysis of the trypsin digests were performed on a Voyager reflector instrument (Applied Biosystems) and a Q-STAR mass spectrometer (Perceptive Biosystems) in positive ion mode. For mass fingerprint analysis, each raw spectrum was opened in Voyager Data Explorer software (version 4.6) and treated using advanced base-line correction and noise removal 2 functions. It was calibrated with two peptides resulting from trypsin autolysis (m/z 842.5100 and 2211.1046). Filter peak list for monoisotopic masses only was enabled, the peak detection threshold was manually adjusted over the background, and then the peak list was copied to Mascot public interface and searched against Swiss-Prot database 49.0 (268,833 sequences, 123,649,849 residues) using the following parameters: trypsin as enzyme, two possible missed cleavages, oxidized methionine as variable modification, MH⁺ monoisotopic masses, and peptide tolerance of 50 ppm. Results were scored using probability-based Mowse score (Protein score is $-10 \times \log(p)$ where p is the probability that the observed match is a random event. Protein scores greater than 67 are significant ($p < 0.05$)). For MS-MS sequence information, each raw file was opened with Analyst Data software (version 1.41) and manually sequenced. Neutral mass of the precursor and sequence information were used to identify proteins in the Swiss-Prot database through Mascot public interface using a mass tolerance of 1 Da for precursor, trypsin as digestion enzyme, two possible missed cleavages, and oxidized methionine as variable modification. Results were scored using probability-based Mowse score (Protein score is $-10 \times \log(p)$ where p is the probability that the observed match is a random event. Protein scores greater than 66 are significant ($p < 0.05$)).

Western Blotting—Co-immunoprecipitated proteins (corresponding to 500 μ g of proteins subjected to immunoprecipitation) were separated by 10% SDS-PAGE. After electrophoresis, nitrocellulose membranes were blocked by Tris-buffered saline, 0.1% Tween containing 6% bovine serum albumin for phospho-(Ser/Thr) Akt substrate antibody or 3% for other antibodies and probed with an appropriate antibody overnight at 4 °C. Then the membranes were rinsed and incubated with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibody (1 h at room temperature). After extensive washes, the reaction was revealed using the Super Signal West Pico chemoluminescent substrate (Pierce) with Eastman Kodak Co. X-Omat AR film.

In Vitro Phosphorylation Assay—2 μ g of pure actin protein (Cytoskeleton, Inc.) was incubated for 30 min at 30 °C in a kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM β -glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 0.2 mM ATP) containing no enzyme or 200 ng of active Akt (Upstate), inactive Akt (Upstate), or PH domain-

deleted active Akt (Upstate). Then the reaction was stopped by boiling in Laemmli buffer, and phosphorylation was revealed by phospho-Akt substrate (PAS) Western blotting.

Transfection with siRNA—MCF-7 cells were incubated for 6 h in serum-free medium with a mixture of 5 μ l of transfectant (siIM-PORTER[®]) and 150 nM SMART pools siRNA in MEM according to the manufacturer's instructions (Upstate). Then 10% FCS was added directly to the medium. After 72 h, the cells were rinsed for 2 h in serum-free medium. The decrease in Akt protein level was assessed by Western blotting with anti-Akt antibody.

Confocal Microscopy—After FGF-2 stimulation, cells were successively rinsed with PBS, pH 7.5; fixed for 20 min with paraformaldehyde 4%; permeabilized for 20 min at room temperature with PBS, pH 7.5, 0.05% saponin, 50 mM ammonium chloride; and blocked for 30 min with PBS, pH 7.5, 0.05% saponin, 2% bovine serum albumin. Then cells were incubated for 2 h at room temperature in a blocking solution containing 1:200 phospho-Akt antibody, rinsed with PBS, pH 7.5, and incubated for 1 h at 37 °C in a blocking solution containing 1:3000 Alexa Fluor 546-goat anti-rabbit IgG and 1:500 Alexa Fluor 488-phalloidin (Invitrogen). Cells were mounted under Mowiol and observed through a Zeiss LSM510 confocal microscope. For studying cortical remodeling of actin, the same protocol using phalloidin was used after 6 h of growth factor stimulation. siRNA directed against Akt was transfected as described previously (9) 48 h before cell treatment by growth factor and visualization of actin cytoskeleton.

Migration Assays—Migration assays were done in 12-well Boyden microchambers (Transwell) with 12- μ m-pore size membranes (Costar). The Transwells were first equilibrated with 1 ml of MEM for 1 h at 37 °C in 5% CO₂. The equilibration medium was then replaced by 1 ml of MEM supplemented or not with 5 ng/ml FGF-2 in the lower chambers, and 40,000 cells in 400 μ l of MEM were loaded on the upper chambers. After a 24-h incubation, the Transwells were rinsed with PBS, and the upper surface of the membranes were scraped to remove cells. The cells remaining on the down side of the membrane were Hoechst-stained and mounted on glass slides with Glycergel (Dako) before counting (15 fields per membrane) under a UV microscope. Each condition was examined in quadruplicate.

RESULTS

Identification of Putative Akt Binding Partners—Co-immunoprecipitates obtained with Akt antibodies were resolved by SDS-PAGE (Fig. 1B). Two experimental conditions were compared. In the first Akt was not activated, and in the second Akt was activated by cell stimulation with FGF-2. After band cutting and trypsin digestion, α -actinin, valosin-containing protein, inhibitor κ B kinase, mortalin, Akt1, tubulin β , cytokeratin 8, actin, 14-3-3 σ , proliferating cell nuclear antigen (PCNA), and heat shock protein HSP27 were identified by mass spectrometry. The identified proteins are listed in Table I, including the corresponding band number, protein name, accession number in the Swiss-Prot database, molecular mass, Mascot score, and percentage of coverage. The fact that neither the intensity of the Akt-corresponding band nor the intensity of the IgG-corresponding band changed from unstimulated to Akt-stimulated conditions was a reliable indication of equal loading. All identifications, primarily done by MALDI-TOF, were validated by sequencing with MS-MS and/or by Western blotting as indicated in Table I. The putative interactions were validated by Akt Western blotting on reverse immunoprecipitates (Fig. 2).

