



**HAL**  
open science

## The Valosin-containing Protein (VCP) Is a Target of Akt Signaling Required for Cell Survival

Franck Vandermoere, Ikram El Yazidi-Belkoura, Christian Slomianny, Yohann Demont, Gabriel Bidaux, Eric Adriaenssens, Jérôme Lemoine, Hubert Hondermarck

► **To cite this version:**

Franck Vandermoere, Ikram El Yazidi-Belkoura, Christian Slomianny, Yohann Demont, Gabriel Bidaux, et al. The Valosin-containing Protein (VCP) Is a Target of Akt Signaling Required for Cell Survival. *Journal of Biological Chemistry*, 2006, 281 (20), pp.14307 - 14313. 10.1074/jbc.m510003200 . hal-03030155

**HAL Id: hal-03030155**

**<https://hal.science/hal-03030155>**

Submitted on 29 Nov 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# The Valosin-containing Protein (VCP) Is a Target of Akt Signaling Required for Cell Survival\*

Received for publication, September 12, 2005, and in revised form, March 15, 2006. Published, JBC Papers in Press, March 21, 2006, DOI 10.1074/jbc.M510003200

Franck Vandermoere<sup>†1</sup>, Ikram El Yazidi-Belkoura<sup>‡</sup>, Christian Slomianny<sup>§</sup>, Yohann Demont<sup>‡</sup>, Gabriel Bidaux<sup>§</sup>, Eric Adriaenssens<sup>‡</sup>, Jérôme Lemoine<sup>¶</sup>, and Hubert Hondermarck<sup>‡2</sup>

From the <sup>†</sup>ERI-8 INSERM, "Growth factor signaling in breast cancer. Functional proteomics," <sup>§</sup>EMI-0228 INSERM, and <sup>¶</sup>UMR-8576 CNRS IFR-118, University of Sciences and Technologies Lille, 59655 Villeneuve d'Ascq, France

The serine/threonine kinase Akt is a key mediator of cell survival and growth, but its precise mechanism of action, and more specifically, the nature of its signaling partners largely remain to be elucidated. We show, using a proteomics-based approach, that the valosin-containing protein (VCP), a member of the AAA (ATPases associated with a variety of cellular activities) family, is a target of Akt signaling. SDS-PAGE of Akt co-immunoprecipitated proteins obtained from MCF-7 breast cancer cells revealed the increase of a 97-kDa band under Akt activation. Mass spectrometry analysis allowed the identification of VCP, and we have shown a serine/threonine phosphorylation on an Akt consensus site upon activation by growth factors. Site-directed mutagenesis identified Ser-351, Ser-745, and Ser-747 as Akt phosphorylation sites on VCP. Confocal microscopy indicated a co-localization between Akt and VCP upon Akt stimulation. Interestingly, small interfering RNA against VCP induced an inhibition of the growth factor-induced activation of NF- $\kappa$ B and a potent pro-apoptotic effect. Together, these data identify VCP as an essential target of Akt signaling.

The valosin-containing protein (VCP)<sup>3</sup> belongs to the AAA (ATPases associated with various cellular activities) family, the members of which are characterized by having highly conserved ATPase domain(s) with high sequence similarities and ring structures consisting of homo-oligomers (1, 2). VCP, also known as VAT in archaeobacteria, CDC48p in yeast, TER94 in *Drosophila*, and p97 in *Xenopus*, is one of the most evolutionarily conserved proteins that is ubiquitous and abundant in cells, accounting for more than 1% of total cellular proteins. Like other AAA proteins, VCP has been shown to be involved in a wide variety of ATP-dependent cellular processes such as ubiquitin-mediated proteolysis, DNA repair, membrane fusion and dynamics of subcellular compartments, gene expression, and cell growth. Admittedly, it acts as a molecular chaperone that unfolds or unwinds proteins, although the detailed mechanisms of VCP function remain to be determined, as well

as its regulation mechanisms in both physiological and pathological conditions.

During the course of studying the molecular partners of the serine/threonine kinase Akt, a key regulator of cell survival that is activated by growth factors, we detected VCP in Akt co-immunoprecipitated material. The interaction between Akt and VCP was further studied, and from the functional point of view, we showed that disruption of VCP using siRNA impaired the cell survival signaling mediated by Akt. We propose that VCP is an important player in the Akt-mediated signaling of cell survival.

## EXPERIMENTAL PROCEDURES

**Materials**—Cell culture reagents were purchased from BioWhittaker. Recombinant human fibroblast growth factor-2 (FGF-2) was from R&D systems. Wortmannin (phosphatidylinositol 3-kinase (PI3K) inhibitor) was from Calbiochem. C2 ceramide analogue (*N*-acetyl-D-sphingosine), 5-fluorouracil, camptothecin, etoposide, Hoechst 33258, G250 Coomassie Brilliant Blue, Me<sub>2</sub>SO, and peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibody were from Sigma. Dynabeads M-280 tosylactivated were from Dynal Biotech. Constitutively active Akt and wild-type Akt pUSE-encoding plasmids were from Upstate Biotechnology.

**Cell Culture, NF- $\kappa$ B Activation, and Induction of Apoptosis**—Breast cancer cell lines MCF-7, T-47D, and BT-20 were obtained from the American Type Culture Collection and routinely grown as monolayers as described previously (3). Apoptosis of MCF-7 cells was induced by treatment with ceramide analogue C2 (10  $\mu$ M for 24 h), previously described as a pro-apoptotic agent for human breast cancer cells (4). The determinations of both the NF- $\kappa$ B activation (luciferase reporter assay) and the proportion of cells in apoptosis (Hoechst staining) were performed as described (3). For apoptosis induction by chemotherapeutic agents, MCF-7 breast cancer cells were treated for 3 h with 5-fluorouracil (100  $\mu$ M), camptothecin (0.1 nM), or etoposide (10  $\mu$ M) and replaced in new culture medium. After 24 h, cells were fixed with cold methanol (−20 °C) for 10 min and washed twice with phosphate-buffered saline (PBS) before staining with 1  $\mu$ g/ml Hoechst 33258 and apoptosis quantification.

