Akt and Mammalian Target of Rapamycin Regulate Separate Systems of Proteolysis in Renal Tubular Cells

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EGF suppresses proteolysis via class 1 phosphatidylinositol 3-kinase (PI3K) in renal tubular cells. EGF also increases the abundance of glycolytic enzymes (e.g., glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) and transcription factors (e.g., pax2) that are degraded by the lysosomal pathway of chaperone-mediated autophagy. To determine if EGF regulates chaperone-mediated autophagy through PI3K signaling, this study examined the effect of inhibiting PI3K and its downstream mediators Akt and the mammalian target of rapamycin (mTOR). Inhibition of PI3K with LY294002 prevented EGF-induced increases in GAPDH and pax2 abundance in NRK-52E renal tubular cells. Similar results were seen with an adenovirus encoding a dominant negative Akt (DN Akt). Expression of a constitutively active Akt increased GAPDH and pax2 abundance. An mTOR inhibitor, rapamycin, did not prevent EGF-induced increases in these proteins. Neither DN Akt nor rapamycin alone had an effect on total cell protein degradation, but both partially reversed EGF-induced suppression of proteolysis. DN Akt no longer affected proteolysis after treatment with a lysosomal inhibitor, methylamine. In contrast, methylamine or the inhibitor of macroautophagy, 3-methyladenine, did not prevent rapamycin from partially reversing the effect of EGF on proteolysis. Notably, rapamycin did not increase autophagosomes detected by monodansylcadaverine staining. Blocking the proteasomal pathway with either MG132 or lactacystin prevented rapamycin from partially reversing the effect of EGF on proteolysis. It is concluded that EGF regulates pax2 and GAPDH abundance and proteolysis through a PI3K/Akt-sensitive proteasomal pathway that does not involve mTOR. Rapamycin has a novel effect of regulating proteasomal proteolysis in cells that are stimulated with EGF.

vation inhibits renal hypertrophy (13), stimulation of CMA could provide an explanation for the effects of diet on renal growth.

Class 1 phosphatidylinositol 3-kinase (PI3K) signaling has been implicated as regulating proteolysis in a number of tissues. This enzyme consists of an 85-kD regulatory subunit (p85) and a 110-kD effector subunit (p110) (14). The p110 subunit places phosphates on the 3 hydroxyl group of inositol (PtH1ns). PtH1ns 3,4 or PtH1ns 3,4,5 mediate downstream signaling by activating 3-phosphoinositide-dependent protein kinase 1 which phosphorylates Akt (15). Blocking class 1 PI3K or Akt activity inhibits cell growth and protein accumulation and makes cells susceptible to apoptosis (15). 3-Phosphoinositide-dependent protein kinase 1 and Akt mediate these responses by phosphorylating other downstream effectors, such as glycogen synthetase kinase (GSK), which regulates glucose metabolism (16), and p70S6 kinase, which stimulates protein synthesis (14). The mammalian target of rapamycin (mTOR) modulates PI3K signaling in two ways. When bound to rictor, it phosphorylates Akt in a rapamycin-independent manner, but when complexed with raptor, it phosphorylates p70S6 kinase, an activity that is inhibited by rapamycin (17). This rapamycin-sensitive pathway has been linked to regulation of macroautophagy by nutrients: in isolated hepatocytes and hepatocellular carcinoma cell lines, rapamycin prevents PI3K/Akt signaling from suppressing macroautophagy (18,19). This finding seems to be tissue specific, because macroautophagy can be regulated by rapamycin-independent pathways in fibroblasts and skeletal muscle (20).

We have shown that signaling through PI3K is required for EGF to suppress proteolysis (21). We also determined that EGF suppressed lysosomal proteolysis, including CMA (7), but we did not establish whether CMA or another system of proteolysis was regulated by PI3K. We now investigate the EGF-induced signaling intermediates that regulate proteolysis and the substrates of CMA in NRK-52E renal tubular epithelial cells. Our results indicate that PI3K and Akt but not the rapamycin-sensitive activity of mTOR are required for EGF to increase pax2 and lysosomal proteolysis, including CMA (7), but we did not establish whether CMA or another system of proteolysis was regulated by PI3K. We now investigate the EGF-induced signaling intermediates that regulate proteolysis and the substrates of CMA in NRK-52E renal tubular epithelial cells. Our results indicate that PI3K and Akt but not the rapamycin-sensitive activity of mTOR are required for EGF to increase pax2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and suppress lysosomal proteolysis. Surprising, rapamycin stimulates a proteasomal system of proteolysis without affecting lysosomal proteolysis in EGF-treated cells.

