EGF Receptor Transactivation Mediates Angiotensin II-stimulated Mitogenesis in Intestinal Epithelial Cells through the PI 3-Kinase/Akt/mTOR/p70S6K1 signaling pathway

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Abstract

The role of epidermal growth factor receptor (EGFR) tyrosine kinase and its downstream targets in the regulation of the transition from the G₀/G₁ phase into DNA synthesis in response to ANG II has not been previously investigated in intestinal epithelial IEC-18 cells. ANG II induced a rapid and striking EGFR tyrosine phosphorylation, which was prevented by selective inhibitors of EGFR tyrosine kinase activity (e.g. AG1478) or by broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM 6001. Pretreatment of these cells with either AG1478 or GM 6001 reduced ANG II-stimulated DNA synthesis by ~50%. To elucidate the downstream targets of EGFR, we demonstrated that ANG II stimulated phosphorylation of Akt at Ser-473, mTOR at Ser-2448, p70S6K1 at Thr-389, and S6 ribosomal protein at Ser-235/236. Pretreatment with AG1478 inhibited Akt, p70S6K1, and S6 ribosomal protein phosphorylation. Inhibition of PI 3-kinase with LY 294002 or mTOR/p70S6K1 with rapamycin reduced [³H]-thymidine incorporation by 50%, i.e. to levels comparable to those achieved by addition of either AG1478 or GM 6001. Utilizing Akt siRNA targeted to Akt1 and Akt2, Akt protein knockdown dramatically inhibited p70S6K1 and S6 ribosomal protein phosphorylation. In contrast, AG1478 or Akt gene silencing exerted no detectable inhibitory effect on ANG II-induced ERK1/2 phosphorylation in IEC-18 cells. Taken together, our results demonstrate that EGFR transactivation mediates ANG II-stimulated mitogenesis through the PI 3-Kinase/Akt/mTOR/p70S6K1 signaling pathway in IEC-18 cells.
Introduction

The sequential proliferation, lineage-specific differentiation, migration and cell death of epithelial cells of the intestinal mucosa is a tightly regulated process that is modulated by a broad spectrum of regulatory peptides (5, 26, 50), but the signal transduction pathways involved remain incompletely understood. The non-transformed IEC-18 cells, derived from rat small intestinal crypt (40), have provided an in vitro model to examine intestinal epithelial cell migration, differentiation and proliferation (16, 17, 28). These cells are undifferentiated as judged by morphologic and functional criteria; they resemble stem cells and thus, may serve as a model to study the crypt stem cell (51). The importance of examining this compartment is highlighted by recent reports suggesting that the initial mutant cell in the pathogenesis of colorectal adenoma may arise from the crypt stem cell compartment (37, 55).

Neuropeptides and vasoactive peptides that signal through G-protein coupled receptors (GPCRs), characterized by seven-transmembrane helices, act as potent cellular growth factors for a variety of cell types (43, 44, 46, 47). In particular, GPCRs and their agonists play a crucial role in the regulation of multiple functions in the gastrointestinal tract, including cell proliferation, motility, and transport. We have recently demonstrated that the vasoactive peptide angiotensin II (ANG II) and the neuropeptide arginine vasopressin induce DNA synthesis and cell proliferation in intestinal epithelial IEC-18 cells (8, 9). Some of the key molecules that mediate the growth-promoting signal of these GPCR agonists in IEC-18 cells have been identified. ANG II induces a dramatic increase in PKC-dependent protein kinase D (PKD)
activation in IEC-18 cells via the AT-1 receptor coupled to Gq (7). ANG II also stimulates PKC-dependent tyrosine phosphorylation of Pyk2, a non-receptor tyrosine kinase that has been implicated as an upstream element in ERK-1/2 activation, in IEC-18 cells (56). Accordingly, this GPCR agonist stimulates PKC-dependent ERK activation in these cells. Interestingly, inhibition of the PKC or ERK pathway in these cells reduces ANG II-induced DNA synthesis by 50-60% (8, 9). These findings not only indicated that ANG II-induced DNA synthesis is partially dependent on ERK and PKC but also prompted us to hypothesize that another pathway, in addition to ERK and PKC, plays a substantial role in AT1-mediated mitogenesis of IEC-18 cells.

