Published in final edited form as:

Exp Cell Res. 2008 January 15; 314(2): 286–296.

Identification of functional domains in AKT responsible for distinct roles of AKT isoforms in pressure-stimulated cancer cell adhesion

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Abstract

Cell adhesion is a critical step in cancer metastasis, activated by extracellular forces such as pressure and shear. Reducing AKT1, but not AKT2, ablates the increase in cancer cell adhesion associated with 15 mmHg increased extracellular pressure. To identify the determinants of this AKT isoform specificity, we exchanged the pleckstrin homology (PH) domains and/or hinge regions of AKT1 and AKT2. Wild type isoforms or these chimeras were overexpressed in Caco-2 cells in the absence or presence of isoform-specific siRNA to suppress endogenous AKT1. Pressure-induced AKT translocation and phosphorylation to the membrane were compared, along with the stimulation of cell adhesion by pressure. Pressure stimulated translocation of AKT1, but not AKT2 to the plasma membrane. Among our chimeras, only the chimeric AKT2 (chimera2), in which both the AKT2 PH domain and hinge region had been replaced by those of AKT1, translocated to the membrane in response to pressure. Similarly, only chimera2 rescued the function of AKT1 in mediating pressure-stimulated adhesion after endogenous AKT1 had been reduced. Pressure also promoted phosphorylation of AKT1 but not AKT2, and expression of a non-phosphorylatable double point mutant prevented pressure-stimulated adhesion. Among the chimeras, pressure promoted only chimera2 phosphorylation. These results identify the AKT1 PH domain and hinge region as functional domains which jointly permit AKT1 translocation and phosphorylation in response to extracellular pressure and distinguish determine the specificity of AKT1 in mediating the effects of extracellular pressure on cancer cell adhesion. These may be useful targets for interventions to inhibit metastasis.

Keywords

adhesion; AKT isoform; AKT1; AKT2; pressure; PH domain; hinge region

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Introduction

Cell adhesion is an important step in the metastatic cascade [1]. Physical forces such as moderate extracellular pressure (15 mm Hg), shear, and fluid flow promote the adhesion of colonic and other cancer cells [2–4]. This phenomenon is important because tumor cells are exposed to increased pressure and shear stress during and after surgical procedures, and during passage through lymphatics or the circulatory system. Indeed, cancer cells subjected to such forces also adhere with greater affinity to endothelial monolayers [3] and surgical wounds [5]. Moreover, delineation of a mechanism by which tumor cells regulate their own adhesion might offer new targets by which metastasis can be inhibited in patients with locally unresectable disease. An improved understanding of the mechanism of pressure-stimulated cancer cell adhesion is therefore desirable.

AKT/protein kinase B is an important Ser/Thr kinase that regulates many biological processes [6–10]. AKT has three mammalian isoforms, AKT1, AKT2, AKT3, which share a highly homologous conserved structure. Each AKT isoform contains four functional domains. These are an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, a hinge region (HR) between the PH domain and the catalytic domain, and a C-terminal regulatory domain/C-tail [11,12]. Although the three AKT isoforms share 80% homology in amino acid sequence [13], they may perform isoform-specific functions in knockout mice [14], and *in vitro* [12, 15]. However, the mechanism of such specificity is not clear.

We have previously reported that reducing AKT1 by siRNA ablated the increase in colon cancer cell adhesion associated with pressure, but that reducing AKT2 by siRNA did not [16]. This suggested an isoform-specific role for AKT1 in this process, although the modest reduction in AKT2 that we were maximally able to achieve with siRNA raised the question of whether further AKT2 reduction might have also prevented the effect. We now sought to confirm AKT1 specificity in modulating pressure-induced cancer cell adhesion and to identify the structural determinants of such isoform specificity. We used a domain swapping strategy, exchanging the PH domain and/or hinge region between AKT1 and AKT2 to determine which regions might be important. In addition, the phosphorylation of AKT at T308 in the catalytic domain and S473 in the C-tail regulatory domain may be required for its full activation [8, 17]. AKT is phosphorylated in response to increased extracellular pressure (15mm Hg) in colon cancer cells [16], as well as in response to other physical forces in other cell types [18–20]. We therefore also examined whether the phosphorylation of AKT in response to increased extracellular pressure is isoform specific and how this is regulated.

Materials and Methods

Materials

We obtained Lipofectamine 2000 and other transfection supplies from Invitrogen (Carlsbad, CA), human transferrin from Roche Applied Science (Indianapolis, IN), and trypsin, horseradish peroxidase(HRP)-conjugated anti-mouse or rabbit IgG, total AKT and AKT2 and Phospho-AKT (Thr308) and Phospho-AKT (Ser473) antibodies, AKT1 (targeting to the PH domain) and AKT2 (targeting to the C-terminal) antibodies from Cell Signaling Technology (Beverly, MA). We obtained antibodies targeting the C-terminal of AKT1 and Rho-GDI from Upstate (Lake Placid, NY), anti-hemagglutinin (HA, clone 12CA5) and HA.11 monoclonal antibodies from Roche Applied Science and Covance respectively, and Protein A or G-sepharose CL4B and E-Cadherin antibody from Amersham Biosciences (UK) and Santa Cruz Biotechnology (Santa Cruz, CA) respectively. pCMV-HA and pEGFP-C1 vectors were obtained from Clontech (Mountain View, CA). pcDNA3 myr HA AKT1 and pcDNA3 myr HA AKT2 plasmids were kindly provided by Dr. Paula Herman (Dana Farber Cancer Institute) through Addgene (Cambridge, MA). Mammalian expression plasmid pEGFP-C1 fused with

