

## Cholesterol Sensitivity of Endogenous and Myristoylated Akt

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### Abstract

The serine-threonine kinase, Akt, has been linked to cholesterol-sensitive signaling mechanisms, suggesting a possible means whereby cholesterol might affect tumor cell growth and survival. However, it has not been shown whether Akt itself, as distinct from upstream components of the pathway (e.g., membrane phosphoinositides), can be directly responsible for cholesterol-mediated effects. Consistent with this possibility, we identified an Akt1 subpopulation in cholesterol-rich lipid raft fractions prepared from LNCaP human prostate cancer cells. Phosphorylation of this Akt subspecies was ablated with methyl- $\beta$ -cyclodextrin, a cholesterol-binding compound, under conditions where nonlipid raft-resident Akt was unaffected. A myristoylated Akt1 (MyrAkt1) fusion protein expressed in LNCaP cells was found to be highly enriched in lipid rafts, indicating that oncogenic Akt is over-represented in cholesterol-rich membranes compared with wild-type Akt. Notably, lipid raft-resident MyrAkt1 exhibited a markedly distinct substrate preference compared with MyrAkt1 immunoprecipitated from cytosol and nonraft membrane fractions, suggesting a redirection of signal transduction when the protein is present in cholesterol-rich membranes. Expression of MyrAkt1 in LNCaP cells overcame their characteristic dependence on constitutive signaling through the phosphoinositide 3'-kinase pathway. This protective effect was substantially diminished with cyclodextrin treatment. Phosphorylation of Akt substrates in lipid raft fractions, but not in cytosol/nonraft membrane fractions, was ablated with cyclodextrin. In addition, in control (LacZ transfected) cells, lipid raft fractions were relatively enriched in phosphorylated Akt substrates. Collectively, these data show that a subpopulation of Akt is cholesterol sensitive and that the oncogenic effects conferred by myristoylation arise, in part, from the tendency of the membrane-targeted form of the protein to reside in cholesterol-rich membrane microdomains. [Cancer Res 2007;67(13):6238–46]

### Introduction

Cholesterol is a critical component of biological membranes. In addition to regulating membrane fluidity, cholesterol is an

important constituent of a class of detergent-resistant microdomains, generally referred to as "lipid rafts" (1). The invaginated, vesicular structures known as "caveolae" are a specialized form of lipid raft that contain caveolin proteins. Noncaveolar, "flat" lipid rafts are also believed to exist, based on experimental and theoretical evidence (2). The structural and biophysical properties of lipid rafts result in the retention and exclusion of certain classes of proteins, such that these microdomains can be viewed as "privileged" sites that promote interaction between discrete subsets of signaling intermediates, thereby serving as platforms for signal transduction (reviewed in ref. 3). In cancer cells, lipid rafts/detergent-resistant microdomains may provide an important subcellular microenvironment in which signals are processed that are central to tumor cell growth, resistance to apoptotic signals, and other aggressive characteristics.

Although elevation in circulating cholesterol levels has long been associated with cardiovascular disease, there is now increasing evidence to suggest a link between cholesterol accumulation and the risk of certain malignancies. Several recent epidemiologic studies have described a reduction in incidence of certain cancers in patients taking 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase inhibitors ("statins") for cardiovascular indications (reviewed in ref. 4). Statins inhibit the rate-limiting step in cholesterol biosynthesis (conversion of HMG-CoA to mevalonate) and thereby reduce synthesis of cholesterol and its isoprenoid precursors, geranylgeranyl pyrophosphate and farnesyl pyrophosphate. The effect of statin therapy on incidence of solid tumors may vary with organ site. Current evidence supports the hypothesis that prostate cancer may be particularly sensitive to this intervention (5, 6). This may be a reflection of aspects of cholesterol metabolism characteristic of prostate cells and tissues, including high endogenous levels of cholesterol seen in the normal prostate, the abnormal accumulation of cholesterol in prostate tumors, and the sensitivity of prostate cancer cells to cholesterol depletion (7–9).

The Akt/protein kinase B family (Akt1, Akt2, and Akt3) of serine-threonine kinases processes signals in tumor cells that mediate tumor cell proliferation, survival, and migratory behavior (10–12). Akt has also been linked to pathways sensitive to changes in membrane cholesterol. Data from our group and others have shown that constitutive and epidermal growth factor-stimulated Akt activation and cell survival are regulated by cholesterol-sensitive signaling mechanisms in prostate cancer cells (9, 13). Elevation of circulating cholesterol levels in mice promoted growth, kinase activation, and survival signaling in human prostate tumor xenografts (14). In cell culture studies, simvastatin preferentially inhibited phosphorylation at a key regulatory site, Ser<sup>473</sup> on Akt1 present in lipid rafts, whereas Akt1 at other locations in the cell was relatively resistant to the effects of the drug (14). These findings suggest that prostate cancer and possibly other tumor cells contain discrete Akt populations that process distinct signals depending on subcellular location. These results also implicate

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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lipid raft microdomains as sites where the signaling effects of cholesterol may influence the regulatory dynamics of Akt, a critical node in cancer cell signaling.

Although evidence has been obtained implicating membrane cholesterol as a direct regulator of signal transduction of relevance to cancer, it has not been shown that Akt itself, as opposed to upstream effectors, such as growth factor receptors or membrane phospholipids, is a direct target for such cholesterol-mediated effects. For example, because statin drugs, in addition to their cholesterol-lowering ability, affect post-translational isoprenylation and activation of proteins, such as Rho, Ras, and Rac, (4), it is possible that inhibitory effects of simvastatin on raft-resident Akt, observed previously (14), are not primarily the result of cholesterol synthesis inhibition. In this study, we provide evidence that Akt itself is cholesterol sensitive as a result of the localization of an Akt subpopulation within lipid raft microdomains. Our results also indicate that the raft microenvironment processes distinct Akt-dependent signals.

## Materials and Methods

**Antibodies and reagents.** The antibodies used in this study include anti-Akt polyclonal antibody (pAb; this antibody recognizes endogenous Akt1, Akt2, and Akt3), anti-Akt1 monoclonal antibody (mAb; clone 5G3), anti-Akt1 mAb 2H10, immobilized Akt IG1 mAb, anti-phospho-Akt (Thr<sup>308</sup>) pAb, anti-phospho-Akt (Ser<sup>473</sup>) pAb, anti-phospho-glycogen synthase kinase-3 $\alpha$ / $\beta$  (p-GSK3 $\alpha$ / $\beta$ ) pAb, and anti-phospho-Akt substrate rabbit mAb (all from Cell Signaling Technology); anti-Akt1 pAb (Upstate Biotechnology, Inc.); anti-Akt1 pAb, anti- $\beta$ -tubulin mAb (clone D10), and anti-G<sub>1 $\alpha$ 2</sub> subunit pAb (Santa Cruz Biotechnology); and anti-HA tag (clone HA.11; Covance, Inc.). GSK3 fusion protein was from Cell Signaling Technology. Protein A-Sepharose and protein G-Sepharose were obtained from Amersham Biosciences. Opti-MEM reduced serum medium was from Invitrogen. Fugene 6 was from Roche Applied Science. All other chemicals were obtained from Sigma Chemical Co. Akt expression vectors encoding wild-type (WT) Akt1 tagged with the T7 epitope or myristoylated Akt1 (MyrAkt1) were generous gifts from Dr. William Sellers (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA) and Dr. Thomas Franke (Columbia University, New York, NY).

