The Cleavage of Akt/Protein Kinase B by Death Receptor Signaling Is an Important Event in Detachment-induced Apoptosis*

Received for publication, March 29, 2001, and in revised form, June 8, 2001
Published, JBC Papers in Press, July 19, 2001, DOI 10.1074/jbc.M102806200

Robin E. Bachelder‡§, Melissa A. Wendt‡, Naoya Fujita¶, Takashi Tsuruo‡, and Arthur M. Mercurio†

From the ‡Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, ¶Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032, Japan, and |Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo 113-0032, Japan

Epithelial cells undergo death receptor-dependent apoptosis when detached from matrix, a process termed anoikis. Activation of Akt/protein kinase B (PKB) by matrix attachment protects cells from anoikis. In this study, we establish a link between anoikis and Akt/PKB-mediated survival by demonstrating that Akt/PKB is cleaved by caspases in matrix-detached epithelial cells by a mechanism that involves death receptors. Reduced levels of Akt/PKB protein were observed in detached Madin-Darby canine kidney cells relative to cells attached to collagen. Equivalent levels of Akt/PKB, however, were detected in matrix-adherent and detached cells after inhibition of caspase activity or expression of an Akt/PKB mutant (D108/H11545) that is resistant to caspase cleavage. The contribution of death domain-containing proteins to Akt/PKB cleavage was evidenced by the ability of dominant negative Fas-associated death domain to restore normal levels of Akt/PKB in matrix-detached cells. Importantly, expression of a cleavage-resistant Akt/PKB mutant protected matrix-detached cells from apoptosis. These studies suggest that members of the death receptor family promote the caspase-mediated cleavage of Akt/PKB and that this event contributes to anoikis.

Epithelial (1) and endothelial (2) cells undergo apoptosis when they are deprived of matrix attachment, a phenomenon that has been termed anoikis. Matrix receptors (integrins) activate key signaling molecules that inhibit this form of apoptosis. One of the most important signaling molecules in this respect is the Akt/PKB ser/threonine kinase (3). The regulation of Akt/PKB kinase activity by integrins and growth factor receptors has been a focus of intense investigation because of its widespread importance in cell survival (4). Phosphatidylinositol 3-kinase (PI3K) is essential for Akt/PKB activation because the D3 phosphoinositide products of PI3K bind to the pleckstrin homology (PH) domain of Akt/PKB (5, 6). This interaction results in the recruitment of Akt/PKB to the cell membrane and an Akt/PKB conformational change, events that are required for its phosphorylation on threonine residue 308 by PI3K-dependent kinase 1 (7–9). After this phosphorylation event, Akt/PKB is phosphorylated at serine residue 473, resulting in the stimulation of its kinase activity toward exogenous substrates (10). The ability of Akt/PKB to phosphorylate and inhibit the activity of proapoptotic proteins such as Bad (11, 12), the Forkhead transcription factors (13, 14), and caspase 9 (15) has been suggested to contribute to its critical role in maintaining cell survival.

Recent studies have implicated the TNF family of death receptors in anoikis (16, 17). These transmembrane receptors contain one or more intracellular death domains, which mediate the recruitment and activation of initiator caspases (e.g. caspase 8) that in turn stimulate the activity of executioner caspases (e.g. caspases 3 and 9). As implied by their name, the executioner caspases promote apoptosis by directing the cleavage of key substrates in cells. The finding that inhibitors of death receptors block anoikis indicates that these receptors regulate the activity of caspases, which in turn cleave key substrates in these cells to promote their apoptosis. In fact, several substrates of caspase activity have been identified in matrix-detached epithelial cells including β-catenin (18) and MEKK (19). An important issue that arises from these findings is whether the caspase-mediated cleavage of such substrates is necessary for anoikis. For example, although β-catenin is a target of caspase activity during anoikis (18), the importance of this cleavage event in the induction of apoptosis has not been established. In addition, MEKK is activated in matrix-detached epithelial cells as a result of its cleavage by caspases (19). However, the expression of a cleavage-resistant MEKK in matrix-detached epithelial cells only partially inhibits their apoptosis, suggesting that other caspase targets are important for anoikis (19).

