Phosphatidylinositol 3-Kinase Activity Is Required for Epidermal Growth Factor to Suppress Proteolysis

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Abstract. Suppression of protein breakdown occurs commonly in cell growth, but the pathways responsible for controlling proteolysis are poorly understood. Protein breakdown in NRK-52E renal epithelial cells treated with epidermal growth factor (EGF) and intracellular signaling inhibitors or dominant negative signaling molecules contained in an adenoviral vector were measured. The tyrosine kinase inhibitor, herbimycin A, eliminated the suppression of proteolysis induced by EGF. In contrast, the Src inhibitor, PP1, had no effect. Expression of dominant negative H-RasY57 blocked the ability of EGF to stimulate downstream targets of Ras and also reduced the ability of EGF to suppress proteolysis. Inhibiting MEK did not influence the ability of EGF to suppress proteolysis, but the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor, LY249002, stimulated basal proteolysis and completely eliminated the proteolytic response to EGF. Use of an adenovirus that expresses a dominant negative p85 subunit of class 1 PI 3-kinase completely blocked the ability of EGF to suppress proteolysis, whereas use of an adenovirus expressing a K227E constitutively active p110 subunit reproduced the reduction in protein breakdown. It was concluded that EGF suppresses proteolysis by a mechanism that involves Ras and class 1 PI 3-kinase.

Both increased protein synthesis and decreased protein breakdown can contribute to protein accumulation in growing cells. Suppression of proteolysis during cell growth has been documented in normal and transformed mammalian cells (1–3). One organ with a pronounced decrease in proteolysis during growth is the kidney. In the hypertrophy that occurs in diabetes, protein synthesis in the renal cortex is twofold to threefold above baseline but returns to normal ~7 d after the onset of diabetes, whereas proteolysis falls by ~30% and remains lower for an extended period (4,5).

Pathways responsible for controlling proteolysis during cell growth are poorly understood, but there is evidence for changes in lysosomal protein degradation (6,7). During cell growth stimulated by epidermal growth factor (EGF), protein half-life increases by ~30% in renal tubular cells in culture (8). Our experiments that used inhibitors of lysosomal function, proteasome, or calcium-sensitive proteases revealed that EGF has its effect primarily on lysosomal proteolysis (8,9). Suppression of protein degradation results in increased accumulation of substrates usually targeted for lysosomal destruction, including membrane proteins, organelles, and substrates of chaperone-mediated autophagy (10–12). Because EGF suppresses the activity of chaperone-mediated autophagy, regulating proteins important for renal cell growth (9), we became interested in understanding the signaling pathway responsible for suppressing protein degradation.

Studies of mechanisms that activate protein synthesis have revealed that two early signaling pathways are responsible for the response to growth factors: phosphatidylinositol 3 kinase (PI 3-kinase) and the mitogen-associated protein (MAP) kinases (13). Both pathways are activated by EGF receptor through its tyrosine kinase activity stimulating the small G protein, Ras (14–16). To determine whether PI 3-kinase and one of its upstream regulators, Ras, are also involved in regulating the rate of proteolysis, we used chemical inhibitors or genetic approaches to investigate how protein degradation is regulated in EGF-treated NRK-52E renal tubular cells.

Materials and Methods

All chemicals or reagents were purchased from Sigma Chemical (St. Louis, MO), except Dulbecco’s Modified Eagle Media, newborn calf serum, trypsin–ethylenediaminetetraacetic acid (EDTA), and penicillin–streptomycin, which were obtained from Life Technologies (Grand Island, NY). Recombinant human EGF was obtained from R&D Systems (Minneapolis, MN), L-[U-14 C] phenylalanine from New England Nuclear, Dulport (Boston, MA), anti–phospho-p42, anti–phospho-p44 MAP kinase, and anti-p85 PI 3-kinase antibodies from Upstate Biotechnology (Lake Placid, NY), anti p110 PI 3-kinase antibodies from Santa Cruz Biotechnology (Santa Cruz, Ca), anti-AKT and anti–phospho-AKT antibodies from New England Biolabs (Beverly, MA), and anti–PY-20 from Transduction Laboratories (San Diego, CA).
Cell Culture