AKT1 T308A/S473A mutant was a generous gift of Dr. M. D. Ringel (Ohio State University, Columbus, OH). All primers were purchased from Integrated DNA Technologies (Coralville, IA). QIAquick Gel Extraction, QIAprep spin Miniprep, QIAquick PCR purification and QIAfilter Plasmid Maxi kits were purchased from Qiagen (Valencia, CA). Actin polyclonal antibody and all other reagents were of analytical grade from Sigma. GAPDH (glyceraldehyde-3-PDH) was obtained from Biodesign International (Saco, ME). The AKT inhibitor SH-6, D-2,3-Dideoxy-*myo*inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl] hydrogen phosphate was from Alexis Biochemicals (San Diego, CA).

Expression Vectors

Mammalian expression vectors HA-AKT1 and HA-AKT2 were constructed by PCR with pcDNA3 myr HA AKT1 and pcDNA3 myr HA AKT2 from Addgene (Cambridge, MA) as template respectively and introducing a 5' *EcoR* I and 3' *Kpn* I site. PCR products were then subcloned into the *EcoR* I/*Kpn* I double digested pCMV-HA cassette to get pCMV-HA-AKT1 and pCMV-HA-AKT2 respectively. We used overlapping PCR to generate four chimeras, chimera1, chimera2, chimera3, and chimera4 (Fig. 4A), with exchanged PH domain and/or hinge region, in a manner similar to that previously described by Shevchuk [21]. The generated chimera constructs were confirmed by restriction analysis and sequencing.

siRNA design

The siRNA specifically targeting human AKT1 was designed by modifying the published siRNA sequence targeting mouse AKT1 [22]. The sequence of siRNA against human AKT1 is 5'-AACCAGGACCAUGAGAAGCUUTT-3' (The 2-nucleotide overhanging of 2'-deoxythymidine is indicated as TT and denoted by underlines.) A non-specific siRNA, siCONTROL Non-Targeting siRNA #1 (NT1 siRNA), the AKT1 siRNA that we had designed, and SMARTpool AKT2 siRNA were all purchased from Dharmacon (Lafayette, CO).

Cell culture

Caco-2 and SW620 colon cancer cells were cultured as described previously [3,23].

Transfection

For transfection with siRNA, Caco-2 cells were plated on p100 dishes at 20–25% confluence on the day before transfection. siRNAs were transfected using the Lipofectamine 2000 according to the manufacturer's instructions. The final concentration for siRNA and Lipofectamine 2000 were 10nM for AKT1 siRNA and 10 or 100nM for AKT2 siRNA and 2.5 µg/ml for the Lipofectamine 2000. Five hours after transfection, the medium was replaced with 15 ml pre-warmed Caco-2 medium without antibiotics. 72 hours after siRNA transfection, the AKT isoform siRNA-treated cells were used for adhesion and expression studies.

For transfection with plasmids, Caco-2 cells were plated on p100 dishes at 30–35% confluence on the day before transfection. For the gain of function experiments, the Caco-2 were firstly transfected by AKT1 siRNA to knock down the expression of endogenous AKT1 and then, 24 hours later, transfected by plasmids expressing AKT2 or chimeras to determine if AKT2 or chimeras can rescue the function of AKT1. Briefly, the constructed plasmids or empty plasmid were transfected into Caco-2 cells at final concentrations of 2 µg/ml plasmid and 5 µg/ml Lipofectamine 2000. Five hours after transfection, the medium was replaced with 15 ml pre-warmed Caco-2 media without antibiotics. Forty eight hours after DNA transfection, the transfected cells were lysed for western analysis or used for adhesion assays. For co-transfection, pEGFP-C1 vectors was used (pEGFP-C1/plamid= 1:4) together with constructed plasmids or empty plasmid as a reporter to mark the successfully transfected cells.

Pressure regulation

15 mmHg pressure was applied as previously described[3,24] within an airtight box with inlet and outlet valves, thumb screws, a pressure gauge and an O-ring for an airtight seal. Temperature was maintained within ± 2 °C and pressure within ± 1.5 mmHg.

Matrix precoating

Six-well plates were precoated with collagen I (12.5 μ g/ml) in ELISA buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.4) and the wells were rinsed three times with sterile phosphate buffered saline (PBS) prior to cell seeding.

