Akt Activation Promotes Degradation of Tuberin and FOXO3a via the Proteasome* 

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Growth factor receptors promote cell growth and survival by stimulating the activities of phosphatidylinositol 3-kinase and Akt/PKB. Here we report that Akt activation causes proteasomal degradation of substrates that control cell growth and survival. Expression of activated Akt triggered proteasome-dependent declines in the protein levels of the Akt substrates tuberin, FOXO1, and FOXO3a. The addition of proteasome inhibitors stabilized the phosphorylated forms of multiple Akt substrates, including tuberin and FOXO proteins. Activation of Akt triggered the ubiquitination of several proteins containing phosphorylated Akt substrate motifs. Together the data indicate that activated Akt stimulates proteasomal degradation of its substrates and suggest that Akt-dependent cell growth and survival are induced through the degradation of negative regulators of these processes.

Metazoan cell size, survival, and proliferation are coordinately regulated by the availability of extrinsic growth factors that govern these processes through their control of intracellular signal transduction cascades. The signals emanating from growth factor receptors determine the size, number, and turnover rate of cells within a given tissue (1). A major pathway that controls cell growth and survival in response to the activation of growth factor receptors is the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (2). Studies of the PI3K/Akt pathway in model organisms such as Drosophila melanogaster and Caenorhabditis elegans have established the importance of this pathway in controlling the number and size of cells within an organism and also in controlling the size and longevity of the entire organism. In vertebrates, constitutively active oncogenic forms of both PI3K and Akt have been characterized in murine and avian retroviruses, and inactivating mutations in PTEN (a phosphatase that opposes the action of PI3K) are prevalent in many types of human tumors (3–5).

Activation of the PI3K/Akt pathway stimulates growth and survival via its actions on a number of Akt substrates, including the FOXO family transcription factors Bad, GSK-3, mTOR, and tuberin. Active FOXO family transcription factors promote the transcription of genes involved in cell cycle arrest and apoptosis such as p27Kip1 and Bim (6–8). One mechanism by which Akt promotes cell survival is by phosphorylating FOXO family transcription factors, which inactivates them and prevents the transcription of proapoptotic molecules. Recently Akt has also been shown to phosphorylate and inactivate a negative regulator of cell size, tuberin (9–12). In conjunction with its binding partner hamartin, increased tuberin protein levels negatively regulate cell size, possibly by inhibiting the phosphorylation and activation of mTOR or p70S6K. Phosphorylation by Akt prevents tuberin-dependent declines in cell size.

In growth factor-dependent cells, the removal of growth factors triggers cellular atrophy and the initiation of programmed cell death (13). Activation of Akt inhibits both of these processes, suggesting that Akt can inhibit mediators of both cell atrophy and cell death (14). In analyzing the effects of Akt activation on downstream targets involved in the regulation of cell size and survival, we show here that the activation of Akt results in a decline in total protein levels of tuberin and FOXO3a. The Akt-stimulated decline in tuberin correlated with a decline in the protein level of its binding partner, hamartin, suggesting that the tuberin-hamartin complex is coordinately regulated by Akt. Inhibition of the proteasome resulted in a recovery in the protein levels of tuberin, FOXO1, and FOXO3a and triggered a general accumulation of Akt substrates. Physiologic activation of the endogenous PI3K/Akt pathway through the IL-3 receptor in the presence of proteasome inhibitors resulted in prolonged phosphorylation of tuberin and FOXO3a. IL-3 stimulation triggered the ubiquitination of a number of potential Akt substrates, indicating that Akt-dependent degradation of substrates may be induced through their ubiquitination. Together the data indicate that activation of Akt targets the substrates of Akt for degradation in the proteasome.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—IL-3-dependent FL5.12 cells were cultured in 350 pg/ml recombinant murine IL-3 (R&D Systems) as described previously (15). Vector control and myristoylated Akt (myrAkt)-expressing cells were transduced with either empty pRevTRE retroviral vector (BD Clontech) or myrAkt pRevTRE, as described previously (16). Bcl-xL was expressed in cells with pSPPV-Bcl-xL, also described previously (16). FOXO1 and FOXO1-3A, generously provided by Drs. Fred Barr (University of Pennsylvania) and Gabriel Nunez (University of Michigan) were cloned into pRevTRE and transduced into an FL5.12 line that carries the rTA transcriptional activator of tetracycline-regulated promoters (17). Clones were selected by immunoblot for the ability to induce FOXO1 and FOXO1-3A protein expression to comparable levels. For regular passage, all cell lines were cultured in the absence of doxycycline. For induction of myrAkt, doxycycline was added to the medium (1 µg/ml, BD Clontech) for 24 h followed by an additional 48 h of culture in doxycycline-supplemented medium with or without IL-3 as appropriate. Bcl-xL-expressing and vector control lines were treated similarly to control for nonspecific effects of doxycycline unless otherwise indicated. Induction of FOXO1 constructs was for 24 h before lysis. Measurement of cellular viability