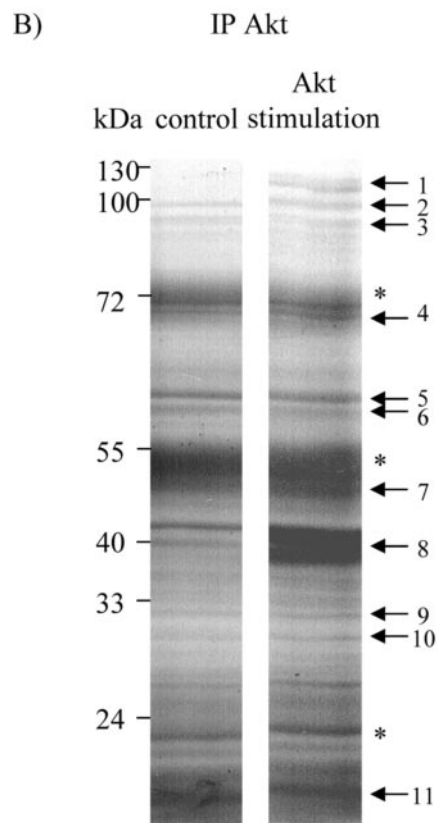
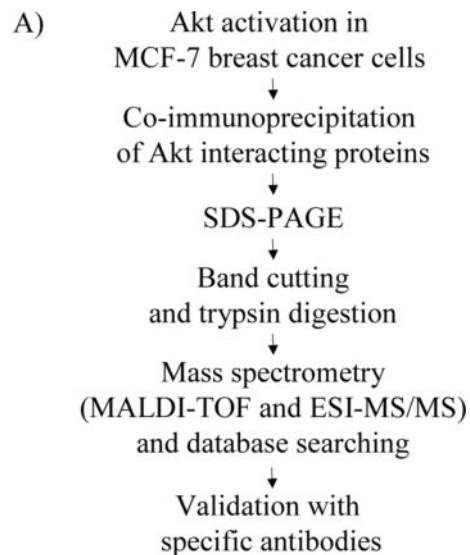


Fig. 1. **Separation of Akt-co-immunoprecipitated proteins.** A, co-immunoprecipitation-based approach to identify putative Akt partners. B, SDS-PAGE gel obtained for Akt co-immunoprecipitates. After a 15-min stimulation by FGF-2, proteins were extracted from breast cancer cells in non-denaturing condition and immunoprecipitated with anti-Akt1 antibody. Co-immunoprecipitated proteins were separated by 8–20% gradient SDS-PAGE and stained with colloidal Coomassie Blue. Bands (1–11, numbered from high to low molecular masses) were cut and trypsin-digested for analysis by mass spectrometry. The positions where IgG were detected are indicated (*). IP, immunoprecipitation.

TABLE I
Identification of putative Akt binding partners

Bands from gradient SDS-PAGE (Fig. 1) were digested by trypsin and analyzed by mass spectrometry through MALDI-TOF mass fingerprint and nano-ESI-MS-MS. For each identified protein, the following information is indicated: band number, protein name, accession number in Swiss-Prot database, molecular mass (MW), Mascot score for mass fingerprint and sequence-based identifications, percentage of protein sequence covered by the matched and sequenced tryptic fragments, mass difference between experimental and theoretical masses for MS-MS analysis, ratio of band intensity (volume on area representing the mean of five independent experiments), and recognition by PAS antibody. I κ B, inhibitor κ B.

band	protein name	MW (kDa)	accession number	mass fingerprints			validation	MSMS					ratio (Akt activated / non activated)	interaction dependent on Akt activation	PAS antibody recognition dependent on Akt activation
				score	matched peptides	sequence coverage (amino acids)		peptide sequence	observed precursor m/z	theoretical precursor neutral mass	delta mass (amu)	score			
1	Alpha-actinin 4	104	O43707	362	48/61	446/911 (49%)	antibody						10.8	yes	no
2	Valosin-containing protein	89	P55072	114	25/59	185/805 (23%)	MS-MS	WALSQSNPSALR	665.1	1328.69	0.6	138	5.7	yes	yes
3	I κ B kinase beta	86	O14920	96	10/12	98/756 (13%)	antibody						2.5	yes	yes
4	Mortalin (GRP70)	73	P38646	80	7/12	129/679 (19%)	antibody						1.1	no	no
5	Akt1	55	P31749	182	12/28	115/480 (24%)	antibody MS-MS	EVIVAKDEVAHLLTENR	963.1	1923.01	1.1	220	1.0	-	-
6	Tubulin beta	49	P07437	81	17/24	146/444 (33%)	antibody						1.0	no	no
7	Cytokeratin 8	48	P05787	77	19/30	198/482 (41%)	antibody MS-MS	LSELEAALQR	565.0	1128.62	0.4	121	1.4	no	no
8	Actin beta/gamma	42	P63261/91	218	26/34	158/375 (42%)	antibody MS-MS	EITALAPSTMK	581.3	1160.61	0.1	593	14.2	yes	yes
								QEYDESGPSIVHR	758.9	1515.70	0.1				
								GYSFTTAAER	566.8	1131.52	0.1				
								SYELPDGQVITIGNER	896.2	1789.89	0.5				
9	Proliferating cell nuclear antigen	28	P12004	36	4/8	42/161 (17%)	antibody	CAGNEDIITLR	602.6	1203.60	0.1	281	2.3	yes	no
10	14-3-3 sigma	28	P31947	70	11/23	55/248 (22%)	antibody	FSASGELGNGNIK	647.3	1292.64	0.0		1.4	no	no
11	Heat shock protein 27	23	P04792	113	8/12	74/205 (38%)	antibody						1.0	no	no