Given the importance of Akt/PKB in inhibiting apoptosis, we hypothesized that this kinase is a critical target of negative regulation by death receptor signaling during anoikis. The results obtained in this study demonstrate that death domain-containing proteins promote the caspase-dependent cleavage of Akt/PKB in matrix-detached epithelial cells. Importantly, our data suggest that Akt/PKB cleavage contributes to anoikis.

* This work was supported by National Institutes of Health Grants CA80789 and CA89209 (to A. M. M.) and CA81697 (to R. E. B.).
† To whom correspondence should be addressed: Beth Israel Deaconess Medical Center, Research North, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-1449; Fax: 617-667-5531; E-mail: rbacheldlcaregroup.harvard.edu.
‡ The abbreviations used are: PKB, protein kinase B; MDCK, Madin-Darby canine kidney; FADD, Fas-associated death domain; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; TNF, tumor necrosis factor; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin; HRP, horse-radish peroxidase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; PI, propidium iodide; poly-HEME, poly-(2-hydroxyethyl methacrylate); annexin-PE, annexin-phycocerythrin.

This paper is available on line at http://www.jbc.org
**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—MDCK cells were provided by Dr. Karl Matlin (Beth Israel Deaconess Medical Center, Boston, MA) and maintained in low-glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The following primary antibodies were used for immunoblots: (a) sheep antiserum specific for the PH domain of Akt/PKB (Upstate Biotechnology; 1:150 final dilution), (b) a mouse monoclonal antibody specific for FLAG (Sigma; 1:1000 final dilution), (c) mouse anti-HA (Roche Molecular Biochemicals; 1:1000 dilution), (d) rabbit anti-actin (Sigma; 1:1000 final dilution), and (e) rabbit anti-p85 (Santa Cruz Biotechnology; 1:1000 final dilution). Akt/PKB activity was assessed using a rabbit antisera specific for Akt/PKB molecules phosphorylated on serine residue 473 (New England Biolabs). HRP-conjugated goat anti-rabbit and mouse IgG (Sigma) were used at a blotting concentration of 1:15,000, whereas HRP-conjugated donkey anti-goat IgG (BIOSOURCE) was used at a final concentration of 1:25,000.

**Transient Transfections**—MDCK cells were co-transfected using the Lipofectamine reagent (Life Technologies, Inc.) with a green fluorescent protein-expressing vector (pEGFP-1; CLONTECH; 1 μg) in addition to either a control vector (pEDNA; 1 μg) or the indicated Akt/PKB-expressing construct (1 μg). Akt/PKB constructs included FLAG-tagged vectors expressing wild type Akt/PKB, an Akt/PKB mutant containing an alanine substitution at aspartatic acid residue 108, an Akt/PKB mutant containing an alanine substitution at aspartatic acid residue 119, and an Akt/PKB double point mutant containing alanine substitutions at aspartic acid residues 108 and 119. The construction of these FLAG-tagged constructs has been described previously (20). To assess the importance of TNF receptor signaling in Akt/PKB proteolysis, MDCK cells were transfected using the Lipofectamine reagent with pEGFP (1 μg) and either a control vector (pEBB; 1 μg) or a plasmid expressing dominant negative FADD (1 μg), both of which were provided by Roya Khosravi-Far (Beth Israel Deaconess Medical Center).

**Matrix Attachment Assays**—Wells of a 6-well tissue culture plate were incubated with either poly-HEMA (1% solution in ethanol) or collagen (25 μg/ml in PBS) overnight. After washing extensively with PBS, the wells were blocked with serum-free media containing 1% bovine serum albumin. MDCK cells transfected with the indicated constructs were plated onto poly-HEMA- or collagen-precoated wells at a density of 1.5 × 10^5 cells/well in serum-free media containing 1% bovine serum albumin. For caspase inhibitor experiments, MDCK transfectants were pretreated for 30 min on ice with either MeSO (1,500 dilution) or Z-DEVD-FMK (4 μM/ml; Calbiochem), a cell-permeable inhibitor of caspase 3 family members. These cells were then incubated on either poly-HEMA or collagen as described above in the continual presence of the indicated caspase inhibitor.