Materials and Methods

Rapamycin and other chemicals were from Sigma Chemical (St. Louis, MO) unless specified. Recombinant human EGF was from R&D Systems (Minneapolis, MN); methyamine, 3-methyladenine, MG132, and lactacystin were from Calbiochem (La Jolla, CA). L-[U-14C] phenylalanine and aprotinin (24,25; data not shown). Total radioactivity that was recovered from labeled proteins was measured after precipitation of proteins with trichloroacetic acid (10% vol/vol). At the end of the sampling period, cell monolayers were solubilized in 1% SDS (1 ml/well) to determine the radioactivity that remained in the cells, and the rate of protein degradation was determined by calculating the slope of the logarithm of [14C]phenylalanine that remained in cell protein at time 0 and at four other time points. The mean of the slope of the degradation curves of the six samples of a single experiment was used in the figures, but all results were confirmed by three repeated experiments on separate days. Only studies with first-degree kinetics were used, and this resulted in the exclusion of a very few studies, because this relationship remained linear with all treatments (Figures 6A and 7A, reference 24,25; data not shown). Total radioactivity that was recovered from cells (calculated from the amount released into the media plus what remained in the cell monolayer) was an indicator of cell viability and did not change with any experimental treatment.

Immunoblotting

Cells were lysed in RIPA buffer (20 mM Tris-HCl [pH 7.4], 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 1% Triton, 10% glycerol, 1% deoxycholate, 10 mM Na3P2O7, 1 mM Na4VO4, 1 mM PMSF, and 10 μg/ml aprotinin). Proteins were separated by SDS-PAGE and transferred to nitrocellulose
membranes. After immunoblotting with specific antibodies, blots were developed using ECL. Developed images were scanned and quantified using BioRad Quantity One software.

**Monodansylcadaverine Staining**

NRK-52E cells were treated with EGF, rapamycin, or EGF plus rapamycin for 16 h with or without 10 mM 3-methyladenine. Cells then were washed twice with Hanks balanced salt buffer and stained with monodansylcadaverine (MDC) as previously reported (26). Briefly, cells were incubated with 0.05 mM MDC for 10 min, then washed four times with PBS. The fluorescence was visualized using a Zeiss Axiovert fluorescence microscope (Thornwood, NY), and were images analyzed using Adobe Photoshop v7.0. Using a reference grid, the number of MDC-positive vesicles per area was calculated for each treatment group.

**Statistical Analyses**

Data are expressed as mean ± SE. Differences between multiple groups were evaluated by ANOVA using the Student-Newman-Keuls test for multiple comparisons. *P* < 0.05 was considered statistically significant.

**Results**

**PI3K Regulates pax2 and GAPDH Abundance**

CMA degrades specific proteins that are important for renal tubular cell growth, including the glycolytic enzyme GAPDH and the paired-box transcription factor, pax2 (7). Because EGF suppresses degradation of pax2 and GAPDH by CMA in renal epithelial cells without significantly increasing their mRNA (27,28), we used the abundance of these proteins as markers of CMA. As expected, EGF significantly increased both pax2 and GAPDH abundance (*P* < 0.001) but not actin, a protein that lacks a KFERQ sequence (Figure 1). The mTOR inhibitor rapamycin (50 nM) did not significantly affect basal levels of GAPDH or pax2 or affect the EGF-stimulated increases in these KFERQ-containing proteins. This concentration of rapamycin was sufficient to prevent the phosphorylation of p70 S6 kinase (Figure 2B) and reduce EGF-induced protein synthesis by approximately 25% (data not shown). In contrast, the PI3K inhibitor LY294002 (25 μM) significantly reversed EGF-induced accumulation of both pax2 and GAPDH. To confirm involvement of class 1 PI3K, we used adenovirus that express a mutant DN p85 with the inner SH2 domain deleted (21,29). Expression of the DN p85 suppressed EGF-induced Akt phosphorylation by 37% and blunted the EGF-induced rise in GAPDH and pax2 (Figure 3). These results suggest that EGF-induced upregulation of pax2 and GAPDH requires class 1 PI3K but not mTOR.