**Cell culture and transfections.** LNCaP human prostate cancer cells were cultured in RPMI 1640/10% fetal bovine serum (FBS). Human embryonic kidney (HEK) 293 cells were cultured in DMEM/10% FBS. All media were supplemented with penicillin/streptomycin and L-glutamine, and cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells in 150-mm dishes at ~80% confluence were transfected using Fugene 6 according to the manufacturer's instructions. In selected experiments, LNCaP cells were transduced with viral supernatants of 293FT cells transfected with pLenti6-MyrAkt1 or pLenti6-LacZ, and stable populations were isolated following selection with 2  $\mu$ g/mL blasticidin.

**Preparation of membrane fractions.** Lipid raft membrane fractions were isolated using two methods. In the first method, lipid rafts were isolated from LNCaP cells using sucrose gradient ultracentrifugation as described (15). In the second method, a procedure involving successive detergent extraction of cell membranes was used essentially as described (13, 14, 16, 17). In some experiments, the cytosolic fraction was isolated before membrane fractionation. Briefly, cell pellets were resuspended in 50 mmol/L HEPES (pH 7.4), 10 mmol/L NaCl, 1 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L Na<sub>3</sub>VO<sub>4</sub> and subjected to mechanical disruption with 12 strokes of a Dounce homogenizer (1,800 rpm). Homogenized samples were centrifuged at 14,000  $\times$  g for 20 min at 4°C and the supernatant was removed as the cytosolic fraction. Membrane pellets were washed with buffer A and lysed as described above to extract Triton-soluble and raft membrane fractions. The protein content of fractions was determined using the Micro-bicinchoninic acid (BCA) assay (Pierce Chemical Co.).

**Cholesterol assay.** Cholesterol determinations were done on 300  $\mu$ L fractions from either membrane preparations or sucrose gradients prepared as described (15). Lipids were solubilized in chloroform, extracted twice through H<sub>2</sub>O, dried, and analyzed with the Infinity cholesterol determination assay kit (Sigma Chemical).

**Immunoprecipitations and Akt kinase assay.** Equal amounts of protein from cytosol and nonraft membrane (C+M fraction) or lipid raft fractions were precleared with protein A-Sepharose or protein G-Sepharose beads for 1 h at 4°C. In selected experiments, membrane fractions were subjected to buffer exchange using BioSpin6 gel filtration columns (Bio-Rad). Antibodies were added to precleared lysates and incubated overnight at 4°C, before addition of 40  $\mu$ L protein A or G beads (50% v/v slurry) for a further 2 h at 4°C. Immunoprecipitates were washed four times with lysis buffer [20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L NaPPI, 1 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/mL leupeptin, 1 mmol/L PMSF] and resuspended in 2 $\times$  SDS loading buffer. To assay Akt kinase activity, a nonradioactive assay kit was used (Cell Signaling Technology). Briefly, immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer [25 mmol/L Tris-Cl (pH 7.5), 5 mmol/L  $\beta$ -glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/L MgCl<sub>2</sub>] to equilibrate the beads before assay. Beads were resuspended in kinase reaction mix, comprising 40  $\mu$ L kinase assay buffer, 1  $\mu$ g GSK3 fusion protein substrate, and 200  $\mu$ mol/L ATP, and incubated for 30 min at 30°C. Reactions were terminated by the addition of 20  $\mu$ L 3 $\times$  SDS loading buffer supplemented with 150 mmol/L DTT.

For determination of Akt kinase activity against myelin basic protein (MBP) or the histone subunit H2B, Akt immune complexes were incubated with 1  $\mu$ g substrate and 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]dATP (3,000 Ci/mmol). Samples were resolved by gel electrophoresis, and gels were fixed with acetic acid and destained before exposure of dried gels to X-ray film to visualize signal. To quantitate incorporation of <sup>32</sup>P, gel slices were excised and radioactivity was determined by scintillation counting. Kinase activity was also assessed using Crosstide as substrate. Briefly, immune complexes were resuspended in kinase buffer [25 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L DTT] containing 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]dATP and 1  $\mu$ g Crosstide (GRPRSSFAEG) in a volume of 30  $\mu$ L and incubated at 30°C for 20 min. To terminate the reactions, 25  $\mu$ L aliquots were spotted onto p81 phosphocellulose paper and the incorporated radioactivity was determined essentially as described (18).

**Preparation of whole-cell lysates and immunoblot analysis.** Cells were washed twice in ice-cold PBS and lysed in a minimum volume of 1 $\times$  cell lysis buffer (Cell Signaling Technology) supplemented with 60 mmol/L octylglucoside and 1 mmol/L PMSF. Protein content was determined using the Micro-BCA protein assay reagent. Cell extracts (10  $\mu$ g/lane) and immunoprecipitates were resolved by 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. Following transfer, membranes were stained with Ponceau S to confirm equal protein loading, where appropriate. Membranes were blocked with PBS/0.1% Tween 20/5% IgG-free bovine serum albumin (BSA) and incubated with antibodies overnight at 4°C. Following incubation with species-specific horseradish peroxidase-conjugated secondary antibodies, signals were detected using SuperSignal chemiluminescent reagent (Pierce Chemical) and exposure of blots to X-ray film. In selected experiments, densitometric analysis of bands was done using the public domain NIH Image (version 1.63) program. Relative Akt kinase activity was determined by dividing the signal for p-GSK3 $\alpha$ / $\beta$  by the signal for total Akt within the same fraction. A value of 100% was assigned to the normalized kinase activity (p-GSK3 signal divided by Akt signal) in the cytosolic/Triton-soluble membrane (C+M) fraction following treatment (e.g., pervanadate) and all other values were expressed relative to this.

**Immunofluorescence microscopy.** To image Akt, LNCaP cells were seeded in chamber slides or coverslips and treated as indicated in legends. Cells were washed once with ice-cold PBS and incubated on ice with tetramethylrhodamine-conjugated cholera toxin B (CTxB) subunit (List Biological Laboratories) or Alexa 594-conjugated CTxB diluted in medium. Cells were fixed in ice-cold methanol or 4% paraformaldehyde and nonspecific binding sites blocked in PBS/0.1% Tween 20/3% BSA (for endogenous Akt) or PBS/0.1% BSA (ectopically expressed Akt1) for 1 h at

room temperature. Cells were incubated with Ser<sup>473</sup>-phosphorylated Akt (S473-P Akt) primary antibody diluted 1:100 in PBS/0.1% Tween 20/3% BSA overnight at 4 °C or PBS/0.1% BSA for 1 h at room temperature followed by FITC-conjugated secondary antibody (1:200) for 1 h at room temperature. Slides were mounted in Vectashield medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.) and analyzed using an LSM 510 META NLO laser scanning confocal microscope or an Axioplan 2 microscope (Carl Zeiss Microimaging, Inc.).