NRK-52E cells (a rat kidney epithelial cell line) (17) and LLCPK1 cells were obtained from the American Type Culture Collection (Bethesda, MD). Passage 19 to 29 NRK-52E cells were grown in 6-well plates or 100-mL dishes with high-glucose Dulbecco’s Modified Eagle Media supplemented with 25 mM Hepes, 25 mM glutamine, and 5% calf serum. Passage 130 to 135 LLCPK1 cells were grown in media 199 with 25 mM glutamine and adjusted to 1.5 g/L Na HCO3. When confluent, were rendered quiescent by serum removal for 48 h before the experiment.

Toxicity of inhibitors or adenoviruses was assessed by trypan blue exclusion as described elsewhere(18). Concentrations of inhibitors or adenoviruses used were usually <25% of the lowest concentration where any toxicity was observed (the exception is the dose response curve for LY290058 [fig 4], where toxicity was observed at 100 μM). Recombinant human EGF was reconstituted at 10−8 M in phosphate-buffered saline that contained 0.1% heat-treated, bovine serum, and the appropriate vehicle, or the same titer of ad.EGFP (in the case of adenoviral experiments) was added to control cells (19).

Protein Turnover

Protein degradation was measured as the release of L-[U-14C] phenylalanine from cells prelabeled, as described elsewhere (8,20). After labeling, 5 mM of L-phenylalanine was added to the media to minimize the reuse of released phenylalanine, and after an initial 4-h washout period to eliminate short-lived proteins and unincorporated phenylalanine from cells prelabeled, as described elsewhere (8,20). Readded every 4 h during the experiment. Media aliquots were removed at intervals and radioactivity measured after TCA precipitation and incubated with agitation at room temperature for 10 min before being added to an ice-cold solubilization buffer that contained 50 mM Na3VO4, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 1% NP-40, 10% glycerol, 2 μg/mL aprotinin, 10 μg/mL antipain, 5 μg/mL leupeptin, 0.5 μg/mL pepstatin, 1.5 mg/mL benzamidine, and 34 μg/mL phenylmethylsulfonyl fluoride (24). After removal of insoluble material by centrifugation, proteins were immunoprecipitated from aliquots of the supernatants with anti-p85 subunit of PI 3-kinase or antiphosphotyrosine antibodies (anti-PY20). Immunoprecipitates were washed successively in (i) phosphate-buffered saline that contained 1% NP-40 and 100 μM Na3VO4; (ii) 100 mM Tris-Cl (pH 7.5), 500 mM LiCl, and 100 μM Na3VO4; and (iii) Tris-Cl (pH 7.5) that contained 100 mM NaCl, 1 mM EDTA, and 100 μM Na3VO4. Pro 3-kinase activity associated with these proteins was measured by resuspending the immunoprecipitates in a solution of 10 mM Tris-Cl (pH 7.5) with 100 mM NaCl, 1 mM EDTA, 15 mM MgCl2, 100 μM NaVO4, 20 μg phosphatidylinositol, 1 mM ethyl-ene glycol-is(β-aminooethyl ether)-N,N'-tetraacetic acid, and 440 μM ATP (which contained γ-32P-ATP). The reaction mixtures were incubated with agitation at room temperature for 10 min before being stopped with 8 M HCl and a 1:1 mix of CHCl3:MeOH. The products were separated by silica gel thin-layer chromatography that used a developing solution of CHCl3:CH3OH:2H2O:NH4Cl (60:47:11:6.2). Results were visualized by phosphorimaging or autoradiography, and products comigrating with a phosphatidylinositol 3-phosphate standard were quantified.