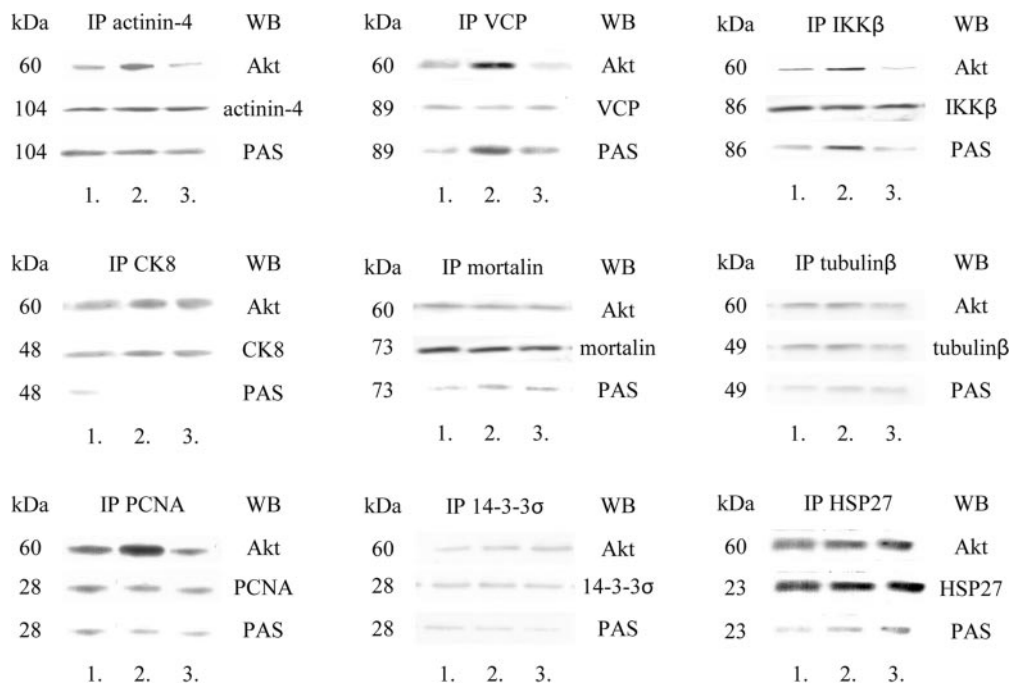


FIG. 2. **Reverse immunoprecipitation and Western blotting analysis.** Reverse immunoprecipitations were performed with antibodies against proteins identified as putative partners of Akt (Fig. 1B and Table I) followed by Akt Western blotting. Variations of interaction were observed between unstimulated control situation (lane 1), Akt activation (by FGF-2 stimulation) without (lane 2) or with (lane 3) inhibition of PI3K/Akt by wortmannin (Akt inhibition). Potential phosphorylation on Akt consensus sites was tested using the PAS antibody. The reverse immunoprecipitation with actin antibodies is shown in Fig. 5. WB, Western blotting; IP, immunoprecipitation; VCP, valosin-containing protein; IKK β , inhibitor κ B kinase β ; CK8, cytokeratin 8.