**Akt Regulates Pax2 and GAPDH Abundance**

In NRK-52E cells, type 1 PI3K activity is required for phosphorylation of the downstream mediator Akt (21). Next, we used a kinase-dead, Akt1 dominant negative protein (DN Akt; Akt1 with T308A, S473A mutations; a gift from Dr. K. Walsh [22]) that was delivered by an adenoviral vector as a tool to examine whether Akt regulates pax2 and GAPDH abundance. Expression of DN Akt suppressed EGF-induced phosphorylation of the Akt downstream effector GSK3 β by approximately 42% (Figure 4A). It also suppressed EGF-induced accumulation

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**Figure 1.** LY294002 but not rapamycin regulates pax2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) abundance. NRK-52E cells were treated with vehicle (C), EGF alone (E; 10 nM) with or without rapamycin (50 nM), or with or without LY294002 (25 μM). Cells were grown and made quiescent as described in Materials and Methods. All reagents were added freshly in serum-free media every 24 h. Western analysis was carried out by using the same amount of protein extracts (10 μg). Representative blots are shown along with densitometry results normalized to actin for all samples. Lines indicate statistical comparisons between groups. (A) Western analysis was performed by using an antibody against pax2. Cells were treated for 96 h (*n* = 4). (B) Western analysis was performed by using antibody against GAPDH. Cells were treated for 96 h (*n* = 4). (C) Western analysis with antibody against actin. Cells were treated for 48 h, but similar results were seen at 96 h (*n* = 3). No significant difference was found between groups (densitometry data not shown).
of pax2 and GAPDH in a dose-dependent manner without changing the level of actin (Figure 4, B through D). These results suggest that Akt is the downstream effector of PI3K that regulates CMA.

To confirm the role of Akt in regulating the abundance of KFERQ proteins, we expressed a constitutively active form of Akt (CA Akt: myristoylated Akt1 [AxCAMyr-Akt], a gift from Dr. K. Walsh [22]) using an adenoviral vector. This virus, unlike the DN Akt, caused detectable toxicity at an MOI of 10 in serum-starved cells, so experiments shown were performed in the presence of 5% calf serum. Similar but more variable results were obtained in serum-starved cells with an MOI of 5 or 10 (data not shown). With CA Akt, GSK3\beta phosphorylation increased with increasing MOI of virus (Figure 5A). CA Akt increased pax2 and GAPDH protein abundance without affecting actin abundance (Figure 5, B through D). These results indicate that Akt activation is sufficient to regulate pax2 and GAPDH abundance. In summary, our results strongly suggest that PI3K acting through Akt regulates the lysosomal pathway of CMA, whereas mTOR does not.

**Effect of Proteolytic Inhibitors on PI3K Downstream Signaling**

To study the effect of these signaling molecules on systems of proteolysis, we used a lysosomal inhibitor that alkalinizes acidic compartments (10 mM methylamine), a proteasome inhibitor that blocks its trypsin-like site (0.5 \( \mu \)M MG132), and a class III PI3K inhibitor that blocks vesicle fusion in macroautophagy (10 mM 3-methyladenine). We previously showed that...
these concentrations of methylamine and MG132 provide maximal proteolytic inhibition and are specific for their proteolytic system in NRK-52E cells (24). Moreover, this concentration of 3-methyladenine is the currently accepted standard for inhibiting macroautophagy in epithelial cells (30). In initial experiments, we examined the effects of proteolytic inhibitors on phosphorylation of several key signaling molecules that are involved in PI3K signaling. Neither methylamine nor MG132 altered the phosphorylation of Akt (Ser473) and p70S6 kinase (Thr389; Figure 2). As expected, 3-methyladenine partially inhibited cell signaling through Akt and p70S6 kinase. This last result is consistent with the observation of Proud and colleagues (31), who reported that this class III PI3K inhibitor also weakly inhibits class I PI3K in fibroblasts.

**EGF and Akt Suppress a Lysosomal System, whereas Rapamycin Affects a Different System**

In prelabeled NRK-52E cells, the release of C14-labeled phenylalanine into the medium is an established method to measure the overall degradation rate of long-lived proteins (7,21,24,32). Phenylalanine is an appropriate amino acid to label because it is not significantly synthesized or degraded in renal epithelial cells (33). To determine whether EGF, proteolytic inhibitors, or cell signaling manipulations alter the kinetics of protein degradation, we plotted the mean values of the counts that remained at each time point (an example is shown in Figure 6A). All treatments resulted in first-order kinetics (Figures 6A and 7A) (21,24). Because statistical comparisons are difficult to visualize when plotting the degradation curves, we expressed most data as the mean slope of the plot of counts that remained versus time (Figure 6B).