Western Blotting

Cells grown in 60-mm tissue culture dishes were washed twice in ice cold phosphate-buffered saline and lysed in RIPA buffer that contained 100 μg/ml phenylmethylsulfonyl fluoride, 2 mm sodium EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. After centrifugation, the protein content in an aliquot of supernatant was determined, and samples were boiled in buffer that contained 1% sodium dodecyl sulfate, and 0.5% β-mercaptoethanol, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters, and 5% fat-free milk protein or 3% bovine serum albumin were used as blocking reagents. Antibodies were detected by use of the ECL system (Amersham, Arlington Heights, IL).

Statistical Analyses

Results are expressed as mean ± SEM. Because there is experiment to experiment variation in the magnitude of responses, results are presented as percentage of the control value measured on the same
day. Differences between two groups were analyzed by the Student’s 
t test and multiple comparisons were analyzed by ANOVA that used 
the Student-Newman-Keuls test for multiple comparisons. Compari-
sions of slopes of lines representing the release of L-[U-14 C] phenyl-
alanine were done by analysis of covariance.

Results

Because EGF typically acts via its tyrosine kinase receptor 
(14), we measured protein degradation in the presence of 
inhibitors of tyrosine kinase pathways. Figure 1A shows a 
typical protein degradation experiment that used herbimycin to 
block autophosphorylation of the EGF receptor (25). Proteol-
ysis decreased 30% with EGF treatment, whereas herbimycin 
only slightly decreased the rate of proteolysis. However, her-
bimycin blocked the ability of EGF to suppress protein degra-
dation, a finding that suggests that EGF suppresses proteolysis 
through a pathway that requires tyrosine phosphorylation (Fig-
ure 1A). Not every tyrosine kinase inhibitor exerted this effect: 
the Src family inhibitor PP1, which does not affect EGF 
receptor autophosphorylation (26), did not impair the ability of 
EGF to suppress proteolysis degradation (Figure 1B). These results 
suggest that EGF suppresses protein degradation via the well-
characterized, receptor-mediated tyrosine kinase pathways 
(14).

To determine whether signaling pathways activated by Ras 
are involved in the control of proteolysis, we used a replica-
tion-defective adenovirus, AdexCAHRasY57, which contains 
the dominant negative Ras (tyrosine is substituted for aspartic 
acid at residue 57, so the Ras protein binds tightly to guanine 
nucleotide exchange factors and inhibits endogenous Ras ac-
tivation) (21). Ad.EGFP, an adenovirus that expresses GFP 
alone, was used as a control. We assessed the effectiveness this 
strategy by examining the phosphorylation of the Ras down-
stream effectors, p42 and p44 MAP kinases (14,27). When Ad.exCAHRas effectively suppressed basal and EGF-induced 
MAP kinase phosphorylation, there was no significant change in 
the baseline rate of protein degradation (Figure 2). However, 
it partially blocked the ability of EGF to suppress proteolysis 
(Figure 2). These results suggest that EGF suppresses proteol-
ysis, in part, by stimulating Ras-linked signaling pathways.

Two major signaling pathways controlled by Ras are the 
MAP kinase and PI 3-kinase system (13,27). To examine 
whether the MAP kinase pathway was involved, we used two 
distinct MEK inhibitors, PD098059 and U0126, because they 
block activation of MAP kinase (28,29). Both PD098059 and 
U0126 suppressed the basal rate of proteolysis, and EGF 
suppressed proteolysis further (Figure 3). These data suggest
that activation of the MAP kinase pathway is not involved in the antiproteolytic response to EGF. To determine whether the PI 3-kinase pathway is involved in EGF-induced suppression of protein degradation, we used the specific PI 3-kinase inhibitor, LY294002 (30). This inhibitor stimulated proteolysis at doses as low as 6 μM and at 25 μM completely blocked the ability of EGF to suppress proteolysis. (Figure 4). We had similar results with the PI 3-kinase inhibitor wortmannin (data not shown), but because of its short half-life in aqueous solutions (30), wortmannin had to be added every 4 h, making it difficult to calculate the exact concentration used. To demonstrate that this effect is not limited to NRK-52E cells, LLCPK1 cells were treated with LY294002. LLCPK1 cells do not show the same linear change in proteolysis as NRK-52E cells, but EGF clearly prolongs cellular protein half-life (Figure 4C). In these cells, only 6 μM of Ly294002 was required to increase basal proteolysis and block the effect of EGF to suppress proteolysis.