## Results

Targeting membrane cholesterol with cholesterol-binding compounds, or by inhibiting cholesterol synthesis endogenously, was shown to attenuate phosphorylation of Akt1 at Ser<sup>473</sup> in androgen-sensitive LNCaP prostate cancer cells (13, 14). The following experiments were conducted to determine whether cholesterol regulates Akt1 directly. We initially assessed the extent to which Akt1 is present in cholesterol-rich (lipid raft) membrane fractions compared with nonraft subcellular compartments. We used two complementary methods to fractionate cells into lipid raft-enriched and nonraft components. First, LNCaP cells homogenized by mechanical disruption in Triton X-100-containing buffer were subjected to sucrose density gradient ultracentrifugation. In the second method, cells were fractionated by successive detergent extraction as described previously (13, 14, 16, 17) to isolate Triton-soluble cytosolic and nonraft membrane components (C+M) and Triton-insoluble, octylglucoside-soluble raft fractions. Equal amounts of gradient, C+M, or raft fractions were resolved by SDS-PAGE and blotted with antibodies to Akt. The fidelity of fractionation was confirmed by blotting of fractions with antibodies to  $\beta$ -tubulin or G $\alpha$ <sub>2</sub> as markers of nonraft membranes/cytosol and lipid rafts, respectively.

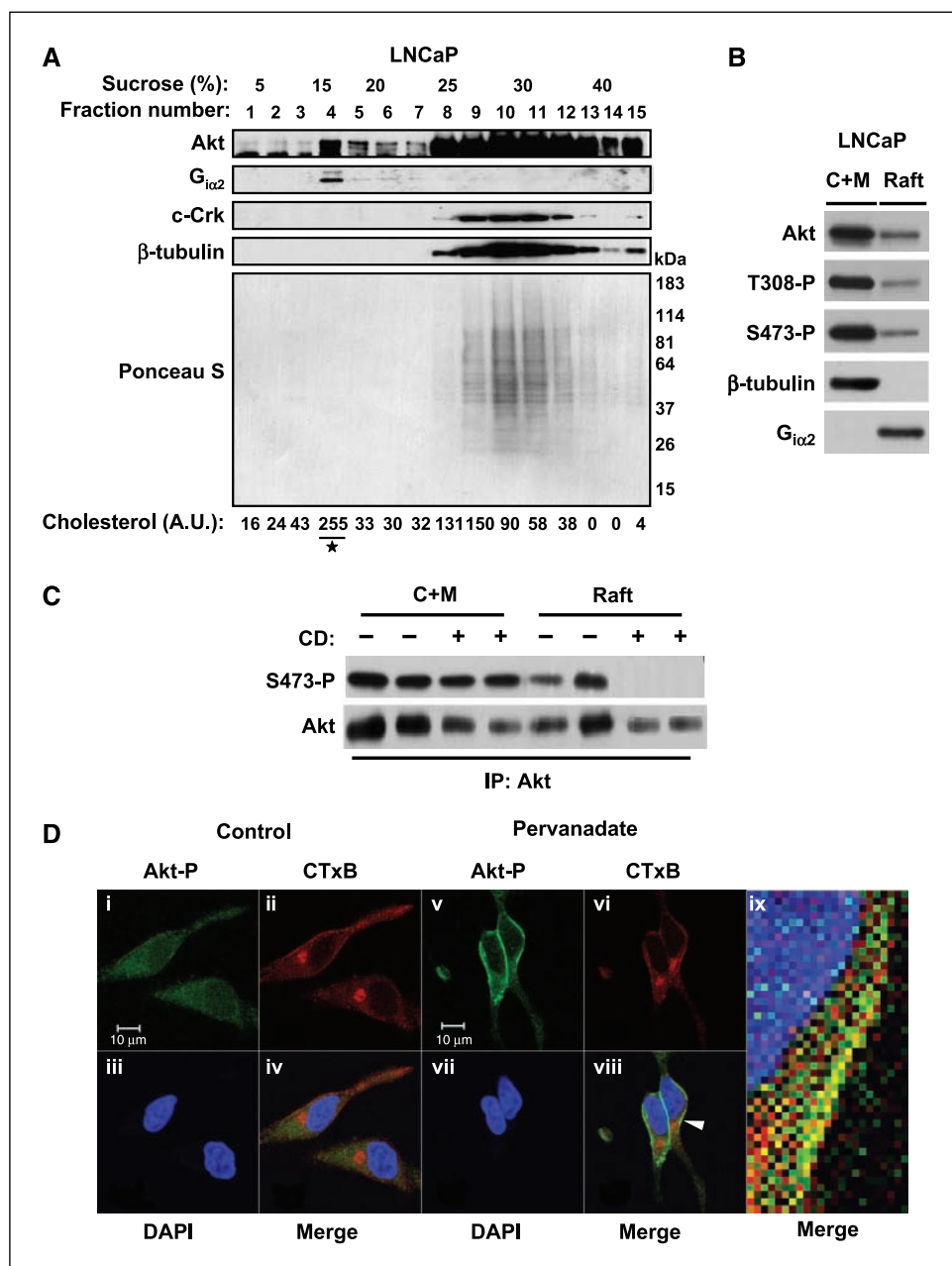
As shown in Fig. 1, the patterns of Akt distribution in cells analyzed by density gradient (Fig. 1A) or by differential solubility in nonionic detergent (Fig. 1B) were similar. The majority of Akt was present in higher density fractions in LNCaP cells (Fig. 1A) that correspond to the cytosol + nonraft membrane (C+M) fraction (Fig. 1B), with only a small proportion of total Akt present in rafts (Fig. 1A, fraction 4; Fig. 1B). Consistent with the PTEN-null status of LNCaP cells, Akt in both C+M and raft fractions was phosphorylated on Thr<sup>308</sup> and Ser<sup>473</sup>, with the extent of phosphorylation commensurate with the level of Akt. To confirm the cholesterol sensitivity of lipid raft-resident Akt, LNCaP cells were treated with the cholesterol-binding agent, methyl- $\beta$ -cyclodextrin, and fractionated into C+M and raft components. Akt was immunoprecipitated from each fraction and blotted with antibodies to total and S473-P Akt. Cyclodextrin treatment did not appreciably alter the amount or extent of phosphorylation of Akt isolated from the C+M fraction. In contrast, cyclodextrin ablated phosphorylation of raft-resident Akt (Fig. 1C). We also assessed the subcellular localization of Akt by immunofluorescence imaging of LNCaP cells treated with pervanadate (0.5 mmol/L), the most potent known Akt activator (19, 20). Under basal conditions, S473-P Akt was located predominantly in the cytoplasm (Fig. 1D, *i-iv*). However, following pervanadate treatment of LNCaP cells, there was a marked translocation of P-Akt to the plasma membrane (Fig. 1D, *v-viii*). Notably, a small amount of this Akt cohort was found to colocalize with the lipid raft marker ganglioside GM1, as visualized by CTxB staining, following pervanadate treatment (Fig. 1D, *ix*), consistent with the existence of a subpopulation of raft-resident Akt.

