The Role of Asp-462 in Regulating Akt Activity*

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Protein kinase Akt, an important downstream target of phosphatidylinositol 3-kinase, is one of the major survival factors in mammalian cells. It has been shown that phosphorylation of the C-terminal hydrophobic motif is required for Akt activation. The activated Akt then phosphorylates several pro-apoptotic proteins and prevents apoptosis mediated by caspases and the mitochondria. Interestingly, Akt has also been implicated to be a direct substrate of caspases in apoptotic cells induced by Fas (Widmann, C., Gibson, S., and Johnson, G. L. (1998) J. Biol. Chem. 273, 7141–7147) and anoikis (Bachelder, R. E., Wendt, M. A., Fujita, N., Tsuruo, T., and Mercurio, A. M. (2001) J. Biol. Chem. 276, 34702–34707).

In this study we showed that cytokine withdrawal resulted in Akt degradation by caspases as well. Furthermore, we demonstrated residue Asp-462 of Akt1 which is just upstream of the hydrophobic motif to be the primary cleavage site. The Akt1 mutant (D462N) that prevented caspase cleavage was more stable during factor withdrawal and enhanced cell survival. The Akt trunca- tion mutant mimicking the caspase cleavage product lost its kinase activity and functioned as a dominant negative to promote cell death. Our results suggest that the balance between Akt and caspase activity controls cell survival. In particular, caspases are able to render Akt inactive and dominantly inhibit the Akt pathway by cleaving off the C-terminal hydrophobic motif. Consequently, the survival signal is quickly down-regulated to allow apoptosis to occur.

Cell survival and apoptosis are tightly regulated processes in multicellular organisms, the disruption of which may lead to diseases such as cancer. The serine/threonine kinase Akt (also referred to as PKB or RAC-PK) has proven to be a critical player in cell survival pathways. Originally identified as the cellular homologue of the retroviral oncogene v-akt (3), Akt is conserved throughout evolution. The activation of Akt occurs through a cascade of events and is thought to be directly downstream of phosphatidylinositol 3-kinase (4–7). A number of studies (8–12) have shown that many growth and survival factors, such as nerve growth factor, platelet-derived growth factor, insulin-like growth factor-1 and cytokines, can activate Akt in vivo. Akt contains an N-terminal pleckstrin homology domain (PH) and a C-terminal kinase domain. The PH domain binds to phosphatidylinositol (thereby recruiting Akt to the plasma membrane where PDK1 is located) (13). Phosphorylation of both Thr-308 and Ser-473 is required for fully activating Akt (4). Thr-308 is believed to be phosphorylated by PDK1 (14–16), although the kinase that phosphorylates the Ser-473 site still remains unclear (17). Interestingly, Ser-473 is within the hydrophobic motif (FXX(F/Y)(S/T)(F/Y)) in the C-terminal tail of Akt. This hydrophobic motif is also conserved among the AGC family of kinases. Basal phosphorylation of the Ser-473 site is important for the activation of Akt (4), presumably necessary for maintaining the proper conformation of the kinase domain and docking to PDK1 (18). A recent report (19) has also identified a novel protein that is capable of binding to the C-terminal domain of Akt and negatively regulates its activity. These data point to the important regulatory role of the C-terminal region of Akt.

Akt likely acts on multiple substrates in vivo, which may include Bad and FKHR family transcription factors (20–23). Phosphorylation of Bad and FKHRs leads to inactivation of its apoptotic activity (21). It has also been found that Akt can phosphorylate and inactivate caspase-9 in vitro, suggesting that Akt may protect cells from apoptosis by inhibiting caspases (24). Interestingly, Akt was reported to be degraded during apoptosis induced by Fas and anoikis, and inhibition of caspase activity prevented its degradation (1, 2, 25). These results suggest a possible negative feedback loop between Akt and caspases that may help to maintain the balance between cell survival and apoptosis.

However, it is still unclear how Akt and caspases may interact with each other in cytokine-induced cell death. The exact site(s) where caspase cleavage may occur in vivo and how this cleavage may affect Akt survival activity remain to be elucidated. In this study, we examined the potential caspase cleavage sites in Akt in vitro and in vivo, and we identified a novel primary cleavage site located between the kinase domain and the hydrophobic motif. Mutation of this site (Asp-462) blocked caspase cleavage and significantly enhanced Akt survival activity against cytokine withdrawal. The Akt cleavage product that did not contain the C-terminal tail lost its kinase activity and could function as a dominant negative in IL-3-dependent 32D cells. Therefore, in addition to phosphorylation of Thr-308 and Ser-473, the activity of Akt is also regulated by caspases.