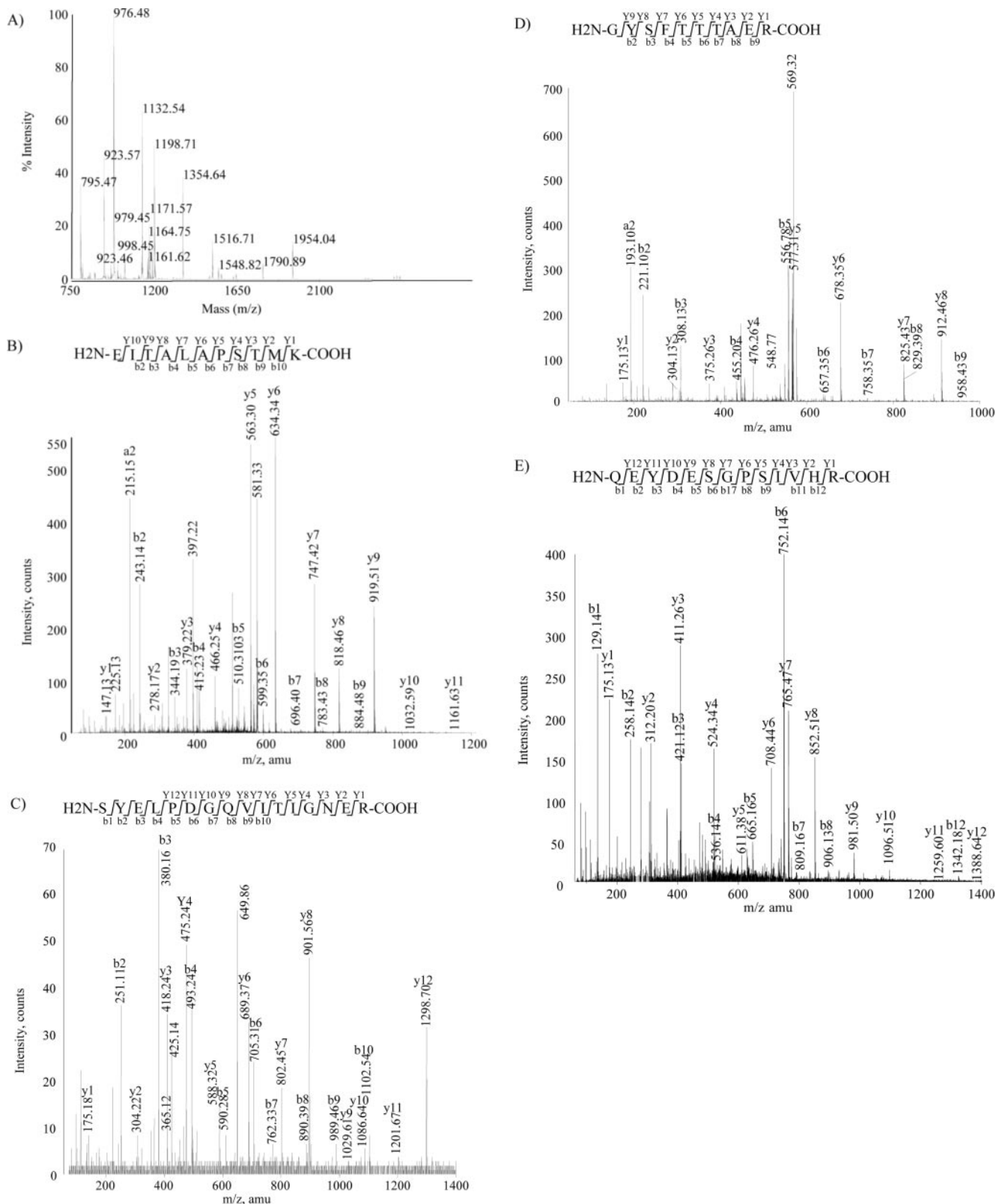


FIG. 3. MALDI-TOF and MS-MS spectra corresponding to actin. Shown are MALDI-TOF (A) and MS-MS analyses (B-E) of band number 9 from gradient SDS-PAGE (Fig. 1B). The band was cut out of the gel and trypsin-digested. After MALDI-TOF and database searching, 21 tryptic peptides matched with theoretical masses leading to a sequence coverage of 42% provided a clear identification of β and γ actin that was confirmed by sequencing of four peptides by MS-MS.

TABLE II
Identification of actin by mass spectrometry

Band number 9 in gradient SDS-PAGE was cut, digested by trypsin, and analyzed by mass spectrometry. Measured masses from MALDI-TOF (Fig. 2A) and theoretical masses from Swiss-Prot database, their difference, and the corresponding sequence in actin are shown for each matched peptide. Peptide sequences obtained by MS-MS (Fig. 2, B–E) are underlined.

MH ⁺ submitted	MH ⁺ matched	Delta mass	Missed cleavages	Peptide sequence consistent with mass
		Da		
976.48	976.44	0.03	0	<u>19AGFAGDDAPR</u> ²⁸
1198.71	1198.70	0.01	0	<u>29AVFPSIVGRPR</u> ³⁹
1171.57	1171.56	0.01	0	<u>40HQGVMVGMGQK</u> ⁵⁰
1187.58	1187.56	0.01	0	<u>40HQGVMVGMGQK</u> ⁵⁰ , oxidation (Met)
1203.56	1203.55	0.00	0	<u>40HQGVMVGMGQK</u> ⁵⁰ , 2 oxidation (Met)
1354.64	1354.62	0.02	1	<u>51DSYVGDEAQSQR</u> ⁶²
800.54	800.53	0.01	1	<u>62RGILTLK</u> ⁶⁸
1515.75	1515.74	−0.00	0	<u>85IWHHTFYNELR</u> ⁹⁵
1954.04	1954.06	−0.02	0	<u>96VAPEEHPVLLTEAPLNPK</u> ¹¹³
998.45	998.48	−0.03	0	<u>184DLTDYLMK</u> ¹⁹¹
1132.54	1132.52	0.02	0	<u>197GYSFTTAAER</u> ²⁰⁶
872.53	872.51	0.01	1	<u>207EIVRDIK</u> ²¹³
1790.89	1790.88	0.01	0	<u>239SYELPDGQVITIGNER</u> ²⁵⁴
1548.82	1548.81	0.01	1	<u>313MQKEITALAPSTMK</u> ³²⁶
1564.82	1564.80	0.01	1	<u>313MQKEITALAPSTMK</u> ³²⁶ , oxidation (Met)
1161.62	1161.61	0.01	0	<u>316EITALAPSTMK</u> ³²⁶
1036.66	1036.64	0.01	1	<u>327IKIIPPER</u> ³³⁵
795.47	795.46	0.01	0	<u>329IIPPER</u> ³³⁵
923.57	923.56	0.01	1	<u>329IIPPERK</u> ³³⁶
1516.71	1516.70	0.01	0	<u>360QEYDESGPSIVHR</u> ³⁷²
1644.81	1644.79	0.01	1	<u>360QEYDESGPSIVHRK</u> ³⁷³