To determine whether raft-resident Akt is active as a kinase, we compared Akt kinase activity between C+M and raft fractions of

LNCaP cells. Cells were serum depleted for 48 h before harvesting because this has been reported previously to increase the activity of both phosphoinositide 3'-kinase (PI3K) and Akt in this cell line (21). Equivalent amounts of protein from C+M or raft fractions were subjected to immunoprecipitation with an anti-Akt antibody that enriches for Akt phosphorylated at Ser<sup>473</sup>. The kinase activity of Akt immune complexes was measured by determining the extent of phosphorylation of a GSK3 fusion protein substrate. Consistent with the PTEN-null status of LNCaP cells, robust Akt kinase activity was detected in the C+M fraction following serum depletion (Fig. 2A). In contrast, the kinase activity of Akt isolated from rafts was attenuated. When normalized to total Akt levels, kinase activity of raft-resident Akt was ~8% of that detected in the C+M fraction. To circumvent the concern that low levels of Akt kinase activity simply resulted from low levels of Akt protein in rafts, we used two approaches to increase the amount of raft-resident Akt. First, we treated LNCaP cells with pervanadate to promote movement of endogenous Akt into rafts (Fig. 2B, *left*). Second, we transiently overexpressed WT Akt1 in LNCaP cells before pervanadate treatment (Fig. 2B, *right*). In either case, Akt was immunoprecipitated from C+M or raft fractions and assayed for kinase activity toward GSK3 (Fig. 2B). Pervanadate treatment induced a marked increase in kinase activity of Akt immune complexes isolated from the C+M fraction of cells expressing endogenous or overexpressed Akt. Despite eliciting a substantial enrichment of Akt in the raft fraction, however, the pervanadate-stimulated activity of raft-resident Akt complexes toward GSK3 was markedly attenuated, displaying only up to ~20% of the activity present in the C+M fraction. The relatively low level of kinase activity in Akt immune complexes isolated from rafts was not due to enzyme inactivation during fractionation because we were able to recover active endogenous Akt from the rafts of PC3 prostate cancer and MC3T3 osteoblast-like cells treated with pervanadate and isolated under identical conditions (Supplementary Fig. S1). These findings suggest that the low Akt activity toward GSK3 observed in LNCaP cells is a reflection of the raft environment specifically in this cell type.

To further investigate the apparent attenuation in activity of Akt immune complexes isolated from lipid rafts, we measured the activity of both WT Akt (Akt1-WT) and constitutively active, MyrAkt1 expressed transiently in HEK293 and LNCaP cells. Unlike endogenous or overexpressed WT Akt, MyrAkt1 partitioned almost equally between the nonraft and raft membrane compartments. MyrAkt1 immune complexes isolated from the C+M fraction of HEK293 cells elicited robust phosphorylation of the GSK3 substrate (Fig. 3A and B), consistent with the reported activity of this Akt fusion protein (22). Mutation of Thr<sup>308</sup> in the catalytic loop to alanine ablated GSK3 phosphorylation in agreement with the absolute requirement for phosphorylation at T308 for Akt kinase activity (Fig. 3A; ref. 23). In contrast, no GSK3 phosphorylation was observed following incubation of the substrate with Akt1-WT or MyrAkt1 immune complexes isolated from rafts (Fig. 3A and B), despite the high level of Akt present in these fractions. A similar result was obtained with LNCaP cells (Fig. 3C). Despite strong Akt kinase activity in the C+M fraction of LNCaP cells, no GSK3 phosphorylation was observed in MyrAkt1 complexes precipitated from rafts and subjected to kinase assay (Fig. 3C, *left*). The lack of kinase activity of raft-localized MyrAkt1 was not due to lack of phosphorylation at Thr<sup>308</sup> or Ser<sup>473</sup> because these sites were heavily phosphorylated in Akt immune complexes isolated from rafts (Fig. 3C, *right*). We confirmed our observations in LNCaP cells in an

**Figure 1.** A population of endogenous Akt1 resides in a cholesterol-rich membrane fraction. **A**, LNCaP cells were subjected to sucrose density centrifugation as described. One milliliter fractions were analyzed for cholesterol content or blotted with antibodies to total Akt,  $G_{i\alpha 2}$ , c-Crk, and  $\beta$ -tubulin. The protein content of individual fractions was visualized by Ponceau S staining of membranes after transfer (*bottom*). *Star*, the cholesterol-enriched lipid raft microdomain (*fraction 4*). *AU*, absorbance units. **B**, LNCaP cells growing in serum were fractionated into cytosolic/Triton-soluble membrane (*C+M*) and lipid raft (*Raft*) fractions as described. Ten micrograms C+M and raft fractions were blotted with antibodies to total Akt, Thr<sup>308</sup>-phosphorylated Akt (*T308-P*), S473-P Akt (*S473-P*),  $\beta$ -tubulin, and  $G_{i\alpha 2}$ . **C**, Akt was immunoprecipitated from LNCaP cells exposed to cyclodextrin (*CD*) and fractionated into C+M and raft fractions using IG1 anti-Akt mAb. Immunoprecipitated (*IP*) eluates were blotted with antibodies to total or S473-P Akt. **D**, serum-starved LNCaP cells treated without (*left, i-iv*) or with (*right, v-viii*) 0.5 mmol/L pervanadate for 15 min were incubated with 10  $\mu$ g/mL tetramethylrhodamine-conjugated CTxB subunit on ice for 30 min, before incubation with anti-S473-P Akt antibody (1:100) and FITC-conjugated secondary antibody (1:200). *Arrowhead*, a region of colocalization of S473-P Akt with CTxB as shown in (*ix*). The image in (*ix*) has been enlarged to show red, green, and yellow pixels.



independent assay using Crosstide as substrate. As shown in Fig. 3D, the activity of MyrAkt1 immune complexes isolated from rafts toward Crosstide was dramatically reduced compared with that precipitated from the C+M fraction.

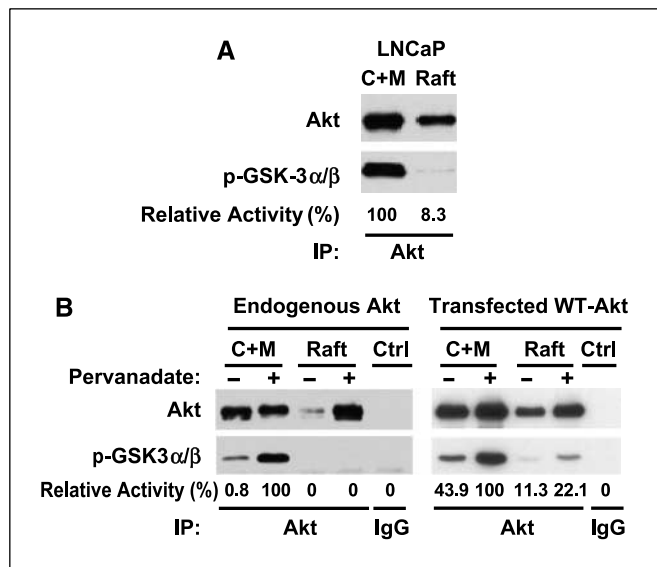
To determine whether the activity of Akt isolated from rafts was also attenuated when assayed against other substrates, we measured the activity of MyrAkt1 immune complexes toward histone H2B or MBP, both of which have been used previously as Akt substrates (24–27). In contrast to the activity observed against GSK3, MyrAkt1 immune complexes isolated from rafts elicited robust incorporation of <sup>32</sup>P into H2B and displayed more than 10 times the activity observed with MyrAkt1 precipitated from the C+M fraction (Fig. 4A). Similar results were obtained with MyrAkt1 transiently expressed in HEK293 cells (Fig. 4B), with raft-resident Akt complexes approximately four times more active against H2B

than was Akt isolated from the C+M fraction. The enhanced activity of raft-resident MyrAkt1 relative to MyrAkt1 in the C+M fraction was also evident when MBP was used as the substrate (Supplementary Fig. S2). Collectively, these findings suggest that raft-resident Akt is functionally distinct from Akt present at other subcellular locations.