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**Akt activation triggers declines in tuberin and hamartin protein levels.** A, activated Akt was expressed under the control of a tetracycline-responsive promoter in FL5.12 cells. Doxycycline (Dox), a tetracycline analogue, was added to vector control (V) and myrAkt (A) cultures during a 3-day time period, and expression of activated Akt was assessed in immunoblots for phosphorylated Akt serine 473 (p-Akt) or total Akt. An actin immunoblot is included as a loading control. B, quantitative analysis of the immunoblot of phospho-Akt standardized to the immunoblot of actin for vector control (Vec) and myrAkt-expressing cells (Akt). Data are presented as the fold induction of phosphorylated Akt. C, vector control and cells expressing activated Akt were cultured in the absence of IL-3 for 48 h. Viability was assessed in triplicate by measuring propidium iodide exclusion in a flow cytometer. D, vector control, Bcl-xL-expressing (Xc), and activated Akt-expressing cells were cultured for 3 days in the presence or absence of doxycycline as indicated, and total cell lysates were prepared for analysis in tuberin, hamartin, and actin immunoblots. Results are representative of three similar experiments. E, after preincubation in the presence or absence of doxycycline as indicated, Bcl-xL- and myrAkt-expressing cells were cultured for an additional 2 days in the absence of IL-3. Note that vector control cells are no longer viable at this time point. Total cell lysates were analyzed in tuberin, hamartin, and actin immunoblots. Results are representative of at least three similar experiments. F, vector control (Ctrl, blue) and myrAkt (red) cells cultured in IL-3 and doxycycline were stained with propidium iodide and the cell-permeant DNA dye Hoechst 33342. Forward scatter was assessed by flow cytometry, and the cell size of G1-phase live cells is plotted. Mean forward scatter for vector control cells and myrAkt cells were 441 and 506, respectively. G, Bcl-xL (Ctrl, blue) and myrAkt (red) cells were cultured, stained, and analyzed as in panel F, except cells were cultured in the absence of IL-3 for 2 days. Mean forward scatter for Bcl-xL and myrAkt cells was 254 and 301, respectively.

**RESULTS AND DISCUSSION**

Stable transfectants expressing constitutively active myrAkt under the control of a tetracycline-responsive promoter were established using the nontransformed IL-3-dependent murine hematopoietic cell line FL5.12 with the intent of identifying and purifying Akt substrates involved in the control of growth.
and survival (Fig. 1, A and B). A low level of myrAkt is expressed in the absence of doxycycline. However, the addition of doxycycline to myrAkt-expressing cells results in a rapid and prolonged increase in Akt activity with concomitant increases in cell size and the induction of growth factor-independent cell survival (Fig. 1C). Disappointingly, using antibodies specific for Akt phosphorylation sites, doxycycline induction of Akt failed to induce an increase in the number or intensity of immunoreactive bands on immunoblots. This result prompted an examination of the expression of specific Akt substrates. The Akt consensus phosphorylation sequence identified tuberin as a potential Akt substrate, which has been confirmed recently in several studies (9, 11, 18, 19). Analysis of tuberin expression revealed a decline in tuberin protein levels as Akt activity was induced (Fig. 1D). A decline in the protein levels of the tuberin binding partner hamartin was also observed in myrAkt cells, and this decline was consistently accompanied by the appearance of a faster migrating species in hamartin immunoblots, suggesting the generation of a hamartin degradation product in cells with activated Akt.