**Determination of Akt/PKB Protein Levels**—Nonadherent cells were collected from poly-HEMA-coated wells, washed twice with PBS, and lysed in Akt/PKB lysis buffer (20 mM Tris, pH 7.4, 0.14 mM NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 μM/ml leupeptin, 50 μM/ml leupepin, and 1 mM sodium orthovanadate). After removing nonadherent cells, collagen-attached cells were washed twice with PBS and lysed by adding 200 μl of Akt/PKB lysis buffer directly to the wells. Cellular debris was removed by centrifugation at 12,000 × g for 10 min at 4 °C, and equivalent amounts of total cellular protein were subjected to reducing SDS-PAGE (8%). These proteins were transferred to nitrocellulose and probed with the indicated Akt/PKB- or FLAG-specific antibody. To confirm equal protein loading, these proteins were subjected to reducing SDS-PAGE (8%). These proteins were transferred to nitrocellulose and probed with the indicated Akt/PKB- or FLAG-specific antibody. To confirm equal protein loading, these proteins were subjected to reducing SDS-PAGE (8%). The percentage of apoptotic cells was determined by assessing the percentage of cells that bound annexin V-FITC but were negative for PI uptake. In addition, the percentage of necrotic cells was assessed by determining the percentage of the total cell population that was positive for PI uptake. At least 5000 cells were analyzed for each sample.

For annexin V-PE stains of GFP transfectants, GFP-positive cells were gated and analyzed for annexin V-PE positivity. Because of an observed overlap between FL1 and FL2 channels at high FL1 intensities, the brightest GFP-positive cells were excluded from analysis. A minimum of 2500 GFP-positive cells were analyzed for each sample. The percentage of anoikis was determined as the difference between the percentage of collagen and HEME-plated cells that were positive for annexin V-PE staining.

**RESULTS**

**Akt/PKB Protein Levels Are Reduced in Matrix-detached MDCK Cells Coincident with the Induction of Apoptosis**—Based on the finding that detachment of adherent cells from matrix stimulates their apoptosis (1, 2), we investigated the hypothesis that the matrix detachment of epithelial cells promotes apoptosis by inhibiting Akt/PKB. Initially, we established a time course for the detachment-induced apoptosis of MDCK cells. Cells were maintained in serum-free medium and either plated on collagen or deprived of matrix attachment by plating on poly-HEMA. These cells were harvested at various times, and the level of apoptosis was determined by staining with annexin V-FITC and PI. As shown in Fig. 1, MDCK cells that had been maintained in suspension for 30 min exhibited significantly higher annexin V staining than MDCK cells plated on collagen. These cells were in the early stages of apoptosis based on their ability to bind annexin V and exclude propidium iodide. After maintaining these cells for 2 h in suspension, we observed an increase in annexin V-positive, PI-negative cells, as well as a 3-fold increase in the percentage of cells stained by propidium iodide, confirming the ability of matrix detachment to induce cell death (Fig. 1).