To verify that PI 3-kinase is involved in the pathway by which EGF suppresses proteolysis, we used a strategy of overexpressing a dominant negative class 1, PI 3-kinase p85 subunit with the inner SH2 domain deleted. This mutation results in a p85 subunit that binds to, but does not activate, the p110 subunit of class 1 PI 3-kinase (22). We delivered this dominant negative p85 using an adenoviral vector, AdTrackp85ΔISH2, which coexpresses GFP, to allow monitoring of transfection efficiency and used ad.EGFP as a control (Figure 5B). Expression of this mutant p85 effectively inhibited both EGF-induced activation of PI 3-kinase and its ability to suppress proteolysis (Figures 5BC). With higher concentrations of the dominant negative PI 3-kinase, we observed a increase in basal protein breakdown as well (Figure 5D).

To determine whether activity of class 1 PI 3-kinase was sufficient to suppress proteolysis in NRK-52E cells, we created an adenovirus that expressed a constitutively active, K227E mutant, p110 catalytic subunit of class 1 PI 3-kinase. This mutant form is catalytically active in the absence of Ras or p85 activation (27). Transfection of NRK-52E cells with this constitutively active p110 construct increased PI 3-kinase activity (measured by immunoprecipitating with anti-p110) compared with cells transfected with Ad.EGFP. It also affected signaling downstream of PI 3-kinase: AKT phosphorylation was increased with expression (Figure 6, A and B). Expression of this protein increased protein half-life by ~33% (Figure 6C), a
result that suggests that p110 activation is sufficient to regulate proteolysis.

Discussion

Growth factors stimulate protein accumulation by both increasing protein synthesis and suppressing protein degradation (2). We have developed a cell culture model system using NRK-52E cells to examine causes and consequences of this response (8,9,18). We used NRK-52E cells for three reasons: (1) they have a similar proteolytic response to growth factors as primary cultures of proximal tubule cells (8); (2) their proteolytic response to growth factors is linear, like primary cultures of proximal tubule cells (8) but unlike many other cell lines (compare Figure 4A with 4C); and (3) they have less variability than primary cultures of proximal tubule cells.
tubule cells, which allowed us to measure accurately smaller changes in proteolysis (8).

Work by ourselves and others has indicated that, in epithelial cells, growth factors specifically suppress lysosomal proteolysis (6–9). A consequence of suppressing this degradation should be accumulation of proteins normally targeted for destruction in the lysosome. In NRK-52E renal epithelial cells, we observe that EGF increases the half-life and abundance of proteins that are substrates for chaperone-mediated autophagy, such as GAPDH or the paired-box transcription factor, PAX2 (9). Indeed, treating NRK-52E cells with inhibitors of lysosomal function causes hypertrophy (18). Because suppressed proteolysis contributes to growth, we sought to understand the mechanisms that control this response.

What is the signaling pathway by which EGF suppresses protein breakdown? After ligand binding, the EGF receptor undergoes tyrosine autophosphorylation, leading to recruitment of proteins that bind to the receptor (31). For example, Grb2 binds to the receptor and, by interacting with Sos, activates Ras. Ras, in turn, activates the MAP kinase pathway (16) and class 1 PI 3-kinase (27). The increases in PI 3-kinase and MAP kinase have been shown to stimulate protein synthesis in response to growth factors (13,16). We found that doses of herbimycin that typically block EGF-receptor autophosphorylation also prevented EGF from suppressing protein degradation and that PI 3-kinase is the pathway that suppresses proteolysis in response to EGF. Inhibiting PI 3-kinase activity with LY294002 or expressing the dominant negative p85 subunit of class 1 PI 3-kinase not only increased the basal rate of proteolysis but also blocked the ability of EGF to suppress protein breakdown (Figures 4 and 5). Conversely, increasing PI 3-kinase activity by expressing a constitutively active PI 3-kinase stimulated the basal rate of proteolysis (Figure 6).