In IL-3-dependent cells, growth factor withdrawal rapidly induces a decline in cell size followed by the induction of programmed cell death. Expression of Bcl-xL prevents the induction of cell death without altering the kinetics of cell atrophy, permitting analysis of cell atrophy in the absence of complicating effects of apoptosis (13). Because tuberin and hamartin function in complex to negatively regulate cell size (20–23), we compared the effect of IL-3 withdrawal and the induction of atrophy on tuberin and hamartin protein expression levels in Bcl-xL-expressing and myrAkt-expressing cells. IL-3 withdrawal had little effect on tuberin and hamartin protein levels. However, induction of Akt in the absence of other components

**Fig. 2.** Akt-induced proteasomal degradation of tuberin. A, cells were cultured with doxycycline in the presence and absence of IL-3. Vehicle control MeSO (−) or 50 μM ALLN (+) was added to cultures 8 h before the preparation of total cell lysates for tuberin and actin immunoblot analysis. Results are representative of three similar experiments. Vec., vector control. B, cells were cultured in the presence of IL-3. 50 μM ALLN (N), 50 μM ALLM (M), or 100 nM epoxomicin (E) was added to cultures for 8 h before the preparation of total cell lysates for analysis in tuberin and actin immunoblots.

**Fig. 3.** Putative Akt substrates are targeted for proteasomal degradation. A, cells were cultured with doxycycline in the presence of IL-3. Eight h after the addition of MeSO (−) or ALLN (+), total cell lysates were prepared and analyzed in an immunoblot using an antisera specific for phosphorylated residues that match the Akt consensus motif. Arrows indicate phosphoproteins that were stabilized by the addition of proteasome inhibitors. Vec., vector control. B, the lysates described in panel A were probed in tuberin, FOXO1, FOXO3a, and actin immunoblots. Results are representative of three experiments. C, vector control and Akt-expressing cells cultured in the presence of doxycycline were treated with vehicle control (−), ALLN, or ALLN + 10 μM LY294002, as indicated. Total cell lysates were probed in immunoblots for FOXO3a phosphothreonine 26 (p-FOXO3a), FOXO3a, or actin.
A. IL-3 (min): 0 10 30 60 90 Epox: + + + + + + p-Tuberin Tuberin p-FOXO3a FOXO3a Actin

Fig. 4. Proteasomal degradation of Akt substrates after IL-3 stimulation. A, FL5.12 cells were withdrawn from IL-3 for 4 h followed by a 30-min incubation in Me$_2$SO (+) or 100 nM epoxomicin (Epox.) (+). Cells were then stimulated with 4 ng/ml IL-3 for the indicated time before lysis. Total cell lysates were prepared for immunoblot analysis with antisera recognizing tuberin phosphothreonine 1462 (p-Tuberin), FOXO3a phosphothreonine 26 (p-FOXO3a), tuberin, FOXO3a, or actin. Results are representative of three experiments. B, doxycycline-inducible clones of either wild type human FOXO1 or human FOXO1 (huFOXO1) with three Akt phosphorylation sites mutated to alanine (FOXO1-3A) were established in FL5.12. These clones were infected with a retroviral vector encoding GFP alone (GFP) or GFP and myrAkt (Akt). GFP-positive cells were isolated and cultured in the presence or absence of doxycycline for 24 h to induce human FOXO1, and FOXO1 protein levels were assessed by immunoblot. Levels of phospho-Akt were measured in cells as a control for the efficiency of retroviral transduction of myrAkt.