Treatment with EGF decreased protein degradation by 24.8 ± 3.9% (P < 0.05; n = 6), and rapamycin significantly attenuated this effect (P < 0.05; n = 6). It is interesting that rapamycin alone had no significant effect on proteolysis (Figure...
6A). Consistent with our previous study (24), EGF did not further suppress protein degradation in the presence of the lysosomal inhibitor 10 mM methylamine (Figure 6B). These results indicate that EGF-induced suppression of proteolysis in renal tubular cells depends on a lysosomal system. When rapamycin is present, EGF no longer suppresses proteolysis to the same extent. However, this effect of rapamycin still is seen when methylamine is present. We interpret these data to mean that although rapamycin increases proteolysis when EGF is present, it does so by activating a nonlysosomal proteolytic pathway. These results are consistent with the Western blotting data showing no effect on KFERQ-containing proteins (Figure 1). It is interesting that rapamycin increases proteolysis when only methylamine is present, suggesting that inhibition of a lysosomal system of proteolysis is sufficient for rapamycin to stimulate proteolysis.

To confirm that Akt is regulating a lysosomal pathway, we measured proteolysis in cells that were treated with the DN Akt1 adenovirus. DN Akt alone had no significant effect on proteolysis, but it reversed EGF-induced suppression of proteolysis by $>50\%$ ($P < 0.01$; Figure 7A). Similarly, the use of the CA Akt1-expressing adenovirus caused a dose-dependent decrease in proteolysis (Figure 7B). In the presence of the lysosomal inhibitor methylamine, we found that DN Akt no longer affected proteolysis in EGF-treated cells (Figure 7C). These results are consistent with Akt influencing a lysosomal system of proteolysis.

**Macroautophagy Is Not Regulated by EGF or Rapamycin**

Because it was reported that growth factor signaling through TOR proteins acts as a negative regulator of macroautophagy (34), we examined proteolysis through this system. We treated cells with 3-methyladenine, which blocks the class III PI3K that is required for macroautophagy. Consistent with our report that EGF regulates a lysosomal pathway that is independent of macroautophagy (namely, CMA), we found that EGF still suppressed proteolysis despite 3-methyladenine treatment (Figure 8) (7). There may be a small change in the degree of the EGF response when 3-methyladenine is present, consistent with the slight decrease in Akt phosphorylation with 3-methyladenine treatment. Rapamycin still reversed the suppression of proteolysis that was produced by EGF when 3-methyladenine was present. Unlike methylamine, the presence of 3-methyladenine was not sufficient for rapamycin to stimulate proteolysis. These results suggest that both EGF and rapamycin affect protein breakdown independent of macroautophagy.

To confirm this conclusion, we further examined macroautophagy in NRK-52E cells by using the autofluorescence chemical MDC. MDC stains double-membrane vesicles, allowing identification of autophagasomes in the cell cytoplasm (26). Quiescent NRK-52E cells show ample granular, cytoplasmic MDC staining, and this staining is decreased by 3-methyladenine (10 mM; Figure 9). In contrast, MDC staining was not affected by EGF and/or rapamycin, indicating that neither treatment alters the number of autophagasomes (Figure 9). Thus, we found no evidence of regulation of macroautophagy in this model.

**Rapamycin Stimulates a Proteasomal System**

Because rapamycin did not significantly affect either CMA or macroautophagy in EGF-treated cells, we tested whether it increases proteasomal proteolysis using MG132 (0.5 $\mu$M). As we have shown previously (24), MG132 strongly reduced proteolysis, but it did not prevent EGF from further suppressing proteolysis (Figure 10A). It is interesting that when MG132 was added, rapamycin no longer reversed the suppression of proteolysis by EGF. As a second test, we treated the cells with the
more specific proteasomal inhibitor lactacystin and again found that the proteasomal inhibitor eliminated the effect of rapamycin (Figure 10B). These results strongly indicate that rapamycin activates a proteasomal pathway in the presence of EGF.

Discussion

Eukaryotic cells have evolved a complex set of mechanisms to modulate protein synthesis and degradation to allow for homeostasis, growth, or atrophy. Type I PI3K and its downstream mediators Akt and mTOR are critical enzymes that control and integrate protein synthesis and degradation (3,35,36). Recent reports have identified type I PI3K as a key regulator of protein degradation, but both the signaling elements that are downstream of type I PI3 kinase and the proteolytic systems vary widely in different cell types. For example, PI3K regulates ubiquitin/proteasome-dependent proteolysis in skeletal muscle by a mechanism that involves Akt and the FoxO transcription factors (37,38), whereas in hepatocytes and in human colon cancer HT-29 cells, regulation of macroautophagy through PI3K involves Akt and mTOR (19,39,40). In NIH 3T3 fibroblasts, regulation of macroautophagy...
agy does not involve mTOR (20). Thus, it is clear that the PI3K signaling pathways regulate different systems of proteolysis through different downstream effectors in different cell types.