**Immunoprecipitation of Akt and Interacting Proteins**—MCF-7 cells were rinsed by PBS, pH 7.5, serum-starved for 3 h in minimum essential medium (minimum Eagle's medium-Earle's salts), and treated for 24 h by 10  $\mu$ M C2 ceramide in fresh minimal essential medium. Proteins were extracted from cells after 10 min of 5 ng/ml FGF-2 stimulation in 100  $\mu$ M pervanadate. 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  $\mu$ M sodium orthovanadate). After centrifugation (10,000  $\times$  g, 5 min), proteins were quantified using Bradford's method (Bio-Rad). Akt and interacting proteins were

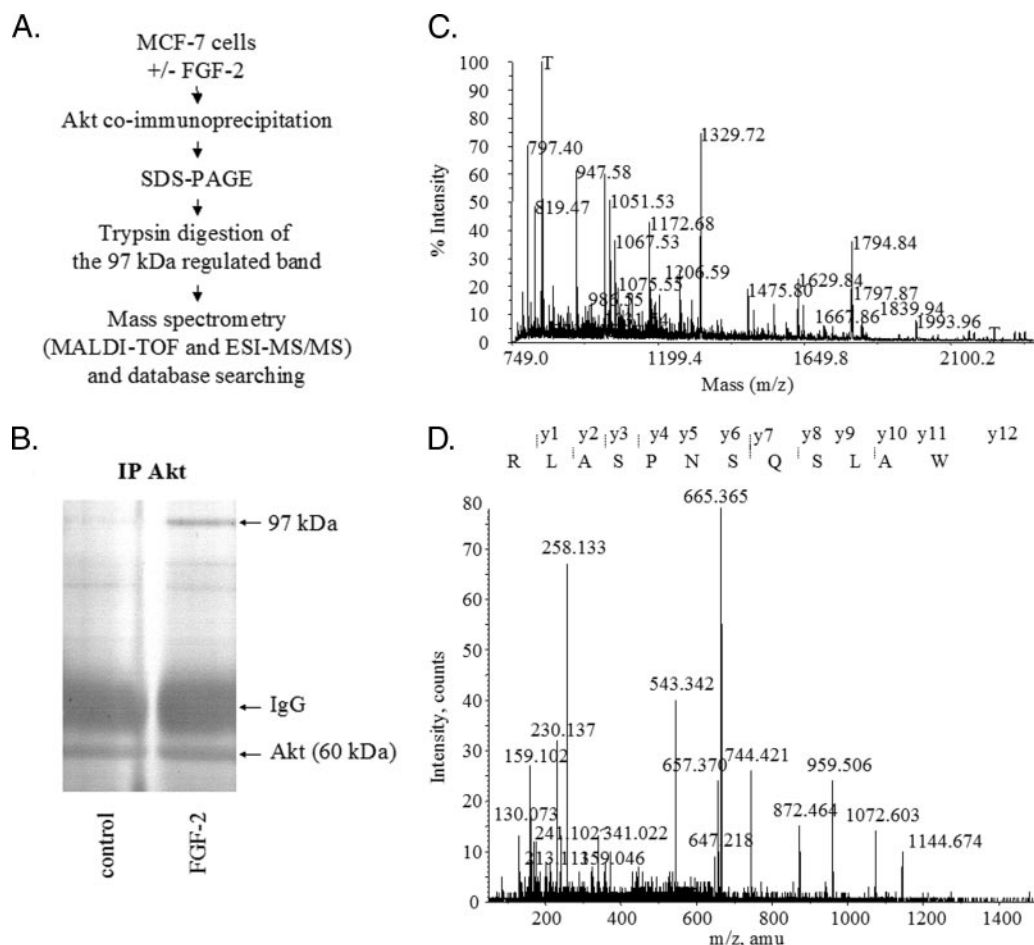
\* This work was supported by grants from the Ligue Nationale Contre le Cancer (Equipe labellisée 2006), the Fondation pour la Recherche Médicale (FRM comité du Nord), the Institut Universitaire de France, and the Region Nord-pas-de-Calais and the Genopole of Lille. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> A recipient of a fellowship from the French Ministry for Research and Education and the Ligue Nationale Contre le Cancer.

<sup>2</sup> To whom correspondence should be addressed: ERI-8 INSERM, bâtiment 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.

<sup>3</sup> The abbreviations used are: VCP, valosin-containing protein; PI3K, phosphatidylinositol 3-kinase; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS-MS, tandem mass spectrometry; PAS, phospho-Akt substrate; siRNA, small interfering RNA.

## VCP Is a Target of Akt Signaling



**FIGURE 1. VCP is co-immunoprecipitated with activated Akt.** *A*, Akt co-immunoprecipitated material was resolved on SDS-polyacrylamide gel. Two experimental conditions were compared: Akt activated by cell stimulation with FGF-2 for 15 min versus the control in which Akt was not activated. The 97-kDa protein band was analyzed by mass spectrometry (MALDI-TOF and MS-MS) after trypsin digestion. *ESI*, electrospray ionization. *B*, silver staining of the SDS-PAGE gel revealed the appearance of a 97-kDa band in the Akt stimulated condition. The levels of both immunoprecipitated (*IP*) Akt and immunoprecipitated IgG were identical under the two conditions. *C*, peptide mass fingerprint of the trypsin-digested 97-kDa band obtained by MALDI-TOF analysis. *D*, ESI-MS-MS spectrum, corresponding to the 1328-Da peptide, allowing the determination of a 12-amino-acid sequence corresponding to VCP.

co-immunoprecipitated from equal quantities of proteins using antibodies covalently bound to magnetic beads, according to Dynal Biotech protocol using 2.5  $\mu\text{g}$  of anti-Akt for 500  $\mu\text{g}$  of total proteins. Immunoprecipitated material was rinsed three times by PBS, pH 7.5, before a 5-min boiling in Laemmli buffer 1 $\times$ .