B. huFOXO1 induction: FOXO1 FOXO1-3A GFP Akt GFP Akt huFOXO1 p-Akt Actin

Because ALLN promoted the accumulation of potential Akt substrates in the 50–90-kDa range, the effects of proteasome inhibitors on protein levels of FOXO family transcription factors were evaluated as a test of this hypothesis. Expression of activated Akt triggered a decline in protein levels of both FOXO1 and FOXO3a, and treatment of cells with ALLN triggered a recovery in protein levels for both transcription factors (Fig. 3A). In lysates from vector control cells the addition of ALLN triggered a significant increase in FOXO3a protein levels, specifically in a slower migrating form of the protein. This result suggested that IL-3 receptor stimulation was sufficient to trigger endogenous Akt phosphorylation and degradation of FOXO3a. To test this possibility, vector control and myrAkt cells were cultured in the presence and absence of ALLN and the PI3K inhibitor LY294002. As shown previously, culture in the presence of ALLN caused an accumulation of a slow migrating form of FOXO3a. The addition of LY294002 prevented IL-3-induced phosphorylation of FOXO3a and caused an increase in the mobility of total FOXO3a protein (Fig. 3C). The addition of LY294002 had little effect on the phosphorylation or mobility of FOXO3a in cells expressing activated Akt. These data indicate that the effects of LY294002 on FOXO3a protein mobility in response to IL-3 depend on inactivation of the PI3K/Akt pathway. Taken together, the results suggest that protein levels of both FOXO1 and FOXO3a are regulated in response to the activation of Akt in a proteasome-dependent manner.

The effects on FOXO3a protein mobility suggested that the activation of endogenous Akt by the IL-3 receptor can influence protein stability. To further explore whether proteasomal degradation of Akt substrates is induced at physiologic levels of Akt activation, we examined the effects of IL-3-induced Akt activation on the expression and phosphorylation status of tuberin and FOXO3a after the addition of IL-3 to growth factor-deprived cells. IL-3 was added to cells that had been pretreated for 30 min with vehicle control or epoxomicin. We have previously demonstrated under these conditions that IL-3 causes a rapid induction of PI3K-dependent Akt activity that is detectable within 5 min and persists for more than an hour (14). Total cell lysates were isolated at several time points and probed in immunoblots with antisera that recognize Akt phosphorylation of tuberin and FOXO3a (26). Similar to ALLN, the addition of epoxomicin to Akt-expressing cells resulted in a recovery in tuberin protein levels (Fig. 2B). Thus, Akt stimulates proteasomal degradation of tuberin.
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The 26 S proteasome degrades proteins that are conjugated to polyubiquitin chains. Akt-induced degradation of substrates in the proteasome may be stimulated by an increase in the ubiquitination of these proteins. To determine whether tuberin ubiquitination is increased in cells expressing activated Akt, ubiquitinated proteins were enriched using affinity matrix consisting of glutathione S-transferase fused to the ubiquitin-binding UBA domains of Rad23 (27, 28). Tuberin was enriched among ubiquitinated proteins isolated from epoxomicin-treated myrAkt cells compared with vector control cells (cultured in the absence of IL-3), indicating that increased Akt activity is sufficient to cause an increase in tuberin ubiquitination (Fig. 5A).

To determine whether activation of the endogenous PI3K/Akt pathway by the IL-3 receptor can cause a generalized increase in the ubiquitination of Akt substrates, vector control and myrAkt cells were stimulated with IL-3 in the presence and absence of epoxomicin and LY294002 for 60 min, a time point at which degradation of tuberin and FOXO3a was observed previously (Fig. 4A). Akt substrates were detected using the Akt substrate antiserum in fractions enriched for ubiquitinated Akt substrates in epoxomicin-treated cells, suggesting that ubiquitinated Akt substrates are processed through the proteasome on stimulation of the endogenous PI3K/Akt pathway with IL-3. The addition of LY294002, a PI3K inhibitor that blocks the activation of Akt by the IL-3 receptor, prevented the accumulation of ubiquitinated Akt substrates in epoxomicin-treated cells, suggesting that activation of Akt was required for ubiquitination of these proteins. Expression of activated Akt-enriched ubiquitinated Akt substrates in only myrAkt cells is marked with an arrow. The blot was stripped and reprobed with anti-ubiquitin as a loading control (bottom).
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Akt targets its substrates for degradation via the proteasome. We propose that proteasomal degradation of substrates may therefore represent a central mechanism by which Akt acts to promote cell growth and survival.

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