Cell adhesion assay

Adherent cells were counted by a modification of the method previously described [16,24]. Briefly, equal number of cells are seeded to collagen I coated 6-well plates under ambient or increased pressure conditions for 30 minutes. Non-adherent cells are washed away, and adherent cells are counted microscopically in at least 20 random high power fields per well after staining. However, plasmid DNA transfection efficiency in Caco-2 cells is relatively low (~25%) [23]. For studies of adhesion in plasmid-transfected cells, we therefore co-transfected cells with the plasmid of interest and with EGFP (Enhanced Green Fluorescent Protein) - encoding plasmid (pEGFP-C1) as a reporter (pEGFP-C1/experimental plasmid= 1:4). After washing away non-adherent cells, and fixing the remainder with 0.01 M NaIO_4 , 0.75 M lysine, 0.0375 M sodium phosphate buffer, pH 7.4, and 2% paraformaldehyde on ice for 1 hour, we counted only adherent EGFP-expressing cells per high power field using fluorescence microscopy. We have previously used similar techniques to successfully demonstrate the impact of a dominant negative FAK construct on pressure-stimulated adhesion [3] and to analyze the effects of cotransfection in Caco-2 cells [23].

Pressure induced inside-out signaling

To investigate whether AKT phosphorylation associated with increased extracellular pressure is specific to AKT1, Caco-2 cells in suspension were subjected to ambient pressure or 15 mmHg increased pressure for 30 minutes in bacteriological plastic plates pretreated with 1% heat inactivated bovine serum albumin in PBS to prevent all adhesion and consequent adhesion-induced signaling. In some studies, cells were pretreated with Akt inhibitor SH-6 (10 μ M) for 1.5 hours. After pressure treatment, the suspended cells were collected by centrifugation. The cell pellet was washed with ice-cold PBS and lysed in lysis buffer as described below.

Western blotting and immunoprecipitation

Transfected or untransfected cells in suspension were exposed to 15 mmHg pressure for 30min in plates pretreated as above to prevent adhesion and consequent adhesion-induced signals.. Cells were then lysed and subjected to Western blotting [3] or immunoprecipitation [24] as described.

Subcellular fractionation and translocation assay

Soluble and particulate cell fractions were prepared as described previously by sonication and serial ultracentrifugation [16]. Rho-GDI and E-Cadherin antibodies were used as protein loading controls for cytosolic and membrane/cytoskeletal fractions respectively.

Statistical Analysis

Results were compared by Student's unpaired t-test and considered statistically significant when $p < 0.05$. All experiments were done independently at least three times unless indicated otherwise. All data are expressed as mean $\pm$ SEM.

Results

Pressure induces phosphorylation of AKT1, not AKT2, and this phosphorylation is required for pressure to stimulate cancer cell adhesion

We have previously reported that reducing AKT1, but not AKT2, by siRNA blocked pressure-stimulated SW620 cell adhesion. Furthermore, pressure induced phosphorylation of total AKT in both SW620 colon cancer cells and primary colon cancer cells isolated directly from surgically resected human cancers [16]. These results prompted us to hypothesize that pressure-induced phosphorylation of AKT might also be isoform specific and might be required for the effects of pressure on cancer cell adhesion. As AKT1 and AKT2 are the predominant AKT isoforms expressed in Caco-2 cells [16], we focused on AKT1 and AKT2 in this study. Because phospho-specific antibodies specific for AKT1 and AKT2 are not available, we studied isozyme-specific phosphorylation using human AKT1 and AKT2 expression plasmids tagged with an HA epitope at the N-terminal of the AKT sequence. Caco-2 colon cancer cells transiently transfected with either HA-AKT1 or HA-AKT2 expression plasmid were treated at ambient pressure or 15 mmHg pressure for 30 minutes in dishes precoated with 1% BSA to prevent adhesion and adhesion-associated signaling. Pressure significantly promoted phosphorylation of HA-AKT1, but not phosphorylation of HA-AKT2, in suspended cells (Fig. 1A, $n=4$, $p<0.05$ for AKT1). Consistent with previous reports [25,26], expressed AKT2 migrated more rapidly during SDS-PAGE than AKT1, although the migration of phosphorylated AKT1 and AKT2 was identical (Fig. 1A). We then sought to determine whether the observed AKT1 phosphorylation is important for the regulation of colon cancer adhesion by increased extracellular pressure.

AKT kinase is activated by two kinases, PDK1 and Ser473 kinase, which phosphorylate threonine 308 and serine 473 of Akt respectively [17]. The AKT1 mutant AKT T308A/S473A, in which these two amino acids are replaced by alanines, cannot be activated by PDK1 and Ser473 kinase. This mutant may interfere with endogenous AKT1 activity [27]. We transiently transfected Caco-2 cells with a plasmid which encoded an EGFP-tagged AKT1 T308A/S473A mutant [28]. This overexpressed AKT1 mutant was confirmed to be nonphosphorylatable at 308 and 473 positions by blotting with phospho-specific AKT antibodies (Fig. 1B). Cell adhesion was then compared under ambient or 15 mmHg increased pressure using EGFP fluorescence to identify the transfected cells and considering only those cells in our assay. Parallel studies utilized Caco-2 cells transfected with a non-insertion EGFP plasmid as controls. Overexpression of the nonphosphorylatable AKT1 mutant blocked stimulation of cell adhesion by increased extracellular pressure (Fig. 1C).