**EXPERIMENTAL PROCEDURES**

**Constructs—**cDNAs encoding wild type and mutant murine Akt1 were cloned into pcDNA3 (Invitrogen) or the retroviral vector pBabe-puro (26). The constitutively active Akt construct (myrAkt) was generated by appending sequences encoding the chicken c-Src myristoylation signal and a FLAG epitope to the 5′ and 3′ end of Akt1 through PCR. The retroviral vector encoding HA- and FLAG- double epitope-tagged Akt1 was generated by ligating the 3′ sequences of Akt1 that contained main; IL-3, interleukin-3; PDK1, 3-phosphoinositide-dependent protein kinase-1; PARP-1, poly(ADP-ribose) polymerase 1; FKHRs, forkhead related transcription factors; HA, hemagglutinin.
Akt C-terminal Cleavage by Caspases

Endogenous Akt is Degraded during Cytokine Withdrawal-induced Apoptosis—Previous studies suggest that the protein level of Akt may be down-regulated during apoptosis in Fas-induced apoptosis and anoikis (1, 2, 25). To investigate further how Akt expression may be regulated post-translationally in cells upon cytokine withdrawal, lysates were collected from 32D cells that were grown in the absence of IL-3 for various times. An antibody (C-20) against the C terminus of Akt1 was used to probe the level of Akt (Fig. 1A). At 16 h, most of the Akt proteins appeared to be degraded. However, this trend could be reversed with the addition of a cell-permeable caspase-3 inhibitor (Ac-AVALLPAVALLAPDEVDEVD-CHO) at a concentration of 100 nM (Fig. 1B). These observations suggest that the protein level of Akt may be regulated during factor withdrawal-induced apoptosis and that caspases may be involved in this process.

In Vitro Cleavage of Akt by Caspase-3—It has been suggested that Akt may be cleaved by caspases such as caspase-3 during apoptosis (1, 2). However, whether such cleavage occurs in cytokine-induced cell death remains unclear. Our findings that the degradation of endogenous Akt could be inhibited by DEVD suggested a role for caspase-3 in regulating Akt protein stability. A comparison of all three mouse Akt isoforms and human Akt1 C-terminal sequences (Fig. 2A) with the consensus motif of caspase-3 substrates (Fig. 2B) revealed several potential cleavage sites in Akt. To determine whether these sites could indeed be cleaved by caspase-3, three of the most likely sites in Akt1 (Asp-494, Asp-456, and Asp-462) were each mutated to Asn. These mutants and myristoylated wild type Akt were translated in vitro and labeled with [35S]Met. The products translated in vitro were subsequently incubated with caspase-3 and resolved by SDS-PAGE. The mutations did not alter the expression levels of the mutants (data not shown). As shown in Fig. 2C, several cleavage products (~60 and 45 kDa, indicated by *) were apparent when Akt was cosedimented with caspase-3. Furthermore, the presence of the caspase inhibitor DEVD appeared to inhibit caspase-3 cleavage of Akt. These observations suggest that several caspase-3 cleavage sites may exist in Akt. Importantly, the cleavage products were no longer present when residue Asp-462 was mutated, suggesting that residue Asp-462 on Akt may constitute an authentic and initial caspase cleavage site. Notably, residue Asp-462 is located between the

RESULTS

Endogenous Akt expression was determined using the FLAG epitope to the XhoI/EcoRI fragment from the pBabe-HA-Akt vector (10). All constructs containing the D462N mutation were generated using the Quickchange mutagenesis kit (Stratagene). Constructs encoding the truncated Akt (D462*) were created by ligating an XhoI/EcoRI cut PCR fragment encoding residues 240–462 of mouse Akt1 to the XhoI/EcoRI cut pBabe-HA-Akt vector. All vectors were sequenced to confirm that no other sequences had been mutated.

Cell Lines—The retroviral packaging cell line BOSC23 was used to generate retroviruses as described previously (27). Briefly, retroviral constructs engineered into the vector pBabe-puro (26) were transfected into BOSC23 cells, and the viruses were harvested 2 days after transfection. The viruses were used to infect either the IL-3-dependent 32D (28) or NIH 3T3 cells. Infected cells were cultured in media containing puromycin (1 μg/ml), and the expression of different Akt proteins was confirmed by Western blotting. pcDNA3 Akt constructs were transiently transfected into 293T cells using the standard calcium phosphate method.