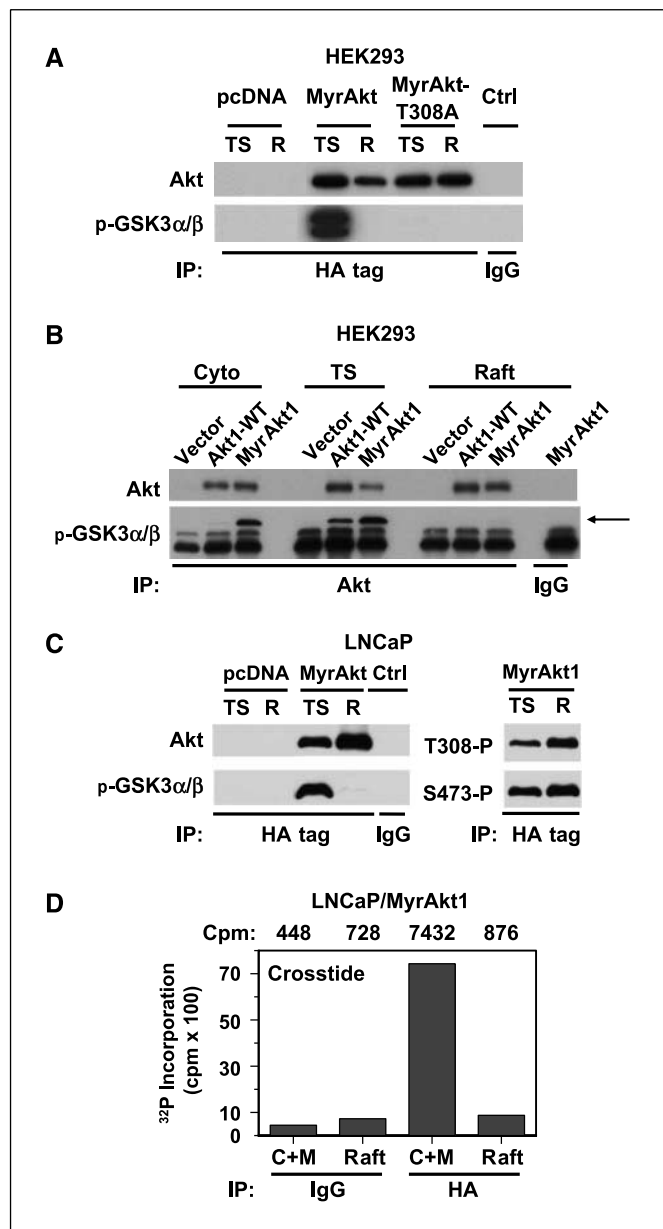
To further understand how signals transmitted from raft-resident Akt differed from signaling downstream of Akt in other locations within the cell, we generated populations of LNCaP cells stably expressing MyrAkt1 (Supplementary Fig. S3). Localization of MyrAkt1 within rafts was confirmed by sucrose density gradient analysis (Fig. 5A), which showed enrichment of the ectopically expressed protein in the light buoyant density fractions. In addition, immunofluorescence imaging showed membrane localization of MyrAkt1 as well as colocalization with the raft-restricted

ganglioside GM1 (Fig. 5B). To assess the cholesterol sensitivity of raft-resident MyrAkt1, membrane cholesterol levels in LNCaP transfectants were manipulated by treatment with either cyclodextrin alone, water-soluble cholesterol alone, or cyclodextrin followed by cholesterol as described in Materials and Methods. As shown in Fig. 5C (top), 5 mmol/L cyclodextrin treatment led to a decrease in membrane cholesterol of ~38% compared with untreated cells and essentially a complete loss of MyrAkt1 from rafts (Fig. 5C, bottom). Cholesterol replenishment restored membrane cholesterol levels to ~80% of the basal level and reestablished the basal distribution of MyrAkt1. Cholesterol treatment in the absence of depletion increased membrane cholesterol by ~12% and led to a modest but detectable increase in MyrAkt1 in rafts. Previous findings from our group showed that treatment of LNCaP cells with PI3K inhibitors induces apoptosis in this PTEN-null cell type (26). To determine whether raft-resident Akt could function to promote cell survival, we exposed LNCaP/MyrAkt1 and control LNCaP/LacZ transfectants to the PI3K inhibitor, LY294002 (10 μmol/L), for 24 h and assessed apoptotic effects by flow cytometry. As shown in Fig. 5D, LNCaP/MyrAkt1 cells were almost completely insensitive to PI3K inhibition, in contrast to LNCaP/LacZ cells that displayed significant induction of apoptosis (Fig. 5D, inset). However, the cytoprotective effect of MyrAkt1 was diminished by depletion of membrane cholesterol before treatment with LY294002, suggesting that antiapoptotic signals are transmitted, at least in part, by the raft-resident cohort of Akt.

To determine the effect of raft-resident Akt on downstream substrates, we used an antibody raised against a phospho-peptide corresponding to the Akt recognition motif RXXXS/T and that specifically recognizes substrates phosphorylated by Akt (28). In LNCaP/LacZ and LNCaP/MyrAkt1 cells cultured in serum, the



**Figure 2.** Akt kinase activity toward GSK3 is attenuated in lipid rafts isolated from LNCaP cells. *A*, C+M and raft fractions from LNCaP cells serum depleted for 48 h were immunoprecipitated with Ig1 anti-Akt mAb and Akt kinase activity against GSK3 determined. Kinase assay eluates were blotted with antibodies to total Akt or p-GSK3α/β. Relative activity was determined as described in Materials and Methods. Data are representative of duplicate determinations. *B*, nontransfected LNCaP cells or cells transiently expressing T7-Akt1 were serum depleted for 24 h and treated without (–) or with (+) 0.5 mmol/L pervanadate. Immunoprecipitation kinase assay and immunoblotting were done as in (*A*). Data are representative of triplicate determinations. *Ctrl*, fraction incubated with isotype control IgG and subjected to immunoprecipitation kinase assay.



**Figure 3.** The activity of constitutively active Akt toward GSK3 is attenuated in lipid rafts. *A*, HEK293 cells transiently expressing MyrAkt1, MyrAkt1-T308A, or vector alone (pcDNA3) were fractionated into Triton-soluble membrane (TS) or raft membrane (R) fractions and immunoprecipitated with anti-HA or isotype control antibodies. Kinase assays were done as described in Fig. 2A. *B*, HEK293 cells transiently expressing WT T7-Akt1, MyrAkt1, or vector alone were fractionated into cytosolic (Cyto), Triton-soluble membrane, or raft membrane fractions (Raft). Akt was immunoprecipitated with anti-Akt1 pAb and analyzed by kinase assay as in (*A*). Arrow, the p-GSK3 substrate. *C*, LNCaP cells transiently expressing MyrAkt1 or vector alone were subjected to immunoprecipitation kinase assay and blotted as in (*A*). Immunoprecipitated eluates were also blotted with antibodies to T308-P Akt or S473-P Akt. *D*, C+M or raft fractions from LNCaP cells transfected as in (*C*) were subjected to immunoprecipitation kinase assay using Crosstide as substrate, in the presence of [<sup>32</sup>P]ATP.