**Gradient Gel Electrophoresis**—Akt immunoprecipitated material was solubilized in SDS solution (0.3% SDS and 1%  $\beta$ -mercaptoethanol) before SDS-PAGE migration and colloidal blue staining (G250 brilliant blue).

**Protein Identification by Mass Spectrometry**—Protein identification was performed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and tandem mass spectrometry (MS-MS). The protein band of interest was trypsin-digested as described previously (5). MALDI-TOF and MS-MS analysis of the trypsin digests were performed on a Voyager reflector instrument (Applied Biosystems) and a Q-STAR (PerSeptive Biosystems) in positive ion mode. For MALDI-TOF, mass measurements were made after peak smoothing and internal calibration using the average mass of the two autolysis trypsin fragment ions at  $m/z$  842.510 and 2211.105. Protein sequence data base searching was performed with MS-Fit and Mascot.

**Western Blotting**—Co-immunoprecipitated proteins were separated by 10% SDS-PAGE and Western blotted with anti-VCP (1/2000), anti-Akt, and anti-phospho-(Ser/Thr) Akt substrate antibodies in experimental conditions described in Ref. 3.

**Confocal Microscopy**—After Akt activation using FGF-2, cells were successively rinsed with PBS, pH 7.5, fixed 20 min with paraformaldehyde 4%, permeabilized for 20 min at room temperature with PBS, pH 7.5; 0.05% saponine; 50 mM ammonium chloride, and blocked for 30 min with PBS, pH 7.5; 0.05% saponine; 2% bovine serum albumine. Then, cells were incubated for 2 h at room temperature in a blocking solution containing 1/50 rabbit phospho-Akt antibody (Cell Signaling) and 1/50 mouse VCP antibody (Abcam) rinsed with PBS, pH 7.5, and incubated for 1 h at 37  $^{\circ}\text{C}$  in a blocking solution containing 1/4000 Alexa Fluor 546 goat anti-rabbit IgG and 1/3000 Alexa Fluor 488 goat anti-mouse IgG. Cells were rinsed with PBS and mounted under Mowiol.

**VCP RNA Interference**—VCP siRNA was designed and validated as described by Wójcik *et al.* (6). Corresponding sequences are forward, 5'-TAAGACGCTGCTGGCCAA-3', and reverse, 5'-CATCGCGGAGTTCTGATTTT-3'. The siRNAs were synthesized using the Silencer<sup>®</sup> siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions, and transfection was performed as described previously (7).

**Site-directed Mutagenesis of VCP**—CDNA of VCP was obtained by PCR on MCF-7 cells from the cDNA data base using the following primers that incorporated the KpnI site and NotI site at the 5' and 3' ends, respectively, of the cDNA of VCP: Vcp-dir+KpnI, 5'-ATCGGTACCGCTCGTAGCCGTTACC-3', Vcp-rev+NotI, 5'-ATGCGGCCGCTGAAGTGTTCAGACTGGAG-3'. The PCR product was inserted in the topoA

TABLE 1

## Identification of VCP, from MALDI-TOF obtained peptide masses

The amino acid sequence from the Swiss-Prot database of each matching peptide, the  $\Delta$  mass between measured and matched masses, and the number of cleavages missed by trypsin are reported. The identification of VCP was confirmed by the full MS/MS sequencing of the underlined peptide.

| MH+ submitted  | MH+ matched    | $\Delta$ mass | Mass     | Peptide sequence consistent with mass   |
|----------------|----------------|---------------|----------|---|
|                |                | <i>Da</i>     |          |   |
| 783.42         | 783.36         | 0.06          | 0        | 506FGMTPSK512 Oxidation (M)             |
| 797.40         | 797.40         | 0.00          | 1        | 360FGRFDR365                            |
| 819.47         | 819.46         | 0.01          | 0        | 149GDIFLVR155                           |
| 837.52         | 837.52         | -0.01         | 0        | 232AIGVKPPR239                          |
| 947.58         | 947.56         | 0.02          | 1        | 148KGDIFLVR155                          |
| 951.50         | 951.47         | 0.03          | 1        | 288KAFFEEAEK295                         |
| 986.54         | 986.53         | 0.02          | 0        | 218EMVELPLR225                          |
| 1033.54        | 1033.52        | 0.02          | 1        | 746SVSDNDIRK754                         |
| 1034.46        | 1034.43        | 0.03          | 0        | 734DHFEEMR741                           |
| 1049.55        | 1049.54        | 0.01          | 0        | 669DVDLEFLAK677                         |
| 1050.48        | 1050.42        | 0.06          | 0        | 734DHFEEMR741 Oxidation (M)             |
| 1051.53        | 1051.52        | 0.01          | 0        | 46MDELQLFR53                            |
| 1067.53        | 1067.51        | 0.01          | 0        | 46MDELQLFR53 Oxidation (M)              |
| 1075.55        | 1075.53        | 0.02          | 0        | 278LAGESESNLR287                        |
| 1118.60        | 1118.57        | 0.02          | 1        | 701ESIESEIRR709                         |
| 1172.68        | 1172.66        | 0.02          | 0        | 240GILLYGPPGTGK251                      |
| 1190.58        | 1190.53        | 0.05          | 1        | 733RDHFEEAMR741                         |
| 1203.65        | 1203.63        | 0.02          | 1        | 278LAGESESNLRK288                       |
| 1206.59        | 1206.52        | 0.06          | 1        | 733RDHFEEAMR741 Oxidation (M)           |
| <u>1329.71</u> | <u>1329.68</u> | <u>0.03</u>   | <u>0</u> | <u>454WALSQSNPSALR465</u>               |
| 1629.84        | 1629.80        | 0.04          | 1        | 754KYEMFAQTLQQR766                      |
| 1645.82        | 1645.79        | 0.03          | 1        | 754KYEMFAQTLQQR766 Oxidation (M)        |
| 1660.83        | 1660.82        | 0.01          | 1        | 363FDREVDIGIPDATGR377                   |
| 1667.85        | 1667.94        | -0.09         | 1        | 212QLAQIKEMVELPLR225                    |
| 1695.87        | 1695.82        | 0.04          | 0        | 600VINQILTEMMDGMSTK614 Oxidation (M)    |
| 1711.87        | 1711.82        | 0.05          | 0        | 600VINQILTEMMDGMSTK614 2 Oxidation (M)  |
| 1823.95        | 1823.92        | 0.03          | 1        | 600VINQILTEMMDGMSTKK615 Oxidation (M)   |
| 1839.93        | 1839.91        | 0.02          | 1        | 600VINQILTEMMDGMSTKK615 2 Oxidation (M) |
| 2156.00        | 2155.97        | 0.03          | 0        | 714QTNPSAMEVEEDDPVPEIR732               |
| 2312.10        | 2312.07        | 0.02          | 1        | 714QTNPSAMEVEEDDPVPEIR733               |
| 2328.08        | 2328.07        | 0.01          | 1        | 714QTNPSAMEVEEDDPVPEIR733 Oxidation (M) |