Akt Interacts with Actin under Growth Factor Stimulation— The most important difference observed between Akt-activated and non-activated conditions concerned protein band number 8 (Fig. 1B) that was found to be up-regulated 14-fold under FGF-2 stimulation. MALDI-TOF and MS-MS analysis allowed the clear identification of actin γ and β (Fig. 3 and Table II). By MALDI-TOF, 26 tryptic peptides matched with theoretical masses leading to a sequence coverage of 42% provided a clear identification of β and γ actin that was confirmed by sequencing of four peptides using MS-MS. Because non-dissociating buffer conditions were used to preserve protein-protein interaction during the immunoprecipitation it is likely that physiologically polymerized actin was also observed. In addition, confocal microscopy allowed the detection of a co-localization between polymerized actin (visualized with phalloidin) and Akt (detected with antibodies) under growth factor stimulation (Fig. 4). Activated Akt co-localized with actin cytoskeleton after 15 min of FGF-2 stimulation in cortical submembrane areas. The interaction between Akt and actin was also validated by reverse co-immunoprecipitation with anti-actin antibody followed by Western blotting with anti-Akt antibody (Fig. 5A). Interestingly recruitment of actin by Akt was sensitively decreased by inhibition of PI3K using wortmannin, showing that the interaction of Akt with actin is dependent on Akt activation through the PI3K pathway.

Actin Is a Substrate of Phosphorylation by Akt—The puta-

tive Akt partners identified here were screened with Scansite software (52) to detect potential Akt phosphorylation sites. The best scores were obtained for putative phosphorylation sites in γ actin, α -actinin-4, inhibitor κ B kinase β , and cytokeratin 8. Interestingly we detected phosphorylation of these consensus sites by using PAS antibody as predicted by computational analysis. The Akt-phosphorylated consensus site is commonly defined as RXXRX(S/T) (10, 11). In fact, the primary sequence of actin does not contain the precise consensus sequence predicted for an Akt substrate: the arginine at position −5 is lacking. However, actin encodes a peptide sequence that is consistent with a pattern of other Akt-phosphorylated targets where arginine at position −5 is not present as in insulin-response element-binding protein 1 (KERCS¹⁰³⁶) (12), cAMP-response element-binding protein (LSRRPS^{133Y}) (13), and ATP-citrate lyase (TPAPSRTAS^{455F}) (14). The PAS antibody that we used here was raised against Akt RXXRX(pS/pT) (where pS is phosphoserine and pT is phosphothreonine) consensus sequence but does not appear to strictly require arginine in the −5 position for phosphoprotein recognition as was obtained for ATP-citrate lyase (14). In actin, the putative phosphorylation sites corresponding to Akt consensus are APPERKYS³³⁸ and ILTERGYS¹⁹⁹. Actin immunoprecipitate was subjected to Western blotting with PAS antibody (Fig. 5B). This antibody detects the consensus site of Akt when it is phosphorylated. The results show that actin was recognized by PAS antibody under cell stimulation by FGF-2

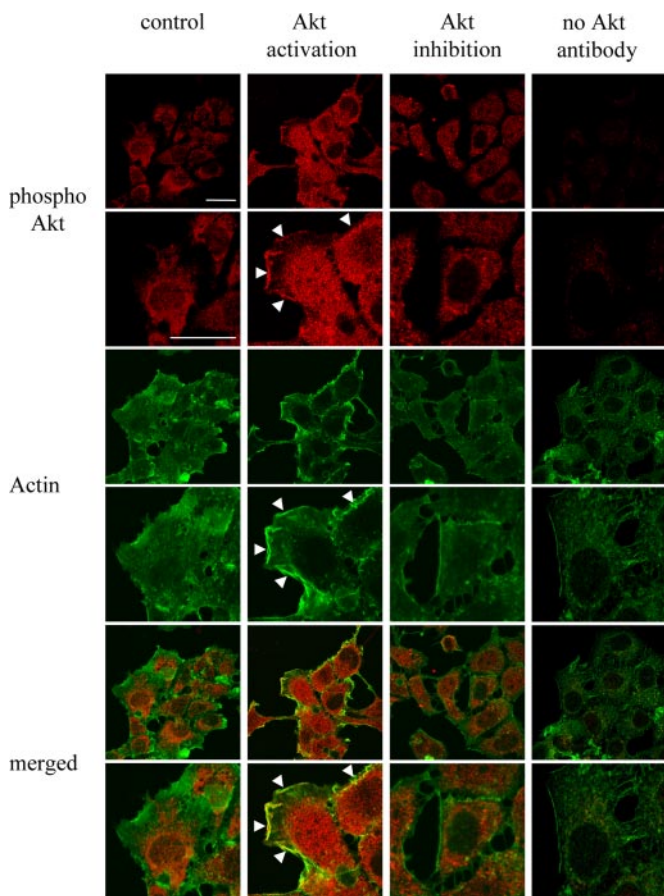


FIG. 4. *In situ* validation of interaction between Akt and polymerized actin by confocal microscopy. MCF-7 breast cancer cells were stimulated for 15 min with 5 ng/ml FGF-2. Immunolocalization of phospho-Akt is revealed in red (antibody), and immunolocalization of actin is revealed in green (phalloidin-Alexa Fluor 488). Merge detection between Akt and polymerized actin appears in yellow, indicating a co-localization in the cortical submembrane area. The areas of co-localization are highlighted with white arrowheads. Antibody control consisted in the same experiment without anti-Akt antibody. Two different magnifications are displayed (a group of cells and one-cell zooms). White horizontal lines show scale bars for 20 μm.

and that actin phosphorylation was inhibited by wortmannin, thereby indicating that actin is a phosphorylation target of Akt signaling. Nevertheless these data obtained with intact cells do not demonstrate a direct phosphorylation of actin by Akt as intermediary kinases could be involved. To investigate a potential direct phosphorylation of actin by Akt, an *in vitro* phosphorylation assay was performed. Recombinant Akt was mixed with actin and ATP. A phosphorylation of actin was detected by Western blotting with PAS antibody specifically when Akt was activated (Fig. 5C). No phosphorylation of actin was detected when Akt was not activated, and only a weak phosphorylation was observed with a PH domain-deleted form of Akt. At this stage, we were not able to directly map the regions of interaction and the phosphorylation sites, but our results suggest that Akt can phosphorylate actin in a PH domain-dependent manner.