number of phosphorylated Akt substrate species was increased in C+M fractions of LNCaP/MyrAkt1 cells compared with LNCaP/LacZ transfectants (Supplementary Fig. S4). Surprisingly, the profiles of phosphorylated Akt substrates were remarkably similar in raft fractions of LacZ-expressing versus MyrAkt1-expressing LNCaP cells under these conditions (Supplementary Fig. S4),

despite the enrichment for activated Akt in this compartment in LNCaP/MyrAkt1 cells. To determine the effect of cholesterol manipulation on signaling from Akt to its substrates, whole-cell lysates, C+M, or raft fractions were prepared from LNCaP/LacZ and LNCaP/MyrAkt1 cells subjected to cholesterol depletion and/or repletion and blotted with the Akt substrate antibody. In control LNCaP/LacZ cells, cyclodextrin treatment elicited a modest reduction in phosphorylation of certain Akt substrates in rafts (Fig. 6A, compare lanes 2 and 4). In marked contrast, cyclodextrin treatment led to ablation of Akt substrate phosphorylation in rafts of LNCaP/MyrAkt1 transfectants (Fig. 6A, compare lanes 6 and 8). Interestingly, signals in the C+M fraction of LNCaP/MyrAkt1 transfectants, which were markedly enhanced relative to LNCaP/LacZ cells (Fig. 6A, compare lanes 1 and 5), were also diminished with cyclodextrin treatment in LNCaP/MyrAkt1 (Fig. 6A, compare lanes 5 and 7), suggesting cross-talk between the raft and nonraft cohorts of Akt. Repletion of membrane cholesterol led to the reappearance of Akt substrate phosphorylation predominantly in

the raft fraction of LNCaP/MyrAkt1 transfectants (data not shown), confirming the cholesterol sensitivity of Akt-dependent signaling in these cells.

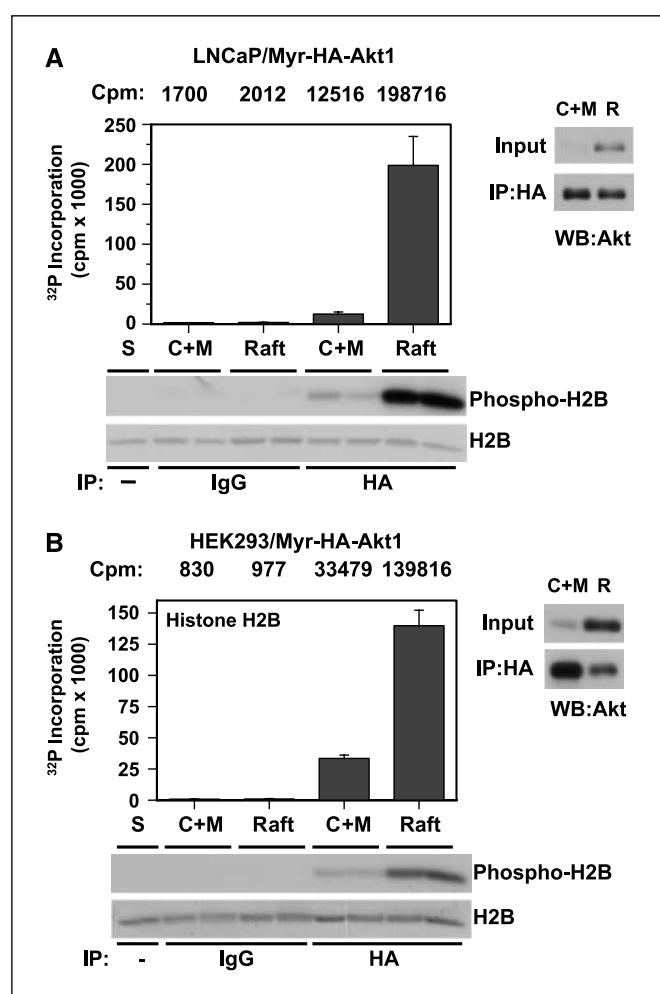
We also assessed the effect of cyclodextrin treatment on known Akt effectors, including p70S6K, FKHR, and GSK3. As shown in Fig. 6B, MyrAkt1-stimulated phosphorylation of p70S6K, FKHR, and GSK3 was diminished by cyclodextrin treatment, consistent with cholesterol-sensitive signaling to these targets. Interestingly, the raft fraction was highly enriched for phosphorylated Akt substrates in the control cells compared with the C+M fraction (Fig. 6A), suggesting that in the PTEN-null background, Akt signaling within cholesterol-rich membranes is extensive.

## Discussion

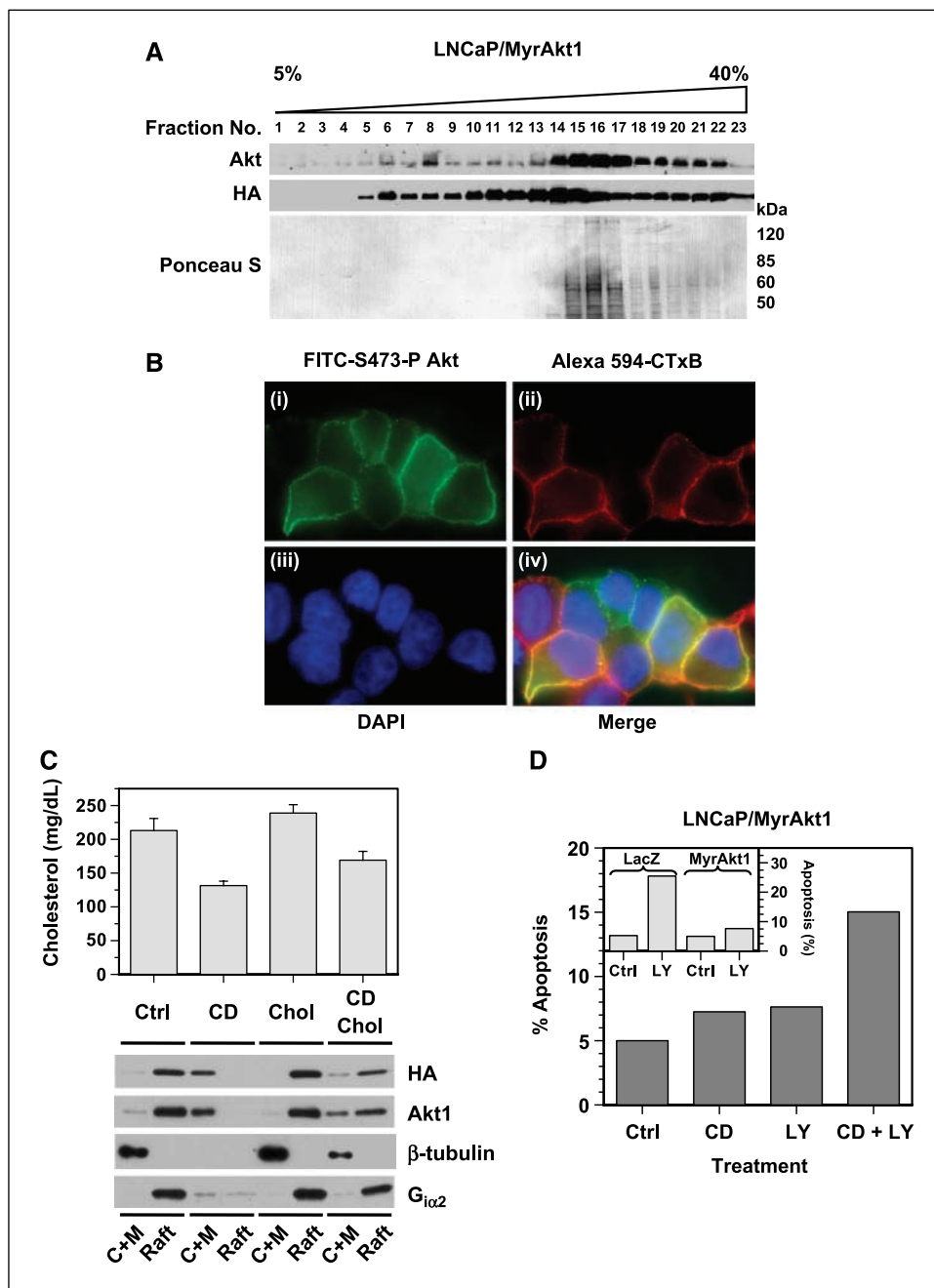
In this study, we show that Akt1 is regulated directly by a cholesterol-sensitive mechanism. The evidence for this conclusion is the following: (a) a population of endogenous Akt resides in a cholesterol-rich membrane fraction; (b) phosphorylation of lipid raft-resident Akt1 can be attenuated by membrane cholesterol depletion; (c) MyrAkt1, which is an oncogene, is overrepresented in lipid raft fractions compared with WT Akt1; (d) localization of MyrAkt1 within rafts is modulated reversibly by manipulation of membrane cholesterol; (e) cholesterol depletion attenuates the cytoprotective effect of MyrAkt1 in LNCaP cells exposed to PI3K inhibition; (f) signaling from raft-resident Akt to its effectors is inhibited by cholesterol depletion and restored by cholesterol repletion; and (g) multiple targets of Akt phosphorylation reside in cholesterol-rich membranes. Together, these findings indicate that a subpopulation of Akt1 is regulated in a cholesterol-sensitive manner. They further imply that the alterations in cholesterol synthesis and homeostasis observed in malignancy play a role in the regulation of signal transduction through the Akt pathway.