Subsequently, we investigated the effects of detachment-induced apoptosis on Akt/PKB expression. MDCK cells were transfected with a HA-tagged, full-length Akt/PKB construct and plated on either collagen or poly-HEMA for 2 h. Equivalent amounts of total cellular protein obtained from these cells were then subjected to immunoblotting with a HA-specific antibody. As shown in Fig. 2A, total cellular levels of HA-tagged Akt/PKB were reduced by 70% in MDCK cells that had been detached...
Nonadherent and adherent cells were collected relative to matrix-attached epithelial cells. MDCK cells were transfected transiently with HA-tagged Akt/PKB. Twenty-four h after transfection, these cells were plated for 2 h on either collagen (Col) or poly-HEME (Susp). Nonadherent and adherent cells were collected from poly-HEME and collagen wells, respectively, and extracted with Akt/PKB lysis buffer (20 mM Tris, pH 7.4, 0.14 M NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL peptatin, 50 μg/mL leupeptin, and 1 mM sodium orthovanadate). Equivalent amounts of total protein from these extracts were separated on 8% SDS-PAGE and immunoblotted with a HA-specific monoclonal antibody (Roche Molecular Biochemicals), followed by HRP-conjugated secondary antibody. To control for protein loading, this blot was stripped and reprobed with an actin-specific rabbit polyclonal antiserum, followed by HRP-conjugated goat anti-rabbit IgG. Similar results were obtained in six independent experiments. B, the levels of endogeuous Akt/PKB are reduced in suspension relative to matrix-attached epithelial cells. MDCK cells were plated for 2 h on either collagen (Col) or poly-HEME (Susp). Nonadherent and adherent cells were collected as described above. Equivalent amounts of total protein from these extracts were then subjected to immunoblotting with an antiserm specific for the PH domain of Akt/PKB (Upstate Biotechnology), followed by the appropriote HRP-conjugated secondary antibody. As a control for protein loading, these samples were also reprobed with a rabbit polyclonal antiserm specific for the p85 subunit of PI3K (Santa Cruz Biotechnology), followed by HRP-conjugated anti-rabbit IgG. Similar results were observed in three separate experiments. C, importance of death domain-containing proteins in reducing Akt/PKB protein levels in matrix-detached epithelial cells. MDCK cells were transfected transiently with HA-tagged Akt/PKB and either a control plasmid or a dominant negative FADD, an inhibitor of death domain-containing proteins (21, 22). These transfectants were plated on either collagen or poly-HEME, and the amount of Akt/PKB in extracts obtained from these cells was assessed by immunoblotting with a HA-specific antibody. Similar to the results observed in Fig. 2A, Akt/PKB protein levels were reduced by 90% in control transfectants that had been plated on poly-HEME relative to those plated on collagen (Fig. 2C). In contrast, Akt/PKB protein levels were not reduced in suspension relative to matrix-attached MDCK cells that expressed dominant negative FADD (Fig. 2C). These results demonstrate that TNF death receptor signaling, which has been shown to be critical in detachment-induced apoptosis (16, 17), is important in reducing the level of Akt/PKB in matrix-detached MDCK cells.

Akt/PKB Is Cleaved by Caspases in Matrix-detached MDCK Cells—Based on the results from previous studies establishing the importance of caspase activity in anoikis (23), we investigated whether the reduction in Akt/PKB protein levels observed in matrix-deprived MDCK cells was caspase-dependent. HA-Akt/PKB-transfected MDCK cells that had been pretreated with either Me2SO or an inhibitor of caspase 3 subfamily proteases (Z-DEVD-FMK) were plated on either collagen or poly-HEME for 2 h. Equivalent amounts of total cellular protein from extracts of these cells were subjected to immunoblotting with a HA-specific monoclonal antibody as described in A. The expression of dominant negative FADD in these transfectants was confirmed by the detection of a HA-reactive band at 28 kDa (data not shown). Similar results were observed in three independent experiments.

Recent reports that members of the TNF death receptor family mediate detachment-induced apoptosis (16, 17), we sought to determine whether these receptors are also important in reducing Akt/PKB protein levels in matrix-deprived cells. MDCK cells were co-transfected with HA-tagged Akt/PKB and either a control plasmid or dominant negative FADD, an inhibitor of death domain-containing proteins (21, 22). These transfectants were plated on either collagen or poly-HEME, and the amount of Akt/PKB in extracts obtained from these cells was assessed by immunoblotting with a HA-specific antibody. Similar to the results observed in Fig. 2A, Akt/PKB protein levels were reduced by 90% in control transfectants that had been plated on poly-HEME relative to those plated on collagen (Fig. 2C). In contrast, Akt/PKB protein levels were not reduced in suspension relative to matrix-attached MDCK cells that expressed dominant negative FADD (Fig. 2C). These results demonstrate that TNF death receptor signaling, which has been shown to be critical in detachment-induced apoptosis (16, 17), is important in reducing the level of Akt/PKB in matrix-detached MDCK cells.