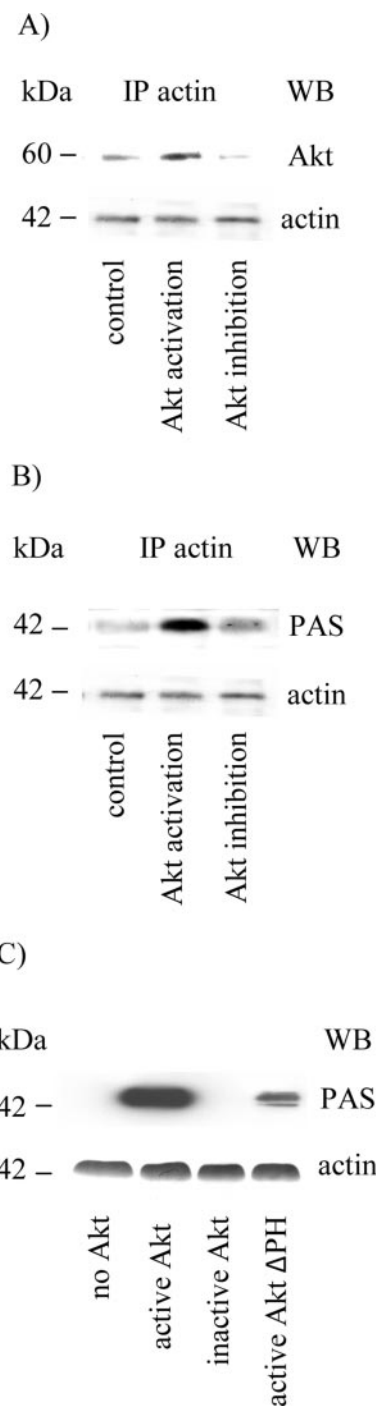


FIG. 5. Phosphorylation of actin by Akt. A, reverse immunoprecipitation was performed with antibody against actin and followed by Akt Western blotting. Variations of interaction between actin and Akt were observed under growth factor stimulation with or without inhibition of PI3K/Akt by wortmannin. B, the same immunoprecipitates were used for Western blotting with PAS antibody, allowing the detection of actin phosphorylation under condition of Akt activation. C, *in vitro* phosphorylation assay was performed by incubating actin for 30 min at 30 °C in a reaction buffer containing either no enzyme, active Akt, inactive Akt, or PH domain-deleted active Akt. Phosphorylation of actin was revealed by Western blotting with PAS antibody. WB, Western blotting; IP, immunoprecipitation.