The initial characterization of Akt revealed that, unlike the proto-oncogene *c-akt*, the oncogenic form (*v-akt*) possessed a myristoylation sequence arising from its fusion to the viral protein Gag (29, 30). Myristoylation of v-Akt was found to promote its membrane association, which in turn enhanced the tumorigenicity of v-Akt-expressing cells in xenograft experiments (31). More recently, a synthetic MyrAkt1 fusion protein was shown to cause prostatic intraepithelial neoplasia when expressed in the mouse prostate (32). The transforming properties of MyrAkt are believed to result from constitutive membrane targeting and constitutive phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup> (25). However, in view of our current findings showing that myristoylation results in overrepresentation of the kinase in cholesterol-rich membranes, localization of Akt within lipid raft microdomains, as opposed to simply plasma membrane localization, is likely to be a key determinant of oncogenicity.

Consistent with this idea, we found that MyrAkt1 isolated from rafts in several cell types displayed attenuated kinase activity toward GSK3 and Crosstide despite (a) robust phosphorylation of the regulatory sites Thr<sup>308</sup> and Ser<sup>473</sup> and (b) measurable activity toward these substrates in MyrAkt1 immune complexes isolated from nonraft fractions. Interestingly, this conclusion is consistent with an unexplained finding in a previous report that a myristoylated phospho-mimetic Akt1 mutant (Akt1-T308D/S473D), which is functionally equivalent to the MyrAkt1 construct used in our analyses, was kinase inactive toward Crosstide, despite robust activation of WT Akt1 under identical conditions



**Figure 4.** Constitutively active Akt1 isolated from lipid rafts displays altered substrate specificity. LNCaP (A) or HEK293 (B) cells transiently expressing Myr-HA-Akt1 were fractionated into C+M or raft fractions. Akt was immunoprecipitated from each fraction with HA antibody and immune complexes were subjected to kinase assay using histone H2B as substrate. Bottom, inputs and immunoprecipitated eluates were blotted (WB) with antibodies to total Akt; top, incorporation of <sup>32</sup>P into histone H2B under each condition. Data are representative of two independent trials. Columns, mean of duplicate determinations; bars, SD.



**Figure 5.** Oncogenic Akt1 is enriched in lipid raft fractions and confers resistance to apoptosis induced by PI3K inhibition. **A**, LNCaP cells stably expressing MyrAkt1 (LNCaP/MyrAkt1) were subjected to sucrose density centrifugation. One milliliter fractions were blotted with antibodies to total Akt and HA. Fractions 5 to 8, raft fraction. **B**, lipid rafts in LNCaP/MyrAkt1 cells were stained with 0.5  $\mu$ g/mL Alexa 594-CTxB for 10 min before staining with anti-S473-P Akt (1:100) and FITC-conjugated secondary antibody (1:100). Nuclei were counterstained with DAPI before imaging. Original magnification,  $\times 63$ . **C**, bottom, LNCaP transfectants were treated with either 5 mmol/L cyclodextrin for 1 h, 45  $\mu$ g/mL water-soluble cholesterol (Chol) alone for 1 h, or cyclodextrin for 1 h followed by cholesterol for 1 h. Cells incubated in serum-free medium served as controls. Ten micrograms of C+M or raft fractions were blotted with antibodies to total HA, Akt1,  $\beta$ -tubulin, or G<sub>102</sub>. Top, membrane cholesterol levels following the indicated treatments. Columns, mean of duplicate determinations; bars, SD. **D**, inset, LNCaP/MyrAkt1 or control cells expressing LacZ (LNCaP/LacZ) were treated without or with 10  $\mu$ mol/L LY294002 (LY) for 24 h and the extent of apoptosis was determined by flow cytometry. LNCaP/MyrAkt1 cells were treated without (Ctrl) or with 5 mmol/L cyclodextrin (1 h), 10  $\mu$ mol/L LY294002 (24 h), or both agents (cyclodextrin for 1 h followed by LY294002 for 24 h) and harvested for flow cytometry. Data are presented as apoptotic cells (sub-G1 peak) expressed as a percentage of the total cell population and are representative of two independent trials.

(33). Based on our own data, we interpret this result to mean that myristoylation targets proteins to lipid rafts (34–37), where the kinase complex is functionally redirected toward alternative substrate targets. In contrast to our observations with GSK3/Crosstide, the activity of raft-resident MyrAkt1 immune complexes toward H2B was 5 to 15 times greater compared with immune complexes precipitated from C+M fractions. These findings suggest for the first time that the raft environment can modulate the activity of raft-resident kinases toward target proteins. The difference in substrate preference cannot be explained by differential phosphorylation of Akt regulatory sites, Thr<sup>308</sup> and Ser<sup>473</sup>, but might result from a change in conformation of the enzyme in rafts. Alternatively, this difference may arise from differential association of Akt with interacting proteins

enriched in distinct subcellular compartments. In support of this second possibility, we recently identified a novel Akt binding partner that associates preferentially with Akt in cholesterol-rich membranes.<sup>5</sup>

These findings emphasize the concept that kinase activity per se is not the sole determinant of Akt oncogenicity. For example, the constitutively active Akt mutant, Akt1-T308D/S473D, displays minimal transforming activity in chick embryo fibroblasts, whereas the kinase-inactive mutant MyrAkt1-T308A forms tumors in chickens, albeit with an extended latency period (25). In

<sup>5</sup> B. Cinar et al., submitted for publication.