Pressure-induced phosphorylation of AKT at Ser473 was blocked by the AKT inhibitor, SH-6

It has been reported that AKT can be phosphorylated/activated either at the plasma membrane or in the cytosol [29]. To clarify the location of AKT phosphorylation in response to increased extracellular pressure, we pretreated Caco-2 cells with SH-6 prior to incubation in suspension at ambient or increased pressure for 30 minutes. SH-6 binds to the PH domain of Akt and traps it in cytoplasm, preventing translocation to the membrane [30]. Pre-treating Caco-2 cells with 10 μ M SH-6 for 1.5 hours blocked the pressure-simulated phosphorylation of AKT at Ser473 without inhibiting endogenous AKT phosphorylation (Fig. 2).

Pressure induces translocation of AKT1, not AKT2 to the plasma membrane and the phosphorylation sites at T308 and/or S473 of AKT1 are required for its translocation

It has been reported that IGF-1-induced Ser473 phosphorylation of AKT greatly requires membrane localization of AKT in human epithelial kidney (HEK) 293 cells [31]. Indeed, translocation of AKT to the plasma membrane is an essential second step in a proposed model of AKT activation [32,33]. We therefore next sought to determine whether, like pressure-

induced phosphorylation of AKT, pressure-induced translocation of AKT is also isoform specific. Consistent with our previous observations in SW620 cells [16], increasing extracellular pressure by 15 mmHg for 30 minutes in suspended Caco-2 cells increased total AKT in a subcellular fraction enriched for plasma membrane and cytoskeletal proteins (Fig. 3A). More importantly, increased pressure was associated with increased AKT1 in this membrane-enriched fraction, but AKT2 did not increase (Fig. 3A). Studies in cells transfected with the non-phosphorylatable AKT1 mutant demonstrated that pressure failed to translocate this nonphosphorylatable AKT1 to plasma membrane, suggesting that pressure-induced translocation of AKT1 requires the availability of the major AKT1 phosphorylation sites at T308 and/or S473 (Fig. 3B).

Construction of chimeras using domain swapping strategy

We next sought to compare the effects of the PH domain and the hinge region upon the specificity of the AKT1 effect. We focused on these regions because their sequences are the most divergent in AKT1 and AKT2. We used a domain swapping strategy to exchange the PH domain and/or hinge region between AKT1 and AKT2 in four chimeras. (Fig. 4A)

The size/length of the PH domain in AKT varies in different reports, from 1–106 [34], 1–111 [35], 1–123 [36,37], 1–129 [11] up to 1–147 [38]. We defined the length of AKT PH domain according to Tschlis [34], i. e., 1–106. We used overlap extension PCR technique to exchange the PH domain and/or hinge region between AKT1 and AKT2 without introducing any unnecessary changes of amino acid residues and potential change of individual domain. The expression of the four chimeras was verified by specific AKT1 antibodies targeting the PH domain or C-tail of AKT1 and AKT2 antibody targeting C-tail of AKT2 (data not shown).

Pressure induces phosphorylation of only chimera2 among the four chimeras

Because extracellular pressure promoted phosphorylation of AKT1, but not AKT2, we next sought to study the effects of pressure on phosphorylation of the chimeras to determine which domains contribute to the isozyme-specificity of AKT phosphorylation induced by extracellular pressure. Pressure stimulated phosphorylation of only chimera2 in which both the PH domain and the hinge region of AKT2 had been exchanged with their counterparts from AKT1, among the four chimeras (Fig. 4B). This suggests that both the PH domain and the hinge region jointly determine the isozyme-specificity of AKT phosphorylation in response to increased extracellular pressure.

Pressure induces translocation to the cell membrane of only chimera2 among the four chimeras

Because pressure translocated AKT1, but not AKT2, to the plasma membrane, we investigated the effects of pressure on translocation of the chimeras after each had been separately and transiently transfected into Caco-2 cells. Among the four chimeras, only chimera2 translocated to the membrane fraction in response to pressure (Fig. 4C, n=4). This further suggests that the distinct response to pressure for AKT1 and AKT2 translocation and phosphorylation requires both the PH domain and the hinge region in AKT1.

The designed AKT1 siRNA specifically and functionally knocked down AKT1

We next sought to determine whether overexpression of AKT2 and/or any chimera can rescue the function of AKT1. To achieve this aim, we designed an AKT1 siRNA targeting the human AKT1 mRNA sequence that codes for the AKT1 catalytic domain, extrapolating from a published AKT1 siRNA targeting the murine AKT1 mRNA similarly coding for the murine AKT1 catalytic domain [39]. This AKT1 siRNA functionally and specifically reduced endogenous AKT1 protein (>70% knock down) at concentrations as low as 10nM (Fig. 5A).