Antibodies and Reagents—For Western blotting of endogenous Akt, the anti-Akt N- (5G3) and C-terminal antibodies (C-20) were purchased from Cell Signaling. The anti-Grb-2 monoclonal antibody was purchased from Transduction Laboratories. The anti-HA and anti-FLAG monoclonal antibodies were purchased from Sigma. Caspase inhibitors Ac-AVALLPAVALLAPDEVDEVD-CHO and Ac-DEVD-CHO were purchased from Oncogene Research Products.

In Vitro Translation and Caspase Cleavage—Myristoylated wild type Akt and Akt point mutants were created by PCR, cloned into the pcDNA3 vector (Invitrogen), and translated in vitro in the presence of [35S]Met using the TNT kit (Promega). The reaction mixtures were subsequently resolved by SDS-PAGE, and analyzed with Western blotting. pcDNA3 Akt constructs were transiently transfected into 293T cells using the standard calcium phosphate method.

Survival Assays and Detection of Akt Degradation—32D cells expressing various forms of Akt were washed twice with RPMI with 10% heat-inactivated fetal calf serum and cultured in the absence of IL-3. At different time points post-IL-3 withdrawal, the cell-permeable caspase-3 inhibitor DEVD was added to cells at 8 h after IL-3 withdrawal. At least three independent transfection/infection experiments were conducted for each construct. Samples of these cells at different time points post-IL-3 depletion were also lysed in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM aprotinin, 1 μM leupeptin, 2 mM β-glycerol phosphate, 500 μM sodium vanadate, and 1 mM dithiothre-itol), resolved by SDS-PAGE, and analyzed with Western blotting.

Immunofluorescence—NIH3T3 cells expressing different HA-Akt mutants were analyzed as described previously (10). Briefly, cells were permeabilized and fixed with paraformaldehyde. The cells were then probed with anti-HA and fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies (Sigma). The localization of Akt was visualized using a Nikon Fluorescence TE200 microscope and a Cool-Snap FX camera.

Akt  is degraded upon cytokine-induced apoptosis. A, lysates from 32D cells collected every 2 h after IL-3 withdrawal were resolved by SDS-PAGE. The level of endogenous Akt was analyzed by an anti-Akt antibody (C20). Grb2 was used as a loading control. B, Akt degradation can be blocked by the caspase-3 inhibitor DEVD. Duplicate sets of 32D cells were grown in the absence of IL-3. At 8 h after IL-3 withdrawal, the cell-permeable caspase-3 inhibitor DEVD was added to one set of the cells to a final concentration of 100 nM. Lysates were collected at 0, 12, 14, 16, and 18 h after IL-3 withdrawal, resolved by SDS-PAGE, and blotted with the anti-Akt C20 antibody. Grb2 was used as a loading control.

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Akt Cleavage Occurs In Vivo and Can Be Blocked by D462N Mutation—Our observations thus far suggested that Akt might be cleaved initially by caspases at residue Asp-462 in vivo upon cytokine withdrawal-induced apoptosis. To test this hypothesis, we examined the levels of endogenous Akt in 32D cells at different time points after IL-3 depletion, using anti-Akt antibodies that recognized either the N terminus (5G3) or C terminus (C-20) of Akt. The C-20 antibody was raised against a peptide epitope C-terminal to Asp-462. If caspases can indeed cleave Akt at Asp-462, the resultant N-terminal Akt protein should no longer be detectable by the C-20 antibody. As shown in Fig. 3A, at 16 h after IL-3 withdrawal, the Akt level appeared to decrease compared with that at 14 h when analyzed with the C-20 antibody. However, this decrease in protein level was not apparent when the same blot was probed with the N-terminal antibody 5G3. These data suggest that a specific cleavage of Akt at Asp-462 likely occurs at about 14–16 h after IL-3 depletion in 32D cells.

To test further the hypothesis that Akt may be cleaved at residue Asp-462 in vivo, expression constructs encoding wild type Akt and mutant Akt with the Asp-462 to Asn mutation (Akt D462N) were generated. Both constructs were also tagged by the HA epitope on the N terminus and the FLAG epitope on the C terminus. Because the Asp-462 site is very close to the C terminus of Akt, cleavage at this site should not markedly affect Akt mobility on SDS-PAGE. The expression of Akt and its variant in 32D cells at different time points after IL-3 withdrawal was then examined using anti-HA and anti-FLAG antibodies. As shown in Fig. 3B, the D462N mutation did not appear to affect Akt expression. At 17 h after IL-3 withdrawal, both Akt and Akt D462N could be detected using the anti-HA antibody, indicating that the N terminus still remained intact. However, Akt D462N but not wild type Akt could be detected with the anti-FLAG antibody, suggesting that the C terminus of wild type Akt had been degraded. This result is consistent with our observations of endogenous Akt levels and suggests that the D462N mutation could block Akt degradation. These data support the notion that Akt may indeed be cleaved in vivo at Asp-462 and that caspase cleavage likely occurs before Akt is further degraded during apoptosis.