Reduction in Akt/PKB Protein Levels during Anoikis Is Dependent on TNF Family Death Receptor Signaling—Based on recent reports that members of the TNF death receptor family mediate detachment-induced apoptosis (16, 17), we sought to determine whether these receptors are also important in reducing Akt/PKB protein levels in matrix-deprived cells. MDCK cells were co-transfected with HA-tagged Akt/PKB and either a control plasmid or dominant negative FADD, an inhibitor of death domain-containing proteins (21, 22). These transfectants were plated on either collagen or poly-HEME, and the amount of Akt/PKB in extracts obtained from these cells was assessed by immunoblotting with a HA-specific antibody. Similar to the results observed in Fig. 2A, Akt/PKB protein levels were reduced by 90% in control transfectants that had been plated on poly-HEME relative to those plated on collagen (Fig. 2C). In contrast, Akt/PKB protein levels were not reduced in suspension relative to matrix-attached MDCK cells that expressed dominant negative FADD (Fig. 2C). These results demonstrate that TNF death receptor signaling, which has been shown to be critical in detachment-induced apoptosis (16, 17), is important in reducing the level of Akt/PKB in matrix-detached MDCK cells.
whether they were maintained in suspension or plated on matrix (Fig. 4B). These results indicate that Akt/PKB is cleaved by caspases during detachment-induced apoptosis at aspartic acid residues 108 and 119. In addition, these data identify an Akt/PKB mutant (D108+119A) that is resistant to caspase cleavage. Collectively, our results support a pathway of detachment-induced apoptosis that consists of TNF death receptor-dependent caspase activation, followed by the caspase-dependent cleavage of Akt/PKB at aspartic acid residues 108 and 119.

**Importance of Akt/PKB Cleavage in Anoikis**—Our results demonstrate that during anoikis, Akt/PKB is cleaved at aspartic acid residues 108 and 119 by caspases. To assess the functional importance of Akt/PKB cleavage in anoikis, MDCK cells were co-transfected with a GFP construct and either wild-type Akt/PKB or the Akt/PKB double point mutant (D108+119A). Expression of these Akt/PKB constructs was confirmed by immunoblotting extracts from these cells with an antibody specific for the FLAG tag (Fig. 5A). These transfectedants were plated on either poly-HEME or collagen for either 2 or 4 h, and the level of apoptosis in GFP-positive cells was quantified by annexin V-PE staining (Fig. 5B). We observed that expression of the Akt/PKB double point mutant (D108+119A) inhibited detachment-induced apoptosis in these cells by ~60% at both of these time points, whereas expression of wild-type Akt/PKB inhibited apoptosis by less than 20% (Fig. 5B). Importantly, the Akt/PKB D108+119A mutant did not exhibit increased activity over wild-type Akt/PKB in transfected MDCK cells (Fig. 5C), confirming that this mutant construct did not promote cell survival because of an intrinsic ability of these mutations to increase Akt/PKB activity. We also observed that neither the Akt/PKB D108A mutant nor the Akt/PKB D119A mutant promoted the survival of matrix-detached MDCK cells (data not shown), consistent with the observation that these mutants are efficiently cleaved by caspases in matrix-detached MDCK cells (Fig. 4B). Collectively, these results suggest that the proteolytic removal of the PH domain of Akt/PKB contributes to detachment-induced apoptosis.

**DISCUSSION**

Although Akt/PKB is known to be essential in promoting cell survival, little attention has been given to the hypothesis that Akt/PKB is regulated negatively by apoptotic signaling pathways. Recent work by our groups foreshadowed this possibility by demonstrating that Akt/PKB is a substrate for caspase 3 (20, 24). In the current study, we demonstrate that the activation of TNF death receptors in response to the detachment of epithelial cells from matrix induces the caspase-mediated cleavage of Akt/PKB. Moreover, our data identify Akt/PKB cleavage as an important event in anoikis. Although previous studies established that the Akt/PKB pathway can inhibit the activity of proapoptotic molecules (11–14), our studies identify a reciprocal form of regulation, namely, the effect of death receptor signaling on Akt/PKB. In addition to its role in anoikis, the regulation of Akt/PKB by death receptors likely has widespread implications for cell survival.

The caspase-mediated proteolysis of Akt/PKB appears to contribute to anoikis because we observed a 60% inhibition of apoptosis in matrix-detached MDCK cells upon the expression of a caspase-resistant Akt/PKB construct. Importantly, the expression of wild-type Akt/PKB in MDCK cells inhibited anoikis only marginally, demonstrating that the restored survival of cells transfected with the caspase-resistant Akt/PKB mutant was not simply an artifact of Akt/PKB overexpression. Because we were unable to assess the ability of the caspase-resistant Akt/PKB mutant to inhibit anoikis at time points later than 4 h (see the figure legends), we cannot rule out the possibility that Akt/PKB cleavage delays anoikis. The ability of caspases to
Negative Regulation of Akt/PKB by Death Receptors