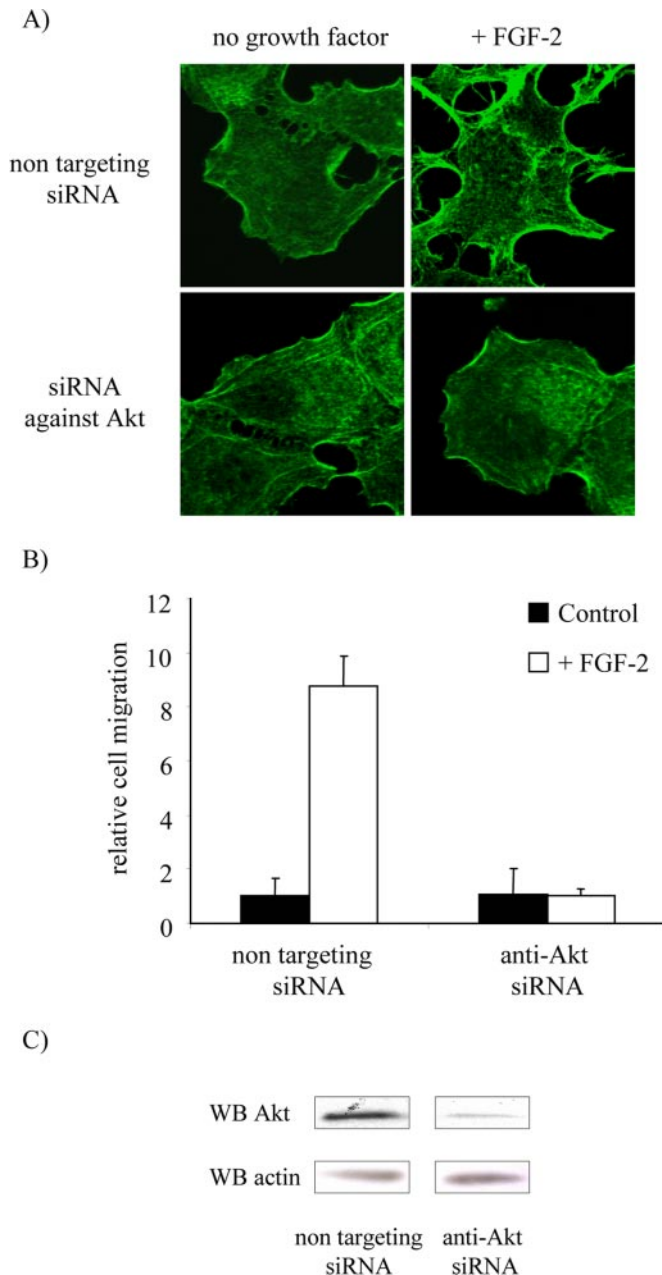


FIG. 6. Cortical remodeling of actin and cell migration are induced by Akt activation. A, MCF-7 cells were stained with phalloidin (green fluorescence) to visualize the actin cytoskeleton. Two situations were studied: cells transfected by siRNA against Akt and cells transfected by non-targeting siRNA. FGF-2 stimulation was performed at 5 ng/ml for 6 h. The cortical remodeling of actin observed under FGF-2 stimulation was inhibited by treatment with siRNA against Akt. B, Transwell experiment performed with MCF-7 cells stimulated or not by 5 ng/ml FGF-2 with or without transfection by siRNA against Akt. FGF-2-induced stimulation of cell migration was inhibited by siRNA against Akt. C, control Western blotting detection of Akt in cells treated by 150 nM siRNA against Akt. WB, Western blotting.

Akt Activation Induces Actin Cortical Remodeling and Is Necessary to Cell Migration—As pharmacological modulation of PI3K has been described to have an impact on the orga-

nization of actin cytoskeleton (15–17), we tested the potential implication of Akt in this process. Actin cytoskeleton was stained with phalloidin (Fig. 6A). FGF-2 cell stimulation induced an increase in cortical localization of actin that was inhibited by RNA interference against Akt, demonstrating an involvement of Akt in the actin cytoskeleton remodeling. In addition, the FGF-2-induced stimulation of MCF-7 cell migration, observed in Transwell experiments, was inhibited by siRNA against Akt (Fig. 6B) indicating the crucial role played by this kinase in the control of breast cancer cell migration. The efficiency of siRNA treatment in decreasing Akt level is shown in Fig. 6C.

DISCUSSION

In contrast to classical approaches to study signal transduction (based on the use of specific antibodies to identify signaling partners), proteomics offers the advantage of allowing the identification of unexpected partners, and this is of considerable potential for defining new therapeutic targets in pathologies such as breast cancer (18, 19). With co-immunoprecipitation and mass spectrometry-based methodology we identified new proteins potentially interacting with Akt. The immunoprecipitation-based methodology that we used has intrinsic limitations for access to the low concentration proteins, probably accounting for the fact that established partners of Akt signaling such as glycogen synthase kinase 3, forkhead, caspases, or Bad were not detected here. More generally, the development of more sensitive methods for analyzing signaling transduction pathways is clearly an issue in proteomics analysis (20). However, it is important to emphasize that no transfection or overexpression of Akt or any other partners were performed here, and therefore our study has the advantage to rely exclusively on physiological levels of protein expression and interaction.

Among putative partners of Akt identified in the present study, we have previously described the inhibitor κ B kinase β (9) and the valosin-containing protein (21) as signaling targets of Akt in breast cancer cells. Some of the identified proteins were found to be constitutively associated with Akt as their interaction was not growth factor-regulated. This is for example the case for the molecular chaperone 14-3-3 σ that targets phosphoproteins and regulates their activities (22). Expression of 14-3-3 σ , which is induced in response to DNA damage and causes cells to arrest in G₂, is lower in breast carcinoma cells than in normal breast epithelium due to frequent hypermethylation of the gene (8, 23). Interestingly it has been shown previously that 14-3-3 σ might be a negative regulator of Akt signaling by masking its phosphorylation targets like tuberin (24), and our data reinforce the hypothesis of a functional interaction between 14-3-3 σ and the Akt-mediated signaling. Other protein chaperones were identified, such as the heat shock protein HSP27 and mortalin. Although the potential interaction with Akt that we report here is in accordance with the oncogenic potentiality of these proteins, further experi-

ments would be necessary to confirm a functional interaction. The PCNA was also found as a putative Akt partner. PCNA was originally characterized as a DNA sliding clamp for replicative DNA polymerases and as an essential component of the eukaryotic chromosomal DNA replisome and turned out to interact with multiple partners involved in metabolic pathways, DNA repair, DNA synthesis and methylation, chromatin remodeling, and cell cycle regulation (25). At this stage, it cannot be ascertained whether Akt directly interacts with PCNA. Because one of the known interacting partners of PCNA is p21^{waf1} (26), which is described as an Akt substrate, we can hypothesize that PCNA could have been co-immunoprecipitated with Akt due to p21 dual interaction. However, further experimentation would also be needed to clarify the potential interaction between Akt and PCNA. Few proteins identified in this study are components of the cytoskeleton. Tubulins, the building block of microtubules, are heterodimers comprising α and β subunits. Tubulin modifications have been reported in breast cancer and are linked to tumor aggressiveness (27). It has been shown that the level of modified tubulins increases in Taxol-resistant MCF-7 breast cancer cells (28), and the structure of tubulin complexed with Taxol has been described (29). Interestingly several kinases such as MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase), ERK (extracellular signal-regulated kinase), and p90RSK interact with tubulin to reorganize cytoskeleton and influence cell division or migration (30, 31), and our data suggest that Akt might belong to this category of tubulin-interacting proteins. However, it should be emphasized that, at this stage, the list of proteins that we established represents putative Akt interacting partners and that further biochemical and physiological analyses are required to validate the existence of functional interactions.