Akt Survival Activity Is Enhanced with the D462N Mutation—One major function of Akt is to protect cells from apoptosis induced by factor withdrawal. It has been shown that
overexpression of Akt can protect IL-3-dependent 32D cells from apoptosis induced by IL-3 depletion (10), and the Akt kinase activity is essential for survival. To examine further how caspase cleavage of Akt might affect the Akt survival activity in vivo, we generated 32D cells expressing either wild type Akt, Akt with the D462N mutation (Akt D462N), or an Akt truncation mutant at residue Asp-462 (Akt D462*, * denotes stop codon) that mimicked the caspase cleavage product. These cells were maintained in the absence of IL-3, and the percentage of live cells was determined using trypan blue exclusion. Error bars indicate S.E. after at least three independent experiments.

**Fig. 4.** The survival activity of Akt is enhanced with the D462N mutation. A, 32D cells expressing wild type Akt, Akt D462N, and Akt D462* were maintained in the absence of IL-3. The percentage of live cells was determined at different time points by trypan blue exclusion. Closed circles, control cells; open circles, wild type Akt; closed squares, Akt D462N; open squares, Akt D462*. Error bars indicate S.E.

**Fig. 5.** The Akt D462* truncation mutant functions as a dominant negative to promote apoptosis. Control 32D cells (○), 32D cells expressing myrAkt (□), and 32D cells co-expressing myrAkt and the HA-Akt D462* mutant (■) were maintained in the absence of IL-3. At different time points after IL-3 withdrawal, the percentage of live cells was determined using trypan blue exclusion. Error bars indicate S.E. after at least three independent experiments.

**Fig. 6.** The D462N mutation does not affect the kinase activity of Akt. FLAG-tagged myrAkt, myrAkt D462N, and myrAkt D462* were expressed in 293T cells. The Akt proteins were immunoprecipitated using an anti-FLAG antibody, and in vitro kinase assays were performed using histone H2B as a substrate. The immunoprecipitates and reaction mixtures were then resolved by SDS-PAGE, transferred to membranes, and analyzed by Western blotting and autoradiography, respectively. The upper panel shows the Western blot of immunoprecipitated Akt proteins probed with an anti-FLAG antibody. The lower panel shows the phosphorylation of histone H2B.

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- **A**
- **B**
- **C**

*Fig. 4.** The survival activity of Akt is enhanced with the D462N mutation. A, 32D cells expressing wild type Akt, Akt D462N, and Akt D462* were maintained in the absence of IL-3. The percentage of live cells was determined at different time points by trypan blue exclusion. Closed circles, control cells; open circles, wild type Akt; closed squares, Akt D462N; open squares, Akt D462*. Error bars indicate S.E.

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Akt was enhanced in cells that expressed the Akt D462N mutant, whereas the Akt D462* truncation mutant was unable to protect 32D cells from IL-3 withdrawal-induced apoptosis.

To investigate further how the D462N mutation might affect Akt function, we examined the survival of 32D cells expressing the constitutively active myristoylated Akt (myrAkt), myrAkt D462N, and myrAkt D462* in the absence of IL-3 as described above. As expected, expression of the myrAkt D462* mutant did not rescue 32D cells from apoptosis (Fig. 4B), whereas cells that expressed myrAkt were able to survive without IL-3. Notably, 32D cells expressing the myrAkt D462N mutant were able to survive even better than the myrAkt cells.

Taken together with our data that DEVD could inhibit Akt degradation during IL-3 withdrawal (Fig. 1B), the above findings suggest that a difference in sensitivity to caspase cleavage may explain the differing survival abilities of Akt and Akt D462N. If this is the case, DEVD should enhance the survival of wild type Akt cells but not Akt D462N cells. Consistence with this prediction, addition of DEVD enhanced the survival of cells expressing wild type Akt but not those expressing Akt D462N (Fig. 4C). These data support our model that the higher survival activity of Akt D462N may be attributed to its resistance to cleavage and subsequent degradation in vivo.