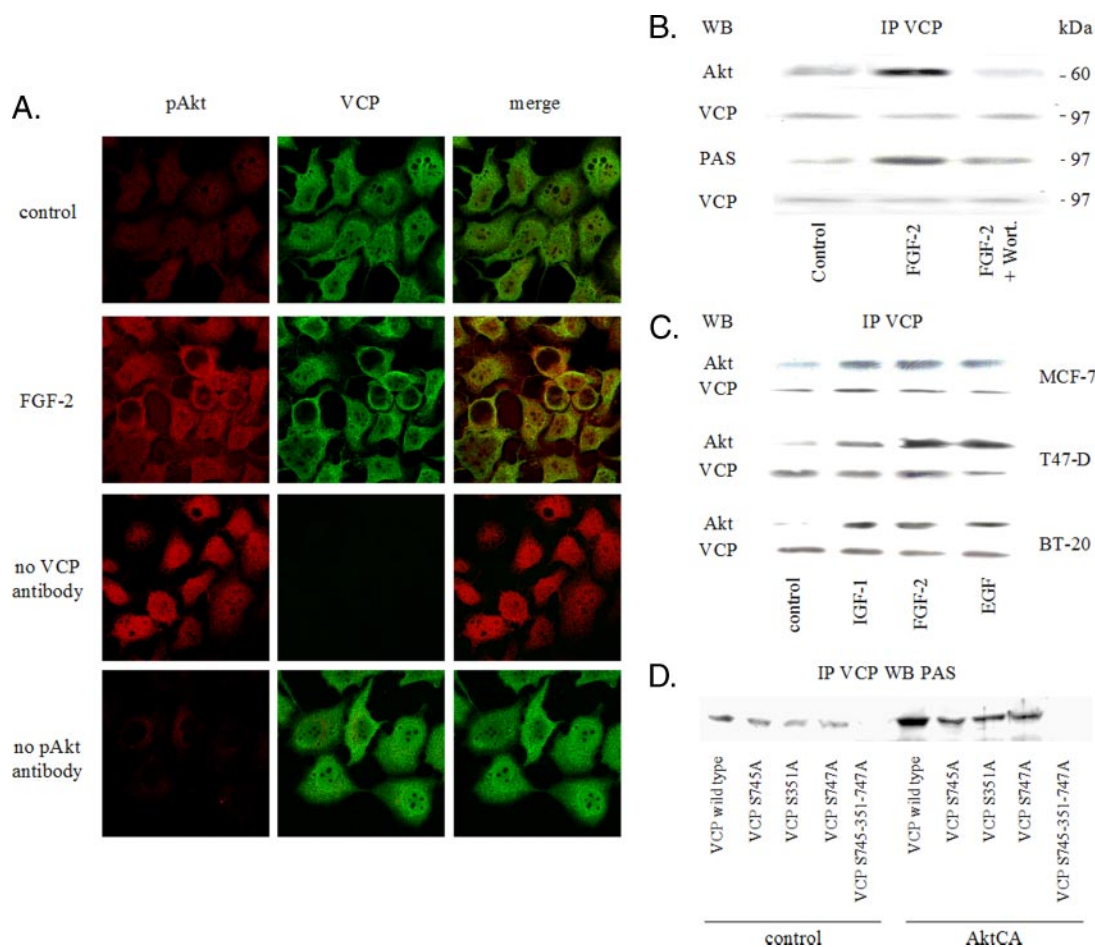
vector and amplified in *Escherichia coli* bacteria. Then, double digestion by KpnI and NotI was used to insert VCP cDNA in CMVTNT vector that allowed expression in mammalian cells. VCP serine 351 mutation to Alanine S351A, S745A, S747A and a triple mutant were carried out using PCR with mutant primers. The primers used to generate the mutants were as follows: S351A, 5'-GACCCAACGCCATTGACCC-3'; S745A, 5'-GCCGACGTGCTGTCAGCG-3'; S747A, 5'-CGACGTTCTGTCGACGATAATGAC-3'; and S745AS747A, 5'CGACGTGCTGTCGCCGATAATG-3'. Cloning and mutation were confirmed by DNA sequencing. Expression of pCMVTNT-VCP, pCMVTNT-VCPS351A, pCMVTNT-VCPS745A, pCMVTNT-VCPS747A, and pCMVTNT-VCPS351AS745AS747A plasmids was carried out by transfection in MCF-7 cells.

## RESULTS

**VCP Is Co-immunoprecipitated with Activated Akt**—The protocol used for identification of VCP in Akt co-immunoprecipitates is summarized in Fig. 1A. Immunoprecipitates obtained with Akt antibodies were resolved on SDS-polyacrylamide gel. 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. Silver staining or Coomassie Blue staining of the gel revealed the appearance of a 97-kDa band in the Akt-stimulated condition (Fig. 1B). The equal loading was assessed by detection of Akt (60 kDa) and IgG (70 kDa). The 97-kDa band was cut out and trypsin-digested before mass spectrometry analysis. Mass spectra from MALDI-TOF (Fig. 1C) and MS-MS (Fig. 1D) allowed the establishment of a peptide mass fingerprint and amino acid sequence, respectively, thereby leading to the identification of VCP. From the peptide fingerprint, a total of 31 experimental masses matched theoretical masses from VCP, leading to a protein sequence covering of 23%, and this identification was reinforced by the sequencing of 12 consecutive amino acids (Table 1).