We then compared the specificities of the designed AKT1 siRNA and AKT2 Smartpool siRNA with regard to their ability to reduce expression of AKT1, AKT2 or chimera 2. For this purpose, we used antibodies for Western blotting specific for the PH domain of AKT1 and the C terminus of AKT2. As shown in Fig. 5B, the AKT1 and AKT2 siRNA specifically suppress the expression of endogenous AKT1 and AKT2 respectively. In further studies, we overexpressed wild type AKT2 or chimera 2 (Fig 5C). A slight perhaps compensatory increase in AKT2 was observed after siRNA reduction of AKT1 (top blot in Fig. 5C, lanes 2 vs. 1). However, substantial AKT2 immunoreactivity was observed when either AKT2 or chimera 2 were overexpressed (top blot in Fig. 5C, lanes 3 and 5 respectively) as compared to vector only controls (lane 1), as would be expected since the antibody we used for this study binds to the C terminal end of AKT2 which is shared by chimera2. Furthermore, siRNA to AKT1 did not prevent the overexpression of either wild type AKT2 or chimera 2 (top blot in Fig. 5C, lanes 4 and 6 respectively vs. lanes 3 and 5). This would be expected since the siRNA to AKT1 targets the mRNA sequence coding for the AKT1 catalytic domain, which is not shared by chimera 2. The ability of the AKT1 siRNA to reduce AKT1 itself in these studies was confirmed in parallel blots for AKT1 (Fig. 5C bottom two rows).

Only chimera 2 can rescue the function of AKT1

We then assessed the adhesion of Caco-2 cells co-transfected with AKT1 siRNA and plasmids encoding AKT2 as well as chimera2, chimera3, and chimera4 respectively together with pEGFP-C1. Studies using a fluorescent non-targeting control siRNA demonstrated that we can expect approximately 90% transfection with the siRNA under these circumstances (not shown), so it seemed reasonable to predict that most cells expressing the chimeras would also experience siRNA knockdown of the endogenous protein. Only chimera2 could rescue the function of AKT1 in mediating pressure-associated increased adhesion (Fig. 5D). We used siRNA targeted to AKT2 as an additional control in these studies, and found no effect of AKT2 siRNA on either basal or pressure-stimulated adhesion, consistent with our previous observations [2]. Therefore, both the PH domain and the hinge region of AKT contribute to the functional difference between AKT1 and AKT2 in pressure-stimulated cell adhesion.

Overexpression of AKT2, but not AKT1 blocks pressure-stimulated Caco-2 cell adhesion

Because AKT2 failed to rescue the function of AKT1 in mediating pressure-stimulated cancer cell adhesion, we next investigated the effect of AKT2 overexpression on pressure-stimulated adhesion, when endogenous AKT1 expression is not suppressed. Overexpression of HA-tagged AKT2 did not affect levels of endogenous AKT1 protein. Similarly, overexpression of HA-AKT1 did not significantly affect endogenous AKT2 protein levels. Overexpression of AKT2, but not AKT1, blocked pressure-stimulated colon cancer cell adhesion compared with overexpressing cells at ambient pressure (Fig. 6A).

Overexpression of chimera1, but not chimera2 blocks the pressure-stimulated cell adhesion

We used the same technique to study the effects of overexpression of chimera1 and chimera2 on pressure-stimulated adhesion. Overexpression of chimera1, but not chimera2, blocked the stimulation of adhesion by increased extracellular pressure (Fig. 6B) in the same manner that we had observed for AKT2 overexpression.

Discussion

We have previously reported that 15 mm Hg increased pressure activates AKT in colon cancer cells in a manner requiring PI-3K, Src and FAK [16]. Our present observations of cell adhesion, AKT translocation and AKT phosphorylation strongly suggest that only AKT1 mediates pressure-stimulated cancer cell adhesion, that the PH domain and the hinge region between the PH domain and the catalytic domain jointly determine this ability of the AKT1 isoform to

become phosphorylated, translocate, and eventually cause increased cancer cell adhesion in response to extracellular pressure, and that phosphorylation of AKT1 at threonine 308 and/or serine 473 is required for the effect.

Increasing evidence suggests that although AKT isoforms are highly homologous AKT nevertheless sometimes exhibits isoform-specific functions [15,20,40]. However, the isoform specificity observed here is not necessarily predictable. For instance, increases in extracellular pressure also stimulate macrophage phagocytosis, but in this case AKT2 appears responsible [41].