The Akt D462* Truncation Product Functions as Dominant Negative—Our data thus far point to a model where caspases would cleave Akt first at residue Asp-462 thereby inhibiting its kinase activity during factor withdrawal. It is therefore conceivable that the resultant cleavage product might act as a dominant negative and further activate the apoptotic machinery. To test this idea, the survival of 32D cells expressing myrAkt with or without Akt D462* was compared. Consistent with previous findings (10), over-expression of the constitutively active myrAkt was able to protect 32D cells from apoptosis better than wild type Akt (Figs. 4B and 5). However, the enhanced survival of cells expressing myrAkt was reduced to a level more comparable with control cells when Akt D462* was co-expressed in these cells. This observation indicates that the Akt D462* truncation mutant likely functions as a dominant negative and raises the possibility that the cleavage product of Akt may further activate the apoptotic machinery in vivo.

Akt Mutants and Their Kinase Activities—It is possible that the higher survival activity of the Akt D462N mutant is the result of an increase in Akt kinase activity and not stability. To test this possibility, FLAG-tagged myristoylated wild type Akt (myrAkt), myrAkt D462N, and myrAkt D462* mutants were transiently expressed in 293T cells. Akt and its mutants were immunoprecipitated using an anti-FLAG antibody and assayed for kinase activities using histone H2B as a substrate. All proteins were expressed at similar levels (Fig. 6). The D462N mutation did not appear to alter the kinase activity. Therefore, the enhanced survival activity of the Akt D462N mutant was likely due to an enhancement of Akt stability against caspase cleavage rather than an increase in its kinase activity. It has been shown that phosphorylation of residue Ser-473 in the C-terminal hydrophobic motif of Akt is necessary for full activation of Akt kinase activity (4). The hydrophobic motif when phosphorylated may help to stabilize a preferred conformation of Akt for further phosphorylation by PDK1 (18). Consistent with this notion, the truncation mutation D462* significantly diminished Akt kinase activity, indicating that the C-terminal region downstream of residue Asp-462 is essential for Akt activity. This finding would also explain the loss of survival activity of the Akt D462* mutant. Taken together, our data suggest that caspase-mediated cleavage of Akt at Asp-462 could inhibit Akt kinase activity and survival function.

The Asp-462 Mutations Did Not Alter the Subcellular Localization of Akt—Previous studies (30, 31) have shown that Akt is primarily localized in the cytoplasm. To examine whether mutation of the potential caspase cleavage site would affect the subcellular localization of Akt, NIH 3T3 cells, and the subcellular localization of these proteins was analyzed by indirect immunofluorescence using an anti-HA antibody.

The subcellular localization of Akt and its mutants was analyzed by immunofluorescence using an anti-HA antibody. Consistent with previous findings, wild type Akt was found to localize mostly in the cytoplasm (Fig. 7). Similar results were also obtained for Akt D462N and Akt D462*. These observations indicate that the mutations did not significantly alter Akt localization.

DISCUSSION

Akt has been reported to be down-regulated during apoptosis induced by Fas ligand or anoikis (1, 2). In this report, we demonstrated that Akt could also be down-regulated during cytokine-induced cell death and it is likely mediated by caspases. In IL-3-dependent 32D cells, we found decreased levels of endogenous Akt at about 14–16 h after cytokine withdrawal. Such degradation is consistent with the time course of apoptosis in these cells, as most of the cells die between 14 and 20 h post-IL-3 depletion. Furthermore, addition of a caspase-3 inhibitor could block Akt degradation in 32D cells upon IL-3 withdrawal. We found that in vitro caspase-3 could cleave Akt at multiple sites, and a single point mutan of residue Asp-462 to Aan was able to block Akt cleavage and degradation both in vitro and in vivo. Interestingly, the D462N mutation enhanced Akt survival activity in vivo, possibly by stabilizing Akt without altering its kinase activity or subcellular localization.

Other caspase cleavage sites (Asp-108 and Asp-119) on Akt have been reported recently (2, 25), but the single mutation of any of these sites could not completely abolish in vitro caspase-3 cleavage. Taken together, these findings suggest that
Akt C-terminal Cleavage by Caspases

caspases at Asp-462 (which deletes the hydrophobic motif) is able to eliminate Akt survival activity before it is completely degraded.

We have shown that the Akt cleavage product could function as a dominant negative. Interestingly, Akt cleavage products at two other caspase-3 cleavage sites have also been reported to function as dominant negative forms of Akt and accelerate the apoptotic process (2, 25). These sites are located N-terminal to the kinase domain, and cleavage is predicted to delete the PH domain. Therefore, cleavage of Akt results in the survival signal being quickly down-regulated thereby allowing the cells to commit suicide.

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