In NRK-52E cells, the suppression of proteolysis by EGF is prevented by inhibiting Akt or mTOR, but the accumulation of the substrates of CMA is blocked only by Akt inhibition. Importantly, PI3K and Akt activity is increased in the renal cortex during diabetic renal hypertrophy (41), a condition whereby CMA is reduced (8). The proto-oncogene Akt seems to be situated uniquely to regulate protein and energy metabolism. Akt activates mTOR, which influences 4EBP-1 and p70 S6 kinase to stimulate protein synthesis, and inactivates glycogen synthetase kinase GSK3 and the FoxO transcription factors to influence glucose metabolism (42). However, it is not immediately clear how Akt regulates CMA. Our data demonstrate that mTOR is not involved in the regulation of CMA. Because rapamycin suppressed the phosphorylation of p70 S6 kinase (35) (Figure 2) but not lysosomal protein degradation, it is unlikely that p70 S6 kinase is involved in the regulation of lysosomal proteolysis. We also found that inhibiting the mitogen-activated protein kinase pathway decreases p70 S6 kinase phosphorylation and suppresses both protein synthesis and degradation (21) (W.S. and H.A.F., manuscript in preparation), further suggesting that p70 S6 kinase phosphorylation in renal tubular cells does not correlate with changes in proteolysis.

Akt also phosphorylates and inactivates subclass O of the F Box (Forkhead) family of transcription factors, FoxO. When dephosphorylated, FoxO proteins increase the transcription of many genes that are important for surviving the unfed state; phosphorylation by Akt prevents this response (43). In skeletal muscle, insulin specifically downregulates proteasomal proteolysis through a process that involves PI3K signaling (25). One way that insulin regulates the ubiquitin-proteasome system occurs through muscle-specific ubiquitin E3 ligases, whose transcription is driven by PI3K, Akt, and FoxO (37,38). Because these effectors are muscle specific, there is at least the possibility that similar signaling might regulate a lysosomal system.

Quiescent NRK-52E cells have considerable activity of their lysosomal proteolytic pathways that can be suppressed by EGF (24), but we have never previously examined macroautophagy. MDC staining shows ample macroautophagic vesicles in control, quiescent NRK-52E cells (Figure 9), indicating that macroautophagy is active. EGF treatment did not alter MDC staining in NRK-52E cells, a result that is consistent with our observation that EGF regulates CMA in these cells (7). Furthermore, rapamycin did not stimulate staining over the baseline levels of activity, even when EGF was present. Furthermore, 3-methyladenine did not greatly alter the effects of EGF or rapamycin on proteolysis (Figure 8). The small inhibition of EGF-induced proteolysis with 3-methyladenine, seen in Figure 8, most likely...
results from the partial inhibition of Akt phosphorylation, seen in Figure 2. Therefore, unlike reports in other epithelial cells (e.g., hepatocytes and intestinal carcinoma cells [18,34,44]), we found no evidence indicating that macroautophagy is significantly regulated in renal epithelial cells.

That rapamycin acts by stimulating protein degradation in EGF-treated cells via a proteasomal pathway is a novel and unexpected finding. Rapamycin had no effect unless growth factors or lysosomal inhibitors also were present (Figure 6). Some studies have suggested that by degrading common substrates, a form of communication between lysosomal and proteasomal pathways exists. Lysosomes may sequester and degrade certain ubiquitinated proteins (45), and monoubiquitination may target plasma membrane protein for rapid entry into the lysosomal pathway (46). This suggests a model that mTOR signaling prevents the proteasome from increasing degradation of proteins that ordinarily would be destroyed by CMA. Rapamycin has been described to accelerate the degradation of many proteasomal substrates, presumably by upregulation of ubiquitin conjugation (47). However, bulk proteolytic flux through the ubiquitin-proteasome pathway can be regulated by multiple factors, including the abundance of ubiquitin, the abundance of 26S proteasomes, the rate of translocation of the 19S cap, and the proteolytic rate of the 20S core particle (48).

**Conclusion**

Growth factors, including EGF, play a significant role in increasing protein synthesis and suppressing protein degradation in renal hypertrophy (49). PI3K and Akt suppress the lysosomal CMA system in EGF-treated renal tubular cells, leading to accumulation of KFERQ-containing proteins, whereas mTOR seems to regulate a separate proteasomal system in these cells. As inhibitors of growth factor-mediated signaling (e.g., rapamycin and Akt inhibitors) become more widespread in clinical medicine, working out the distinct signaling pathways that regulate proteolytic systems in different tissues may lead to greater understanding of the mechanism of action and adverse effects of the pharmacologic agents.

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