Competing models have been proposed to explain the specificities of AKT isoforms in mediating various effects [12]. These include different expression levels of the AKT isoforms, different substrates of each AKT isoform, and differential compartmentalization of isoforms via binding to unique adaptor proteins. It is unlikely that different expression levels of AKT1 and AKT2 account for the distinct roles of AKT in pressure-induced colon cancer cell adhesion since HA-tagged AKT2 acted as a dominant negative to suppress the effect while HA-tagged AKT1 expressed at a comparable level did not. Substrate specificity is more difficult to disprove since AKT could phosphorylate more than 9000 substrates [42]. However, AKT phosphorylation of its substrates is believed to involve the catalytic domain, which is highly conserved between AKT1 and AKT2. Indeed, substitution of the PH domain and hinge region from AKT1 by AKT2 conferred AKT1 function in pressure-induced cell adhesion upon AKT2, even though the AKT2 chimera retained its original catalytic domain. It therefore seems less likely that substrate specificity distinguishes the two isoforms in this regard. Instead, our present results seem more consistent with the third model, differential compartmentalization of isoforms via binding to unique adaptor proteins, since only AKT1 and the chimera sharing the PH domain and hinge region of AKT1 translocated to the membrane-enriched fraction in response to extracellular pressure. Increases in extracellular pressure may activate or otherwise make available an adapter protein that specifically binds to the PH domain and hinge region of AKT1, sequestering the AKT1 to the plasma membrane where it becomes active. Several candidate adapter proteins have been described, including Hsp90, JNK Interacting Protein-1 (JIP1) and Grb10 [17,43,44]. Although JIP-1 has been reported to bind preferentially to AKT1 over AKT2 in HEK293 cells, its specificity in epithelial cancer has not yet been characterized. The isoform specificity of other candidate adaptor proteins in this setting, such as HSP90 and Grb 10 also awaits clarification beyond the scope of the current investigation. Although reducing AKT2 does not block pressure-stimulated cell adhesion [16], overexpressing AKT2 can do so. The overexpressed AKT2 may compete with AKT1 for binding to the mechanisms that sequester AKT1 to the plasma membrane, but be unable to translocate.

Although AKT is known to be phosphorylated by physical forces such as cyclic strain in mesangial cells [18], flow in vascular endothelial cells [19], fluid shear in human umbilical venous endothelial cells [20] and 60mm Hg rise in aortic pressure in rat heart[45], the isozyme specificity of such phosphorylation has not been studied. Extracellular pressure promotes phosphorylation of AKT1, but not AKT2.

The location of Akt activation is not completely understood. Akt has been reported to be regulated by translocation from the cytosol to the plasma membrane in cells activated by many growth factors [17]. At the plasma membrane, PDK1 and other kinases may phosphorylate Akt at Thr308 and Ser473 residues of Akt1, and at the equivalent phosphorylation sites for Akt2 and Akt3 [8]. However, AKT has also been reported to be phosphorylated directly within the cytosol in neutrophils stimulated by PGE2 or other agents that increase cAMP without a requirement for translocation [29] Furthermore, AKT phosphorylated at the membrane after stimulation by many growth factors rapidly translocates back into the cytosol and nucleus within a few minutes[46].

Our present data supports the model that AKT1 is phosphorylated at the plasma membrane in response to pressure after translocation (Fig. 7). SH-6 binds to the PH domain of Akt and keeps it in cytoplasm [30]. One recent report indicates that although SH-6 inhibits PDGF-induced Akt phosphorylation, SH-6 does not inhibit endogenous Akt phosphorylation or activity in lung cancer cells [47]. In our model, SH-6 similarly blocked the pressure-stimulated phosphorylation of AKT at Ser473 without reducing basal AKT phosphorylation. Taken together with our translocation experiments, the results of this SH-6 study strongly suggest that pressure-induced phosphorylation of AKT takes place at the membrane after translocation from the cytosol.

We did observe that, in contrast to wild type AKT1, the nonphosphorylatable T308A/S473A AKT1 mutant failed to translocate to the membrane-enriched fraction in response to extracellular pressure. This is consistent with a recent finding that this nonphosphorylatable construct does not translocate from the cytosol to the nucleus in hepatocellular carcinoma SMMC-7721 cells stimulated by PDGF [46]. This could imply that the AKT1 phosphorylation sites are required for its translocation to the membrane in response to pressure. Alternatively, however, these point mutations could interfere with AKT binding to the adapter proteins involved in its translocation.

Among the four chimeras, only chimera2, which spliced the PH domain and hinge region of AKT1 into AKT2, was phosphorylated in response to increased extracellular pressure, and only chimera2 translocated into the membrane-enriched fraction in response to pressure. This further suggests that AKT1 phosphorylation in this system requires both the AKT1 PH and hinge region. This may be necessary for AKT1 translocation to the membrane where its phosphorylation/activation occurs. AKT1 can also be specifically phosphorylated or activated by other stimuli in other cells. For instances, EGF promotes Ser 473 phosphorylation only in AKT1 in some, but not all, human esophageal squamous cancer cell lines [48], and exercise activates only AKT1 in rat skeletal muscle [49].

AKT is conventionally thought to act primarily within the cytosol or nucleus after translocation back from the membrane where it has been activated [32]. However, in this system, AKT1 may act at the membrane, possibly at the focal adhesion complex, to further modulate adhesion in response to extracellular pressure. AKT has previously been reported to phosphorylate targets at the plasma membrane as well as within the cytosol or nucleus [50].

Overexpression of the dominant-negative (non- phosphorylatable) Akt1 mutant significantly ablated the increase in colon cancer cell adhesion stimulated by increased extracellular pressure, thus indicating the direct involvement of pressure-induced Akt1 phosphorylation for pressure-stimulated cell adhesion. This is consistent with a previous report suggesting that overexpression of a dominant-negative Akt mutant inhibited shear stress-induced suppression of apoptosis in endothelial cells [20].