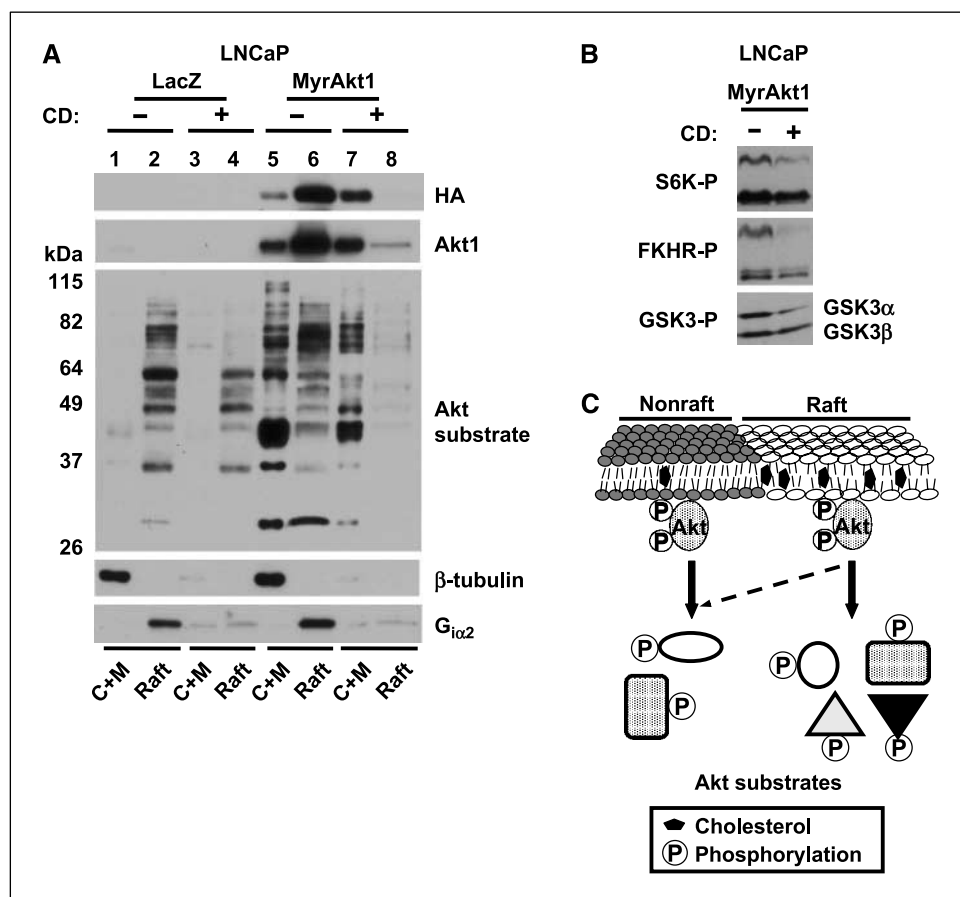
contrast, Akt3 possesses high kinase activity but poor transforming ability (38) consistent with the possibility that Akt exerts signaling activities independently of its kinase function. In support of this hypothesis, two recent studies show that Akt modulates the activity of the transforming growth factor- $\beta$  effector Smad3 in a manner that is independent of Akt kinase activity (39, 40). In those studies, kinase-active and kinase-inactive forms of Akt were found to interact directly with Smad3 and thereby inhibit its activity.

Another implication of our findings is that signals processed by raft-resident Akt are distinct from those transduced by Akt located elsewhere in the cell. Consistent with this possibility, localization of MyrAkt1 to rafts seemed to sensitize Akt signaling to cholesterol depletion. Whereas signaling to Akt targets in rafts of LNCaP/LacZ cells did not change appreciably in response to cyclodextrin treatment, phosphorylation of Akt substrates in rafts of MyrAkt1-expressing cells was essentially ablated. Under these conditions, the cytoprotective effect of MyrAkt1 in LNCaP cells exposed to PI3K inhibition was also diminished, suggesting that raft localization alters the nature of Akt signaling to its effectors. Consistent with these effects, changes in membrane lipid composition, either through the generation of sphingosine-1-phosphate or by exogenous application of omega-3 fatty acids, have been shown to redirect signaling in other cell types (41, 42). These observations may be consistent with recent findings showing increased sensitivity to apoptosis induced by cholesterol depletion in cells harboring higher amounts of cholesterol-rich membranes (9). Our findings may also partially explain the reported reduction in

incidence of tumors/aggressive tumors that accumulate cholesterol, such as prostate cancer, in patients on long-term statin therapy (5, 6).

Several recent studies have reported a link between elevated cholesterol levels and cancer. Li et al. (9) showed that breast and prostate tumor cells contained higher levels of membrane cholesterol and rafts/caveolae compared with their normal counterparts. In addition, elevated synthesis of the cholesterol precursor, mevalonate, resulting from higher expression and/or activity of HMG-CoA reductase, has been reported in several tumor types. Direct administration of mevalonate was found to promote the growth of orthotopic mammary tumor xenografts in nude mice (43). Interestingly, Akt was shown recently to induce sterol-regulatory element binding protein-dependent transcription of multiple genes involved in fatty acid and cholesterol metabolism, including HMG-CoA reductase, HMG-CoA synthase, and fatty acid synthase (44). In that study, a variant of MyrAkt1 fused to the hormone-binding domain of the estrogen receptor (MyrAkt-ER) was used to induce gene expression changes in retinal pigment epithelial cells. Together with our current findings showing enrichment of MyrAkt1 in rafts, the existence of a functional link between raft-resident Akt, regulation of cholesterol metabolism, and membrane lipid composition is strongly suggested. Our results suggest the existence of a potential feedback mechanism, whereby signals transmitted by raft-resident Akt can lead to enhanced cholesterol synthesis, expansion of the cholesterol-rich compartment, and increased localization of Akt to rafts.

**Figure 6.** Phosphorylation of Akt substrates is sensitive to cholesterol depletion. **A**, 10  $\mu$ g C+M and raft fractions from LNCaP/LacZ and LNCaP/MyrAkt1 transfectants treated without (-) or with (+) 5 mmol/L cyclodextrin were blotted with antibodies to HA, Akt1,  $\beta$ -tubulin,  $G_{i\alpha 2}$ , and phosphorylated Akt substrates. **B**, 10  $\mu$ g whole-cell lysates prepared from LNCaP/MyrAkt1 transfectants treated as in (A) were blotted with antibodies to phosphorylated forms of p70S6K, FKHR, and GSK3. The S6K-P antibody is known to cross-react with phosphorylated p85S6K; the FKHR-P antibody cross-reacts with phosphorylated FoxO4; and the GSK3-P antibody recognizes both GSK3 $\alpha$  and GSK3 $\beta$  phosphorylated at Ser<sup>21</sup> and Ser<sup>9</sup>, respectively. **C**, the model summarizes the findings of the study. Akt present in lipid rafts displays a distinct substrate preference to Akt in the nonraft compartment, consistent with a redirection of Akt-dependent signaling when the protein resides in cholesterol-rich membranes. Following cholesterol depletion, signaling from raft-resident Akt is essentially ablated, whereas signaling from Akt in the nonraft compartment is largely unaffected.





In summary, we have obtained evidence that cholesterol is a direct regulator of Akt-dependent signaling in caveolin-negative cells and that localization of Akt to lipid raft microdomains alters the substrate preference of the Akt kinase complex. These findings suggest a direct mechanistic link between cholesterol and cell survival signaling in tumor cells and may be functionally relevant to the reported chemopreventive benefit of long-term use of cholesterol-lowering drugs in certain cancers.

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