A

Flag-Akt

p85

pcDNA

D108+119A

wt

B

Anti-Pser473Akt

Anti-Flag

Anti-p85

wt

Akt

D108+119A

Wild-type Akt

% Inhibition of Annoxi

0

2 hours

4 hours

C

Immunoblot

FIG. 5. Expression of the D108+119A cleavage-resistant mutant of Akt/PKB inhibits detachment-induced apoptosis. MDCK cells were transiently co-transfected with a green fluorescent protein-expressing vector and either pcDNA, the caspase-resistant Akt/PKB mutant (D108+119A), or wild-type Akt/PKB. A, confirmation of exogenous Akt/PKB expression. The expression of the transfected constructs at 24 h was assessed by analyzing equivalent amounts of total protein from extracts of these cells on 8% SDS-PAGE and immunoblotting with a FLAG- or p85-specific antibody, followed by the appropriate HRP-conjugated secondary antibody. B, expression of the Akt/PKB D108+119A mutant inhibits anoikis. Twenty-four h after transfection, these cells were plated on either collagen or poly-HEME for 2 or 4 h, stained with annexin V-PE, and analyzed by flow cytometry. Specifically, the percentage of GFP-positive cells bound by annexin V-PE was determined. The brightest GFP-positive cells were excluded from analysis because of an observed overlap between FL1 and FL2 channels at high FL1 intensities. A minimum of 5000 GFP-positive cells were analyzed for each sample. The data are reported as the mean percentage of inhibition of anoikis (± S.D. from three experiments) obtained by expression of the indicated Akt/PKB construct (percentage of annexin V-positive cells on collagen minus the percentage of annexin V-positive cells in suspension). The effects of the Akt/PKB double mutant protein on anoikis at later time points could not be determined because the control transfectants were equally apoptotic on poly-HEME and collagen (data not shown), most likely because they were maintained in serum-free medium. Of note, we did not observe an ability of either the D108A or D119A single point mutants to inhibit anoikis at either 2 or 4 h after matrix detachment (data not shown). C, Akt/PKB D108+119A mutant does not exhibit elevated activity relative to wild-type Akt/PKB. MDCK cells were transiently transfected with either a control plasmid (pcDNA), FLAG-tagged wild-type Akt/PKB (WT Akt), or a FLAG-tagged Akt/PKB mutant lacking the caspase 3 cleavage sites at amino acid residues 108 and 119 (D108+119A). Equivalent amounts of protein from extracts of these cells were immunoblotted with either an antibody specific for serine 473-phosphorylated Akt/PKB (New England Biolabs) (top panel) or a FLAG-specific antibody (Sigma) (middle panel), followed by the appropriate HRP-conjugated secondary antibody. To control for protein loading, these blots were also probed with an antiserum specific for the p85 subunit of PISK (bottom panel). These bands were quantified, and relative Akt/PKB activity was determined as the ratio of the signals of serine 473-phosphorylated Akt/PKB relative to total Akt/PKB. Similar results were observed in three independent experiments. Similar activity is evident with wild-type Akt/PKB and the D108+119A mutant.

delay detachment-induced apoptosis by inducing the cleavage of Akt/PKB may contribute to a postcommitment amplification step in anoikis, similar to that which occurs upon the caspase-mediated cleavage of bcl-2 (25, 26). Nonetheless, our findings suggest that the ability of apoptotic proteins to impact signal-

ing molecules associated with cell survival is an important determinant of cell fate. Moreover, our data indicate that the serine/threonine kinase Akt/PKB is a relevant target of caspase activity in the induction of apoptosis.