Our finding that the PH domain and the hinge region jointly determine the ability of AKT1 to mediate the effects of pressure on cell adhesion is consistent with a recent report suggesting that both the PH domain and the hinge region are essential for opposing roles for AKT1 and AKT2 in cell migration [12]. However, the PH domain and the hinge region seem not always important in distinguishing AKT1 and AKT2. For instance, the regulatory domain at the C-terminal end of AKT contributes to the functional differences between AKT1 and AKT2 in regulating the transcription factor CREB [51]. Clearly, AKT isoform specificity is complex and varies with the signal pathway under study.

In summary, our observations identify the PH domain and hinge region of AKT1 as structural determinants in permitting AKT1 translocation and phosphorylation that in turn mediate pressure-stimulated colon cancer cell adhesion (Fig. 7). There are manifest differences between

tumor cell adhesion in vitro to matrix proteins or endothelial cells, or even to a murine surgical wound, and local wound recurrence or metastasis from an intact cancer in a patient. Nevertheless, these results raise the possibility that the stimulation of cancer cell adhesion by physical forces might be inhibited by a PH-domain-dependent AKT1 specific inhibitor or a small molecule inhibitor designed to mimic the structural determinants of one of the dominant negative constructs described here.

Acknowledgements

Grant support: This work was supported in part by NIH grant 2R01DK060771 and VA Merit Research support to Marc D. Basson.

This work was supported by National Institutes of Health Grant 2R01DK060771 and a VA Merit Research Award (each to M. D. B.). We thank Dr. Matthew D. Ringel (Ohio State University) for the generous gift of EGFP fused AKT1 mutant (T308A/S473A) and Dr. Paula Herman (Dana Farber Cancer Institute) for providing the plasmids of pcDNA3 myr HA AKT1 and pcDNA3 myr HA AKT2 through Addgene (Cambridge, MA). The technical assistance of Drs. Vijayalakshmi Thamilselvan, Lakshmi S. Chaturvedi, and Matthew A. Sanders is gratefully acknowledged.

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Figure 1. Pressure induces phosphorylation of AKT1, not AKT2

A. Caco-2 cells were transfected with plasmids expressing HA-tagged AKT1 or AKT2, the cells were harvested, and AKT1 or AKT2 was immunoprecipitated from the cell lysates with anti-HA antibody followed by phospho-AKT (Ser473) Western blotting. The top panel represents typical blots, and the graph summarizes densitometric analysis of the ratios of phospho-AKT1 or AKT2 to total AKT in each experiment ($n=4$, *, $p<0.05$, NS: not significant). **B.** The plasmid expressing EGFP-fused AKT1 dominant negative mutant (T308A/S473A) was verified to express the AKT1 mutant which is nonphosphorylatable at either Thr308 or Ser473 site. Caco-2 cells were transfected with either empty EGFP plasmid or the AKT1 mutant plasmid. Phosphorylation of AKT1 at Thr308 or Ser473 site was assessed by Western blotting for phospho-AKT (Thr308) or phospho-AKT (Ser473) antibody, followed by reprobing with AKT1 antibody after stripping the membranes. **C.** Nonphosphorylatable AKT1 mutant blocks pressure-stimulated Caco-2 cell adhesion. Caco-2 cells were transfected with plasmids expressing EGFP only or EGFP-tagged phosphorylatable AKT1. Cell adhesion was assessed by fluorescence microscopy ($n=3$, *, $p<0.05$, NS: not significant).

Figure 2. AKT inhibitor, SH-6 blocks the pressure induced phosphorylation of AKT at Ser473
 Caco-2 cells were pretreated with 10 μ M AKT inhibitor, SH-6 or a DMSO vehicle control for 1.5 hours at 37 °C. The SH-6-treated Caco-2 or control cells were then incubated in suspension under ambient or increased pressure. The cells were then harvested and lysed on ice in lysis buffer followed by phospho-AKT (Ser473) Western blotting. GAPDH was used as loading control. The top panel represents typical blots and the lower graph summarizes densitometric analysis of ratios of phospho-AKT to GAPDH (n=6, *, $p < 0.05$, NS: not significant).

Figure 3. Pressure induces translocation of AKT1, not AKT2, to the cell membrane, and the phosphorylation sites at T308 and/or S473 are required for AKT1 translocation

A. Pressure induces translocation of endogenous AKT1, not AKT2. AKT or AKT1 protein levels in a membrane-enriched subcellular fraction were significantly increased in pressure-treated cells (shaded bars) compared to control cells at ambient pressure (open bars). E-Cadherin served as a control for the membrane-enriched fraction. Typical blots are shown in the upper panel and densitometric analysis of ratios of AKT1, AKT2 or total AKT to E-Cadherin in the lower graph ($n=5$, $*$, $p<0.05$, NS: not significant). **B.** Pressure fails to induce the translocation of nonphosphorylatable AKT1 mutant in which Thr308 and Ser473 were mutated to alanine ($n=4$, $*$, $p<0.05$, NS: not significant).