The major partner of Akt that we identified is the cytoskeleton component actin. In case of cell stimulation by FGF-2, the association between actin and Akt was found to be strongly up-regulated, and using a series of complementary approaches (reversed immunoprecipitations, confocal microscopy, *in vitro* phosphorylation assay, and siRNA) we were able to show that actin is a functional target of Akt signaling. The actin network is a rather complex structural and functional system of all eukaryotic cells that responds to multiple extracellular stimuli and contributes to cell-cell and cell-substrate interactions by providing a structural framework and by modulating signal transduction cascades (32). It also generates movements to carry out many fundamental cell processes, such as lamellipodial and growth cone extension, chemotaxis, endocytosis, exocytosis, and cytokinesis. Regulation of the actin cytoskeleton plays a central role in cell growth, differentiation, and motility and is correlatively related to cancer development and invasion. Actin remodeling can result from the activation of oncogenic signaling pathways (e.g. Ras and Src) or the inactivation of several actin-binding proteins that have tumor suppressor functions such as gel-

solin (33). These changes are thought to be integrally involved in the abnormal growth properties of tumor cells, their ability to adhere to tissue, and their increased ability to metastasize. The family of p21-activated kinases has been implicated in the rearrangement of actin cytoskeleton by acting downstream of the small GTPase Rac under PI3K stimulation and resulting in the dissolution of stress fibers and the redistribution of microfilaments via cdc42 (34). It has been found that p21-activated kinase 1 is stimulated by activated Akt through a GTPase-independent mechanism as well as by PDK1 through a PI3K-independent mechanism (35). Another signaling adapter protein that has been reported to interact with actin is Src homologous and collagen (Shc). Shc binds to actin under growth factor stimulation, allowing the activation of downstream signaling leading to cell orientation toward proliferation *versus* differentiation (36). Similarly the phosphorylation of EDG-1 by Akt specifically regulates coupling to Rac activation, cortical actin changes, and chemoattractant-induced cell migration. Akt phosphorylation of EDG-1 may constitute a "specificity switch" to cell migration. Actin remodeling through Akt activation was rather attributed to p70S6K, a downstream Akt signaling kinase (37). Here we have suggested that Akt is able to directly phosphorylate actin, adding further comprehensive information to PI3K/Akt-dependent regulation of actin organization.

The possibility that actin filaments and their regulatory proteins could be selectively targeted in cancer chemotherapy is attractive (38). Interestingly gelsolin, an actin capping/severing protein, is commonly down-regulated in many tumor cells and functions as a tumor suppressor in breast epithelial cells (39, 40). Major cytoskeleton filaments, including actin, are degraded during the execution phase of apoptosis, and a direct link between actin depolymerization and DNA degradation has been suggested (41), leading to the idea that the derangements of actin filaments may, by themselves, initiate cell death signals (42, 43). Interestingly cytochalasin D, the best studied and most widely used agent that acts on actin and exerts a powerful effect on actin filaments, has been reported to enhance cytokine withdrawal and drug treatment-induced apoptosis (43, 44). In our experiments, confocal microscopy showed a substantial cortical reorganization of actin under condition of growth factor-activated Akt. Such cortical reorganization can be induced by PI3K stimulation (16), and Akt as downstream effector of PI3K constitutes a major effector in PI3K-mediated actin remodeling. Another cytoskeletal protein identified here is α -actinin-4, which is a non-muscle α -actinin isoform up-regulated upon enhanced cell movement (45, 46). Actinin-4 has been described as steadily shifting from the cytoplasm to the nucleus upon inhibition of PI3K or actin depolymerization; the cytoplasmic localization of actinin-4 was closely associated with an infiltrative histological phenotype and correlated significantly with a poorer prognosis of breast cancer (45). In our experiments, we found that actinin-4 interacts with Akt, but we did not detect any change

in phosphorylation of actinin-4 induced by Akt. Actinin-4 might not directly interact with Akt but could be associated with actin and therefore co-immunoprecipitated with it. Nevertheless it is interesting to note that regulation of actinin-4 is related to cell movement and migration, and in breast cancer, it has been reported that Akt mediates the invasive effect of growth factors (47). The relationship between Akt and the control of cell migration has been suggested by the demonstration that the PI3K/Akt pathway is involved in anchorage-independent growth and metastasis of liver cancer cells (48) similar to thyroid carcinoma cells (49). In addition, Akt mediates Ras down-regulation of RhoB, a suppressor of transformation, invasion, and metastasis (50). In breast cancer, it has been demonstrated that Akt is frequently expressed in a high percentage of tumor cells (3). Growth factor-induced metastasis gene expression in breast cancer has been shown to be related to Akt signaling (47), and moreover, the activation of Akt by Her2/PI3K plays an important role in mediating multidrug resistance in human breast cancer cells (4, 51). Our data, showing a cortical reorganization of actin associated with a stimulation of cell migration dependent on Akt activation, indicate a relationship with invasive capacities of breast cancer cells that is potentially germane to the metastatic process. However, it should be noticed that a direct link has not been proved here. Indeed Akt activates other downstream pathways, including for example Rac, that may also be responsible for mediating changes in invasive capacities of breast cancer cells.

In conclusion, our proteomics exploration of Akt signaling in breast cancer cells led to the demonstration that the cytoskeleton protein actin is a major cellular partner of this kinase. Cytoskeleton remodeling occurs at all levels of cell dynamics, and our data suggest that the many key regulatory functions played by Akt in cell physiology could be at least partly mediated through interaction with and subsequent phosphorylation of actin.

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** Present address: UMR CNRS 5180, Université Claude Bernard, 69622 Villeurbanne, France.

‡ To whom correspondence should be addressed: ERI-8 INSERM, batiment SN3, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq, France. Tel.: 33-3-20-43-40-97; Fax: 33-3-20-47-40-38; E-mail: hubert.hondermarck@univ-lille1.fr.

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