Our results indicate that caspases cleave Akt/PKB at aspartic acid residues 108 and 119 during anoikis. Specifically, we observed that an Akt/PKB double point mutant containing alanine substitutions at aspartic acid residues 108 and 119 is resistant to caspase cleavage during anoikis (Fig. 4B). Both aspartic acid residues 108 and 119 are cleaved by caspases during anoikis, as indicated by the fact that the mutation of both sites, but not single mutations at either residue, conferred resistance to caspase cleavage in matrix-detached MDCK cells (Fig. 4B). The caspase-mediated cleavage of Akt/PKB at these sites is expected to produce an Akt/PKB fragment of ~44 kDa. In previous work, we identified a 44-kDa Akt/PKB cleavage product resulting from the incubation of recombinant caspase 3 with Akt/PKB in vitro (20, 24). In contrast, we were not able to detect an Akt/PKB fragment in matrix-detached MDCK cells either by immunoblotting extracts from these cells with Akt/PKB-specific antibodies or by silver staining (data not shown). Our inability to detect this fragment in vivo likely reflects the fact that Akt/PKB is cleaved by multiple proteases in vivo, resulting in the generation of fragments that are too small to be detected by routine methods. This hypothesis is supported by the fact that Akt/PKB can be cleaved at multiple sites in vitro by recombinant caspases 3, 6, and 7 (20).

Although the activity of some caspase targets is enhanced by cleavage (19), other signaling molecules are inactivated after their caspase-dependent proteolysis (25, 26). The caspase-dependent cleavage of Akt/PKB at amino acids 108 and 119 generates two fragments of predicted molecular masses of 44 and 16 kDa, corresponding to a PH-deficient kinase domain and an isolated PH domain, respectively (Fig. 4A). The importance of the PH domain of Akt/PKB in the stimulation of its serine/threonine kinase activity is controversial. Although an Akt/PKB deletion mutant lacking amino acids 1–117 exhibits reduced activity relative to wild-type Akt/PKB (27), the deletion of amino acids 4–129 has been reported to enhance the activity of this kinase (28, 29). Importantly, our previous results indicated that the PH-deficient Akt/PKB fragment resulting from its cleavage at amino acids 108 and 119 does not exhibit reduced activity relative to wild-type Akt/PKB (20). However, the subsequent cleavage of Akt/PKB by other caspases, which likely occurs based on our inability to detect the predicted Akt/PKB fragments in vivo, may inhibit its kinase activity.

The ability of death receptor signaling pathways to impact Akt/PKB has numerous implications for cell survival. It is possible that a critical level of Akt/PKB activity must be maintained for cell survival and that caspases induce anoikis by reducing Akt/PKB activity below this threshold. In addition, the caspase-mediated proteolysis of Akt/PKB may render cells resistant to secondary survival signals. In fact, the ability of caspases to induce the cleavage of Akt/PKB in matrix-detached cells may explain why the growth factor-mediated stimulation of Akt/PKB is significantly enhanced in matrix-attached cells relative to epithelial cells in suspension (3, 30). This ability of caspases to impact Akt/PKB signaling irreversibly explains why tumor cells, which must grow in matrix-deficient environments, must acquire mutations that prevent this death receptor-associated negative regulation of the Akt/PKB pathway. For example, oncogenic Ras inhibits the expression of the TNF death receptor family member Fas (31, 32), a finding that may explain why Ras-transformed epithelial cells are resistant to anoikis (3).
It is also interesting to consider the possibility that the caspase-directed cleavage of Akt/PKB results in the generation of an Akt/PKB fragment that promotes apoptosis. In this manner, Akt/PKB would be converted by caspases from an anti-apoptotic to a proapoptotic signaling molecule, similar to theнер, Akt/PKB would be converted by caspases from an anti-caspase-directed cleavage of Akt/PKB results in the generation will be important for the development of therapeutics that inhibit the activity of proteins necessary for cell survival.

In addition to its central importance in the maintenance of cell survival, Akt/PKB is amplified in numerous tumors (33–36), and its overexpression can promote cellular transformation (37). Although the Akt/PKB signaling pathway has been shown to protect cells from numerous apoptotic stimuli, our studies highlight the impact of apoptotic signaling molecules on Akt/PKB. Specifically, we show that in the absence of integrin signaling, Akt/PKB is cleaved by death receptor-dependent pathways. The identification of such signaling pathways that inhibit the activity of proteins necessary for cell survival will be important for the development of therapeutics that inhibit tumor growth.

Acknowledgments—We thank Katherine Davis for excellent technical assistance, Alex Toker for providing the Akt/PKB constructs, and Roya Khoosavi-Far for providing the FADD construct.