Figure 4. Domain structure of AKT and chimeras between AKT1 and AKT2 and pressure induces phosphorylation and translocation of only chimera2 among the four chimeras

A. Each AKT isoform consists of four domains: N-terminal PH domain, hinge region, catalytic domain and a C-terminal regulatory tail domain (*C-tail*). All constructs have an N-terminal HA tag. The PH domain and hinge region between AKT1 and AKT2 were first exchanged to produce chimera1 and chimera2. Chimera 3 and chimera4 were made by exchanging only the PH domain or hinge region from AKT2 with that from AKT1. **B.** Pressure induces phosphorylation of only chimera2 among the four chimeras. Caco-2 cells transfected with plasmids expressing the indicated HA-tagged chimeras were incubated in suspension under ambient or increased pressure and then harvested. The four chimeras were then immunoprecipitated from cell lysates with anti-HA antibody followed by phospho-AKT (Ser473) Western blotting. The top panel represents typical blots and the lower graph summarizes densitometric analysis of ratios of the phospho-chimeras to total AKT. (n=4, *, $p<0.05$, NS: not significant). **C.** Pressure induces translocation of only chimera2 among the four chimeras to the cell membrane. Caco-2 cells transfected with the indicated chimera expression plasmids were incubated under ambient or increased pressure in suspension and then harvested and fractionated. Protein levels of the HA-tagged chimeras in the membrane-enriched fraction were then assessed by HA 12CA5 Western blot. Pressure induced translocation of chimera2, but not of other chimeras to the membrane-enriched fraction. E-Cadherin served as a control for the membrane fraction. Typical blots are shown in the upper

panel and densitometric analysis in the lower graph of ratios of the indicated HA-tagged chimeras to E-Cadherin (n=4, *, $p<0.05$, NS: not significant).

Figure 5. The designed AKT1 siRNA specifically and functionally knocked down AKT1 expression and only chimera 2 can rescue the function of AKT1 after suppression of endogenous AKT1 by the AKT1 siRNA

A. Dose response of AKT1 siRNA. Caco-2 cells were transiently transfected with the designed AKT1 siRNA at 10–100 nM 72 hours prior to lysis and Western blot. AKT1 Smartpool siRNA (100 nM) served as a positive control and 100 nM nontargeting NT1 as a negative control. The AKT1 siRNA was designed to target to AKT1 RNA corresponding to the catalytic domain of AKT1. **B.** AKT1 and AKT2 siRNA specifically suppressed the expression of endogenous AKT1 and AKT2 respectively. Caco-2 cells were transiently transfected with the designed AKT1 siRNA or AKT2 Smartpool siRNA at 10 nM 72 hours prior to lysis and Western blot. Nontargeting NT1 served as a negative control. **C.** The AKT1 siRNA did not affect protein levels of AKT2 or chimera2. Note that the AKT1 monoclonal antibody targets the PH domain of AKT1, and therefore cannot detect chimera2. The AKT2 monoclonal antibody targets to C-terminal of AKT2, and therefore recognizes chimera2. The empty vector (Vector) was used as control. **D.** Only chimera 2 can rescue the function of AKT1. Caco-2 cells transfected with either NT1 or AKT1 siRNA were then co-transfected with either pCMV-HA control plasmid or indicated AKT2/chimera2/chimera3/chimera4 expression plasmids together with EGFP expression plasmid at 1:4 lower concentration to mark successfully transfected cells. SMARTpool AKT2 siRNA transfected Caco-2 cells were used as an additional control. 72 hours after siRNA transfection, cell adhesion was assessed by fluorescence microscopy. (n=6, *, $p<0.05$, NS: not significant).

Figure 6. The overexpression of AKT2 or chimera1, but not AKT1 or chimera2 blocks pressure-stimulated Caco-2 cell adhesion

A. Caco-2 cells were co-transfected with indicated AKT1/AKT2 expression plasmids or with a non-insertion vector together with EGFP expression plasmid at 1:4 lower concentration to mark successfully transfected cells. Cell adhesion was assessed by fluorescence microscopy (n=4, *, $p < 0.05$, NS: not significant). **B.** Overexpression of chimera1, but not chimera2 blocks pressure-stimulated Caco-2 cell adhesion. Caco-2 cells were co-transfected with indicated AKT2/chimera1/chimera2 expression plasmids or with non-insertion vector together with EGFP expression plasmid to mark the successfully transfected cells. The empty vector and the

AKT2 expression plasmid were used as controls for this experiment. Cell adhesion was assessed by fluorescence microscopy (n=6, *, $p<0.05$, NS: not significant).

Figure 7.

Schematic diagram represents a model based on the present report. Pressure specifically stimulates AKT1 to translocate from the cytosol to the plasma membrane, where AKT1 is phosphorylated. The phosphorylated AKT1 then returns to cytosol and mediates cancer cell adhesion through acting its downstream substrates. AKT2 remains in the cytosol while the pressure stimulation. The PH domain and hinge region of AKT1 are responsible for the specificity of the distinct roles of AKT1, as opposed to AKT2, in mediating pressure-stimulated cell adhesion.