**The Interaction between Akt and VCP Is Dependent on Akt Activation and Leads to VCP Phosphorylation**—The interaction between Akt and VCP was further confirmed by confocal microscopy showing a cellular co-localization of VCP and activated Akt (pAkt) upon growth factor stimulation (Fig. 2A). Importantly, reverse co-immunoprecipitation with anti-VCP antibody, followed by anti-Akt Western blotting, led to the same result (Fig. 2B), hence confirming the interaction between Akt and VCP upon fibroblast growth factor-2 stimulation. The interaction was further confirmed in different breast cancer cell types and under stimulation by insulin-like growth factor 1 and epidermal growth factor (Fig. 2C). Moreover, the interaction between VCP and Akt was abolished by treatment of the cells with the PI3K/Akt pharmacological inhibitor wortmannin (Fig. 2B), thus indicating the requirement of Akt activation for interaction with VCP. Using anti-phospho-Akt substrate (PAS) antibody, which recognizes phosphorylated serine or threonine in a consensus site for Akt, we have been able to show that Akt activation resulted in VCP phosphorylation (Fig. 2B). This Western blotting experiment showed that VCP contained a serine or a threonine phosphorylated, upon growth factor stimulation, on a site corresponding to Akt phosphorylation consensus. Interestingly, treatment by wortmannin resulted in a decrease in VCP phosphorylation, confirming that VCP is a target of Akt signaling.

**Identification of Akt Phosphorylation Sites on VCP**—In our study, we demonstrated that FGF-2 stimulates both the interaction between Akt and VCP and the phosphorylation of VCP on a site recognized by the PAS antibody. The Akt-phosphorylated consensus site is commonly defined as RXXRXX(S/T) (8, 9). We performed a bioinformatics search to identify putative Akt phosphorylation sites on VCP using the Scansite software. The putative Akt phosphorylation sites on VCP are AATNRPNS351, AMRFARRS745, and RFARRSVS747. To test the involvement of Ser-351, Ser-745, and Ser-747, we performed



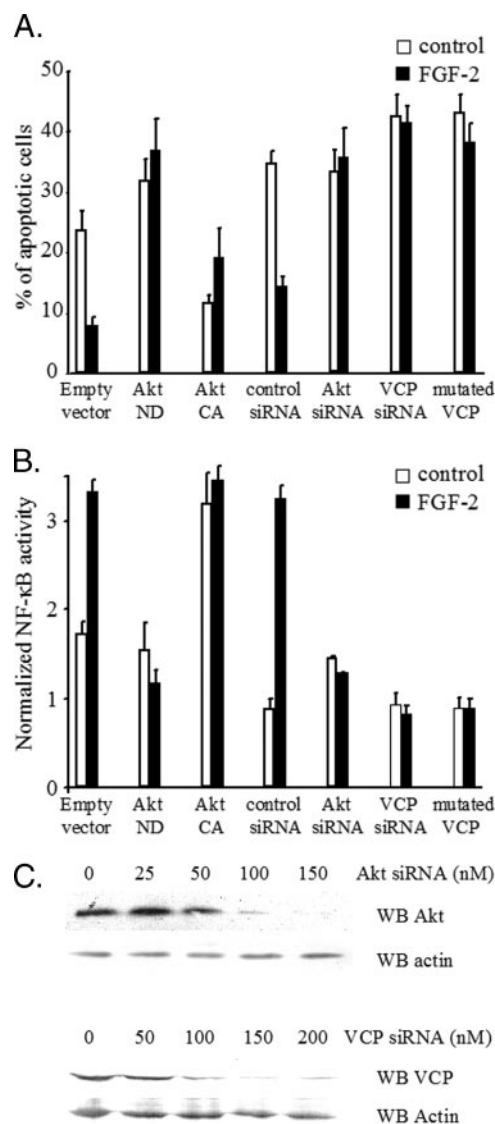
**FIGURE 2. Interaction between Akt and VCP is dependent on Akt activation.** *A*, confocal microscopy revealed the co-localization of VCP and Akt upon FGF-2-induced stimulation of Akt. Activated Akt appears in red, and VCP appears in green. *B*, reverse co-immunoprecipitation (IP) with anti-VCP was separated by SDS-PAGE before Western blotting using anti-Akt. A band of 60 kDa, corresponding to Akt, was found in Akt-activated conditions, confirming the interaction between Akt and VCP. The 97-kDa band corresponding to VCP was found in Akt-activated conditions, confirming that interaction between Akt and VCP correlated with phosphorylation of VCP on an Akt consensus site. The PI3K/Akt pharmacological inhibitor wortmannin inhibited this phosphorylation. *C*, the same co-immunoprecipitation experiment was reproduced with stimulation of Akt by three different growth factors: FGF-2, insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF). *D*, MCF-7 cells were transfected with pCMVTNT plasmids encoding VCP wild type, VCP S351A, VCP S745A, or VCP S351AS745AS747A and cotransfected with empty vector or vector encoding constitutively active Akt (AktCA). Immunoprecipitated VCP was then resolved by SDS-PAGE before Western blotting using the PAS antibody.

site-directed mutagenesis and generated pCMVTNT-VCP<sup>S351A</sup>, pCMVTNT-VCP<sup>S745A</sup>, and pCMVTNT-VCP<sup>S747A</sup> and pCMVTNT-VCP<sup>S351AS745AS747A</sup>. These VCP wild-type and mutant cDNAs were transfected in MCF-7 cells in parallel with Akt constitutively activated (AktCA), and after immunoprecipitation, phosphorylation on Akt consensus site was detected with the PAS antibody. The results (Fig. 2D) show that wild-type VCP was phosphorylated on PAS consensus to a greater extent as compared with S351A, S745A, and S748A mutants. The triple mutant S351A,S745A,S747A was phosphorylated to an even lesser extent as no phosphorylation was detected. These results indicate that Ser-351, Ser-745, and Ser-747 are Akt phosphorylation sites on VCP.