REFERENCES
1. Frisch, S. M., and Francis, H. (1994) J. Cell Biol. 124, 619–626
2. Meredith, J. E. J., Fazeli, B., and Schwartz, M. A. (1993) Mol. Biol. Cell 4, 953–961
3. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) EMBO J. 16, 2783–2793
4. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927
5. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 663–668
6. Frech, M., Andjelkovic, M., Ingleby, E., Reddy, K. K., Falck, J. R., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 8474–8481
7. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
8. Anderson, K. E., Coadwell, J., Stephens, L. R., and Hawkins, P. T. (1998) Curr. Biol. 8, 684–691
9. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
10. Toker, A., and Newton, A. C. (2000) J. Biol. Chem. 275, 8271–8274
11. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 215–227
12. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) Science 278, 687–689
13. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. R., Arden, K. C., Ikenberry, S. J., and Green, M. E. (1999) J. Biol. Chem. 274, 16741–16746
14. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1999) Science 282, 1318–1321
15. Rytomaa, M., Martinis, L. M., and Downward, J. (1999) Curr. Biol. 9, 1043–1046
16. Frisch, S. M. (1999) Curr. Biol. 9, 1047–1049
17. Steinhausen, U., Badock, V., Bauer, A., Behrens, J., Wittmann-Liebold, B., Dorken, B., and Bommert, K. (2000) J. Biol. Chem. 275, 16345–16353
18. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) Cell 90, 315–325
19. Rokudai, S., Fujita, N., Hashimoto, Y., and Tsuruo, T. (2000) J. Cell. Physiol. 182, 290–296
20. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
21. Chinnaiyan, A. M., Pepper, C. G., Seldin, M. F., O’Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4961–4965
22. Frisch, S. M., Vuori, K., Kelaita, D., and Sicks, S. (1996) J. Cell Biol. 133, 1377–1382
23. Bachelder, R. E., Hibick, M. J., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. R., and Mercurio, A. M. (1999) J. Cell Biol. 147, 1063–1072
24. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) Science 276, 1966–1968
25. Clem, R. J., Cheng, E. H., Karp, C. L., Kirsch, D. G., Ueno, K., Takahashi, A., Kastan, M. B., Griffin, D. E., Earnshaw, W. C., Veluiona, M. A., and Hardwick, J. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 554–559
26. Andjelkovic, M., Jakubowiec, T., Cron, P., Ming, X.-F., Han, J.-W., and Frisch, S. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1046–1043
27. Frech, M., Andjelkovic, M., Ingley, E., Reddy, K. K., Falck, J. R., and Hemmings, B. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5069–5074
28. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) EMBO J. 14, 4288–4295
29. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) J. Biol. Chem. 271, 21920–21926
30. Fujii, Y., and Walsh, K. (1999) J. Biol. Chem. 274, 6349–6354
31. Fenton, R. G., Hixon, J. A., Wright, P. W., Brooks, A. D., and Sayers, T. J. (1998) Cancer Res. 58, 3391–3400
32. Peli, J., Schroter, M., Rudaz, C., Hahne, M., Meyer, C., Reichmann, E., and Tschopp, J. (1999) EMBO J. 18, 1824–1831
33. Staal, S. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5034–5037
34. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altamore, D. A., Watson, D. K., and Testa, J. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3641–3641
35. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altamore, D. A., Wan, M., Dubeau, L., Scambia, G., and Masciullo, V. (1995) Int. J. Cancer 64, 280–285
36. Nakanishi, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J., and Roth, R. A. (1999) J. Biol. Chem. 274, 21523–21532
37. Cheng, J. Q., Altamore, D. A., Klein, M. A., Lee, W. C., Kruh, G. D., Lissy, N. A., and Testa, J. R. (1997) Oncogene 14, 2793–2801
The Cleavage of Akt/Protein Kinase B by Death Receptor Signaling Is an Important Event in Detachment-induced Apoptosis

Robin E. Bachelder, Melissa A. Wendt, Naoya Fujita, Takashi Tsuruo and Arthur M. Mercurio

J. Biol. Chem. 2001, 276:34702-34707.
doi: 10.1074/jbc.M102806200 originally published online July 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102806200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 25 of which can be accessed free at http://www.jbc.org/content/276/37/34702.full.html#ref-list-1