Another component of the pathway is Ras, because inhibition of Ras activity also diminished the ability of EGF to suppress proteolysis. Because Ras activates only class 1 PI 3-kinase (27,30,32), this result also points to a central role of class 1 PI 3-kinase in controlling protein degradation in these renal tubular cells. We were surprised to find that inhibition of the activity of the other major downstream target of Ras, the MAP kinase pathway, reduced the rate of basal proteolysis but did not affect the ability of EGF to suppress proteolysis. These results suggest that protein synthesis and degradation are regulated differently: both MAP kinase and PI 3-kinase activity can stimulate protein synthesis (13,33,34), but our results suggest that MAP kinase activity stimulates protein breakdown, whereas PI 3-kinase activity suppresses it. We speculate that selective activation of PI 3-kinase and MAP kinase pathways may provide the cell with the ability to differentially regulate protein synthesis and degradation.

Although there have been no previous studies of signaling pathways controlling proteolysis during growth, our results seem to contradict the report of Petiot et al. (35), which indicated that wortmannin or LY294002 decreased protein breakdown induced by nutrient deprivation in HT-29 colon cancer cells. However, they reported that the downstream lipid products of class 3 PI 3-kinase stimulated proteolysis, whereas adding those of class 1 PI 3-kinase decreased bulk proteolysis (35). These findings are consistent with our results. We speculate that the wortmannin or LY294002 they added suppressed class 3 PI 3-kinase isoforms that were activated by nutrient deprivation, which led to the difference in outcomes (30,32).

The downstream pathways that regulate proteolysis are not known, but class 1 PI-3 kinase products PtdIns 3,4 or PtdIns 3,4,5 can mediate downstream signaling through PDK-1 (phosphoinositol-dependent kinase), which phosphorylates AKT (32). AKT phosphorylates other downstream effectors, such as glycogen synthetase kinase, to stimulate protein synthesis (30). Class 1 kinase products also control the trafficking of membranes and proteins in the liver, including membrane insertion of glucose transporters in response to insulin (30). The fact that AKT phosphorylation correlated with proteolysis better than PI-3 kinase activity in Figure 6 should not be taken as strong evidence that this pathway regulates proteolysis because of the limitations of measuring PI-3 kinase activity when a constitutively active P110 has been introduced. Because we could not use anti-phosphotyrosine or anti-p85 antibodies to immunoprecipitate p110 in these circumstances, we used an anti-p110 antibody. Therefore, differences in the PI-3 kinase measurement could represent differences in the efficiency of immunoprecipitation of active PI-3 kinase between the mutant and native proteins and thus not reflect in vivo type I PI-3 kinase signaling as well as phospho-AKT.

In conclusion, we present the first evidence that type 1 PI 3-kinase controls the anti-proteolytic response to a growth factor and provide a potential mechanism for coordinate regulation of protein breakdown and synthesis. Further investigation will be needed to test the hypothesis that MAP kinase and PI 3-kinase pathways have opposing effects on proteolysis, to examine the role of other PI 3-kinase isoforms, and to determine downstream signaling mechanisms.

Acknowledgments

The authors thank Patryce Curtis and Li Ling Shen for their technical help and Drs. William Mitch, S. Russ Price, Douglas Eaton, and Mario Marrero for advice, support, and/or critical reading of the manuscript. This work was supported by NIH Grants K08 DK02496 (to H.A.F.) and NIH RO1 DK37175 (to J.D.), a Veterans Administration Merit Review Award (to H.A.F.), a fellowship Grant from the National Kidney Foundation of Georgia (to S.S.), and an American Heart Association Scientist Development Award (to J.D).

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