**VCP Is Functionally Involved in Akt Antiapoptotic Signaling and Is Required for Cell Survival**—The functional involvement of VCP in Akt signaling was shown by the treatment of MCF-7 cells with siRNA directed against Akt or VCP. Treatment by 100 nM siRNA directed against Akt or VCP resulted in a strong reduction of the pro-survival effect of FGF-2 for breast cancer cells (Fig. 3A). Similarly, transfection with the triple VCP mutant S351A,S745A,S747A resulted in a reduction of FGF-2 anti-apoptotic activity, confirming the functional involvement of the detected phosphorylation site.

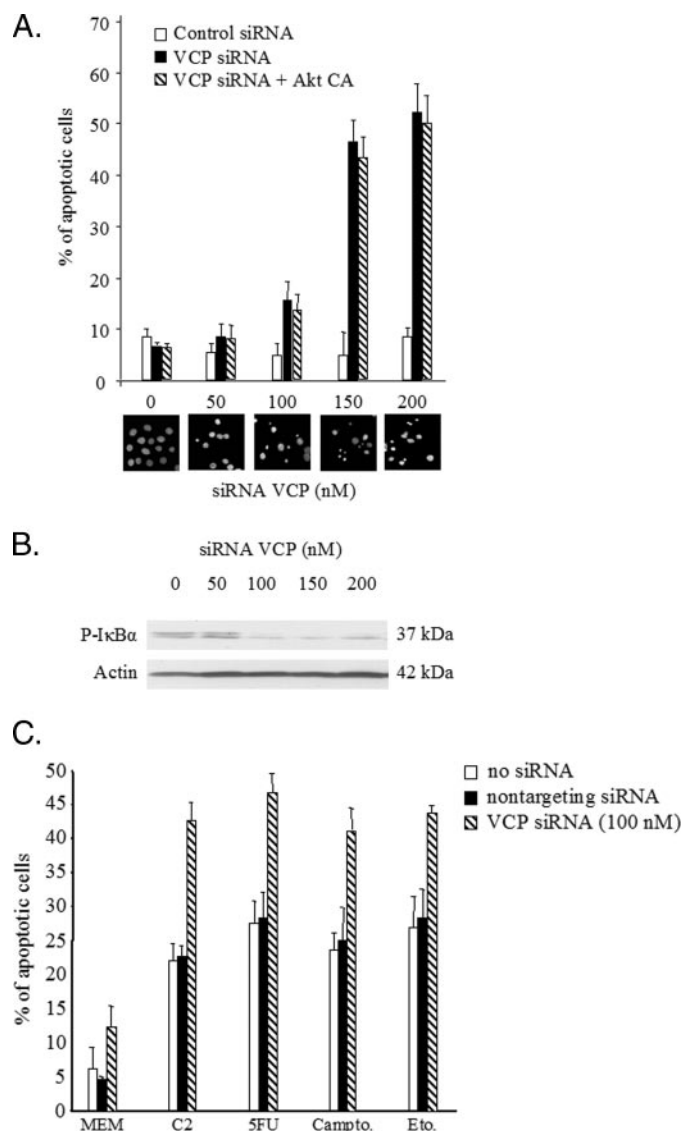
Negative dominant Akt also resulted in the inhibition of FGF-2 survival effect, in contrast with constitutively activated Akt. It has been previously demonstrated that the Akt-mediated anti-apoptotic signaling in breast cancer cells requires the activation of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) (7). We have evaluated the impact of VCP on NF- $\kappa$ B activation induced by Akt. Treatment with 100 nM siRNA directed against Akt or VCP, as well as transfection with dominant negative Akt or VCP mutant S351A,S745A,S747A, resulted in a strong inhibition of NF- $\kappa$ B activation by FGF-2 (Fig. 3B). The efficiency of cell treatment by siRNA directed against Akt and VCP was demonstrated by Western blotting showing the disappearance of Akt and VCP with increasing concentrations of siRNA (Fig. 3C). Together, these data show that VCP is involved in the growth factor-stimulated antiapoptotic signaling mediated by Akt.

Interestingly, high concentrations of siRNA against VCP were shown to induce a potent apoptosis in breast cancer cells that was not rescued by overexpression of a dominant active Akt mutant (Fig. 4A). Cell treatment with increasing concentrations of siRNA against VCP resulted in increase of breast cancer cell death, and it is worth noting that for identical concentrations, no apoptosis was induced by the control siRNA. For example, 50% of apoptosis was observed



**FIGURE 3. VCP is functionally involved in Akt-mediated antiapoptotic signaling.** *A*, quantification of apoptosis with Hoechst staining revealed that siRNA against VCP or Akt (100 nM) inhibited the antiapoptotic effect of FGF-2. *ND*, negative dominant; *CA*, constitutively active. *B*, transfection of MCF-7 cells with siRNA directed against Akt or VCP or transfection with plasmid encoding a negative dominant Akt resulted in a strong reduction of NF- $\kappa$ B activation by FGF-2. In contrast, transfection with plasmid encoding a constitutively active form of Akt mimicked the stimulation of NF- $\kappa$ B induced by FGF-2. *C*, Western blotting (WB) detection of Akt and VCP levels in cells treated by increasing concentrations of siRNA. The efficiency of siRNA was tested 48 h after cell transfection.

for 150 nM siRNA against VCP after a 48-h treatment, whereas no induction of cell death was detected with control siRNA. In conclusion, VCP depletion dramatically induced, by itself, a massive apoptosis in breast cancer cells, demonstrating the crucial role played by this protein in cell survival. Interestingly, siRNA directed against VCP were found to induce a decrease in the level of phospho-I $\kappa$ B $\alpha$  (Fig. 4B). This is consistent with the decrease of NF- $\kappa$ B activation that was also observed when cells were treated with siRNA against VCP (Fig. 3B). Moreover, we have shown that depletion in VCP resulted in a potentiation of the proapoptotic effect of various chemotherapeutic drugs (Fig. 4C). The proapoptotic effect of 5-fluorouracil, camptothecin, and etoposide obtained with MCF-7 cells was potentiated by siRNA directed against VCP (at the 100 nM dose that has no apoptotic effect by itself). This demonstrates the significance



**FIGURE 4. VCP is required for breast cancer cells survival.** *A*, dose effect of siRNA against VCP on breast cancer cell apoptosis. MCF-7 cells were transfected with increasing amounts (0, 50, 100, 150, 200 nM) of siRNA directed against VCP (or control siRNA) and cotransfected with plasmid encoding constitutively active Akt (AktCA). After Hoechst staining, apoptotic cells were counted, and results are expressed as the mean  $\pm$  S.D. of five independent experiments. *B*, Western blotting detection of phospho-I $\kappa$ B $\alpha$  (P-I $\kappa$ B $\alpha$ ) in MCF-7 cells treated with increasing concentrations (same as before) siRNA directed against VCP. *C*, MCF-7 cells were transfected with 100 nM siRNA against VCP or with nontargeting siRNA and were treated with minimum Eagle's medium (*MEM*), C2, 5-fluorouracil (*5FU*), camptothecin (*campto.*), or etoposide (*eto.*) before serum deprivation. The percentage of apoptotic cells was determined after Hoechst staining.

of VCP under conditions of apoptosis induced by anti-cancer treatment drugs.

## DISCUSSION

The serine/threonine kinase Akt is increasingly appearing as a general mediator of cell survival signaling, with high potentialities as a drug target in various diseases characterized by a deregulation of the balance between cell apoptosis and cell growth, such as cancer (10). Akt is mainly, but not exclusively, activated through the PI3K pathway that is stimulated by a wide range of tyrosine kinase growth factor receptors. Although Akt signaling remains incompletely known, it noticeably promotes cell survival by indirectly activating the pro-survival transcription factor NF- $\kappa$ B, through the stimulation of the inhibitor I $\kappa$ B kinase. Akt activation of I $\kappa$ B kinase results in the phosphorylation and subsequent

degradation of I $\kappa$ B through the ubiquitin-mediated proteasome proteolysis, allowing NF- $\kappa$ B to translocate into the nucleus, where it stimulates target gene expression (11). Due to its involvement in several pathologies such as cancer, the signaling initiated by Akt is the subject of increasing attention, and we indicate here the involvement of VCP in the growth factor-induced antiapoptotic signaling mediated by the Akt/NF- $\kappa$ B pathway. In contrast to classical approaches to studying signal transduction (based on the use of specific antibodies to identify signaling partners), proteomic approaches have 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 (12). Interestingly, VCP has previously been shown to associate directly with the  $\alpha$  form of I $\kappa$ B (I $\kappa$ B $\alpha$ ) and is co-purified with the mammalian 26 S proteasome involved in the ubiquitin-dependent proteasome degradation pathway of I $\kappa$ B $\alpha$  (13, 14). In breast cancer cells, growth factors such as FGF-2 and nerve growth factor have been shown to stimulate cell growth and survival through the activation of a PI3K/Akt/NF- $\kappa$ B-mediated pathway (7, 15, 16). In this context, our results indicate that the NF- $\kappa$ B activation and the subsequent effects on cell survival are functionally regulated through the recruitment and phosphorylation of VCP by the Akt pathway.

The Akt-phosphorylated consensus site is commonly defined as RXXR(S/T) (8, 9). In fact, the primary sequence of VCP does not contain the precise consensus sequence predicted for an Akt substrate; the arginine at position -5 is lacking. However, VCP 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 (KERCSQ1036) (17), cAMP-response element-binding protein LSRRPS133Y (18), and ATP-citrate lyase (TPAPS\_TASF) (19). The bioinformatic analysis performed with Scansite indicated three potential Akt phosphorylation sites in VCP, and we have used site-directed mutagenesis to confirm their involvement. We demonstrated that Ser-351, Ser-745, and Ser-747 on VCP are phosphorylated on a site recognized by the PAS antibody corresponding to the Akt consensus site. The phosphospecific Akt substrate antibody (PAS) that we have used here was raised against Akt RXXRXX(pS/pT) consensus sequence but does not appear to strictly require arginine in the -5 position for phosphoprotein recognition, as was obtained for ATP-citrate lyase (19). Interestingly, in rat hippocampal tissue, a functional integration between Akt and VCP that also leads to VCP phosphorylation on the same corresponding serine residues has very recently been shown (20). Therefore, these three serine residues appear to be generally crucial for VCP regulation by Akt.

VCP is known to interact with other adaptor proteins, which are proposed to function in various cellular pathways. The list of proteins known to interact with VCP includes clathrin (21), the p47 membrane fusion required for organelle biogenesis (22), the VCP p97/p47 complex-interacting protein (VCPPIP135) (23), the small VCP-interacting protein (24), and the breast/ovarian cancer susceptibility gene product (BRCA1) (25). BRCA1 is of particular interest in the context of breast cancer as this protein is one of the most prominent players identified in breast tumorigenesis, with 50% of familial breast tumors exhibiting BRCA1 mutations (26). BRCA1 presumably acts as a DNA-unwinding factor in DNA damage repair, although VCP is predominantly found in the cytoplasm and less abundantly in the nucleus. However, VCP can be translocated to the nucleus, and *in vitro* studies have revealed that VCP, via its N-terminal region, binds to amino acid residues 303–625 in the BRCA1 protein (25). Interestingly, the cell growth regulator heregulin has been shown to induce phosphorylation of BRCA1 through PI3 kinase/Akt in breast

cancer cells (27), and therefore, our data suggest that VCP might participate in the interaction between Akt and BRCA1, leading to DNA damage repair function and an antiapoptotic effect under growth factor stimulation. However, our study also suggests that other known biological activities of VCP, such as those related to intracellular trafficking, ubiquitin-mediated proteolysis, and activation of transcription (28), might be regulated by Akt through the activation of VCP. In our study, a potent induction of apoptosis was observed in breast cancer cells treated with siRNA against VCP, thereby demonstrating the crucial role played by this protein in the survival of cancer cells. VCP has been described as an important regulator of protein ubiquitination, and interestingly, it was reported before that VCP is involved in the ubiquitin proteasome-mediated degradation of I $\kappa$ B (13). In our study, we demonstrated that siRNA directed against VCP induced a decrease in the level of phospho-I $\kappa$ B. As the phosphorylation of I $\kappa$ B is the germane event leading to the proteolytic proteasome-mediated degradation of I $\kappa$ B responsible for the release and nuclear translocation of NF- $\kappa$ B, the decrease in phospho-I $\kappa$ B that we observe with siRNA directed against VCP is consistent with the concomitant inhibition of NF- $\kappa$ B and provides a biochemical basis for the cell survival activity of VCP. In addition, the potential of VCP as a therapeutic target is particularly well illustrated by the potentiation of the proapoptotic effect of chemotherapeutic drugs that we have obtained with anti-VCP siRNA. Hence, VCP targeting appears as a rational approach to sensitize breast cancer cells to chemotherapy.

In conclusion, we identified VCP as an essential new target in the Akt signaling pathway that is necessary to its related antiapoptotic effect, and therefore, this protein appears as a new player to be considered in cancer cell survival, with a correlative potential as a target for cancer therapy.

### REFERENCES

- Dougan, D. A., Mogk, A., Zeth, K., Turgay, K., and Bukau, B. (2002) *FEBS Lett.* **529**, 6–10
- Lupas, A. N., and Martin, J. (2002) *Curr. Opin. Struct. Biol.* **12**, 746–753
- El Yazidi-Belkoura, I., Adriaenssens, E., Dolle, L., Descamps, S., and Hondermarck, H. (2003) *J. Biol. Chem.* **278**, 16952–16956
- Cuvillier, O., Nava, V. E., Murthy, S. K., Edsall, L. C., Levade, T., Milstien, S., and Spiegel, S. (2001) *Cell Death Differ.* **8**, 162–171
- Vercoutter-Edouard, A. S., Lemoine, J., Le Bourhis, X., Louis, H., Boilly, B., Nurcombe, V., Revillon, F., Peyrat, J. P., and Hondermarck, H. (2001) *Cancer Res.* **61**, 76–80
- Wojcik, C., Yano, M., and DeMartino, G. N. (2004) *J. Cell Sci.* **117**, 281–292
- Vandermoere, F., El Yazidi-Belkoura, I., Adriaenssens, E., Lemoine, J., and Hondermarck, H. (2005) *Oncogene* **24**, 5482–5491
- Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) *FEBS Lett.* **399**, 333–338
- Obata, T., Yaffe, M. B., Lepar, G. G., Piro, E. T., Maegawa, H., Kashiwagi, A., Kikkawa, R., and Cantley, L. C. (2000) *J. Biol. Chem.* **275**, 36108–36115
- Luo, J., Manning, B. D., and Cantley, L. C. (2003) *Cancer Cell* **4**, 257–262
- Karin, M., Yamamoto, Y., and Wang, Q. M. (2004) *Nat. Rev. Drug. Discov.* **3**, 17–26
- Hondermarck, H. (2003) *Mol. Cell Proteomics* **2**, 281–291
- Dai, R. M., Chen, E., Longo, D. L., Gorbea, C. M., and Li, C. C. (1998) *J. Biol. Chem.* **273**, 3562–3573
- Dai, R. M., and Li, C. C. (2001) *Nat. Cell Biol.* **3**, 740–744
- Descamps, S., Toillon, R. A., Adriaenssens, E., Pawlowski, V., Cool, S. M., Nurcombe, V., Le Bourhis, X., Boilly, B., Peyrat, J. P., and Hondermarck, H. (2001) *J. Biol. Chem.* **276**, 17864–17870
- Welm, B. E., Freeman, K. W., Chen, M., Contreras, A., Spencer, D. M., and Rosen, J. M. (2002) *J. Cell Biol.* **157**, 703–714
- Villafuerte, B. C., Phillips, L. S., Rane, M. J., and Zhao, W. (2004) *J. Biol. Chem.* **279**, 36650–36659
- Du, K., and Montminy, M. (1998) *J. Biol. Chem.* **273**, 32377–32379
- Berwick, D. C., Hers, I., Heesom, K. J., Moule, S. K., and Tavare, J. M. (2002) *J. Biol. Chem.* **277**, 33895–33900
- Klein, J. B., Barati, M. T., Wu, R., Gozal, D., Sachleben, L. R., Jr., Kausar, H., Trent, J. O., Gozal, E., and Rane, M. J. (2005) *J. Biol. Chem.* **280**, 31870–31881

21. Pleasure, I. T., Black, M. M., and Keen, J. H. (1993) *Nature* **365**, 459–462
22. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997) *Nature* **388**, 75–78
23. Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P., and Kondo, H. (2002) *J. Cell Biol.* **159**, 855–866
24. Nagahama, M., Suzuki, M., Hamada, Y., Hatsuzawa, K., Tani, K., Yamamoto, A., and Tagaya, M. (2003) *Mol. Biol. Cell* **14**, 262–273
25. Zhang, H., Wang, Q., Kajino, K., and Greene, M. I. (2000) *DNA Cell Biol.* **19**, 253–263
26. Venkitaraman, A. R. (2002) *Cell* **108**, 171–182
27. Altiok, S., Batt, D., Altiok, N., Papautsky, A., Downward, J., Roberts, T. M., and Avraham, H. (1999) *J. Biol. Chem.* **274**, 32274–32278
28. Wang, Q., Song, C., and Li, C. C. (2004) *J. Struct. Biol.* **146**, 44–57