Recently, it has been shown that a variety of GPCR agonists also induce a rapid increase in epidermal growth factor receptor (EGFR) tyrosine autophosphorylation in several cell types (10-12, 29, 52), termed transactivation (6, 36). This receptor cross-talk can occur via extracellular release of EGFR ligands at the cell surface (36) by matrix metalloproteases that belong to the ADAM (a disintegrin and metalloprotease) family of zinc-dependent proteases. GPCR-induced EGFR transactivation leads to the phosphorylation of specific tyrosine residues within the EGFR cytoplasmic domain, which act as docking sites for effector molecules resulting in activation of downstream signaling pathways. However, it is increasingly recognized that the contribution of EGFR transactivation to agonist-induced mitogenic signaling is strongly dependent on cell context. The experiments presented in this study were designed to examine the role of EGFR tyrosine kinase and its downstream targets in mediating the transition from the G0/G1 phase into S phase in response to the potent mitogen ANG II in the normal intestinal epithelial IEC-18 cells.
In contrast to many cell types in which EGFR contributes to mitogenesis via the Ras/Raf/MEK/ERK pathway, the results presented in this study demonstrate that EGFR transactivation mediates ANG II-induced DNA synthesis in IEC-18 cells via activation of the PI 3-kinase/Akt/mTOR/p70S6K1 pathway.
Material and Methods

Cell culture

IEC-18 cells were purchased from American Type Culture Collection. Stock cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere containing 10% CO₂ and 90% air at 37°C. For experiments, cells were plated in 100 mm dishes at 3x10⁵ cells/dish in DMEM containing 5% FBS and were allowed to grow to confluency (5-7 days) and then changed to serum-free DMEM for 18-24 h prior to the experiment.

Western Blot Analysis for protein kinase phosphorylation

Serum-starved cultures of IEC-18 cells grown on 100 mm dishes were washed twice with DMEM and then treated as described in the individual experiments. The cells were lysed in 2x SDS-PAGE sample buffer. Following SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore) and blocked by 3-6 h incubation with 5% non-fat milk in phosphate-buffered saline, pH 7.2. Membranes were then incubated overnight with the respective phosphospecific antibodies (see Materials). The same membranes were stripped and probed in a similar fashion with the respective polyclonal antibody for total protein.

Bound primary antibodies to immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase conjugated anti-mouse, anti-rabbit or anti-goat antibodies. Autoradiograms were scanned using a GS-710 scanner (Bio-Rad), and the labeled bands were quantified using Quantity One software program (Bio-Rad).
Assays of EGFR tyrosine phosphorylation

Serum starved cultures of IEC-18 cells were washed twice with serum-free DMEM and then incubated for 1 h at 37°C with or without inhibitors, as indicated. Cells were then treated for 5 min with agonists. Cell stimulation was terminated by transferring the cultures to ice, aspirating the medium, and lysing the cells in 1 ml ice-cold modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris·HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Pefabloc SC, 1 mg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium pervanadate (Na₃VO₄), and 1 mM sodium fluoride (NaF)). The cell suspension was then mixed for 20 min at 4°C to lyse the cells, and cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. To measure EGFR tyrosine phosphorylation, the supernatants were transferred to new microtubes and mixed with 10 µg/ml polyclonal α-EGFR at 4°C overnight. Immunocomplexes were captured by adding 100 µl protein-A-agarose beads for 1 h, then agarose beads were collected by pulse centrifugation (0.5 min at 14,000 rpm). The beads were washed three times with ice-cold RIPA buffer and subsequently solubilized in 100 µl 2X SDS-PAGE sample buffer. Tyrosine-phosphorylated EGFR was detected by immunoblotting using 4G10 mAb.

Assay of DNA Synthesis

Confluent and serum-starved cultures of IEC-18 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1, v/v), and various additions as described in the figure legends. After 15 h of incubation at 37 °C, [³H]-thymidine (0.2 µCi/ml, 1 µM) was added and the cultures incubated for a further 4 h at 37 °C. Cultures were then washed twice with phosphate buffered saline (PBS) and
incubated in 5% trichloroacetic acid at 4 °C for 20 min to remove acid-soluble radioactivity, washed with ethanol, and solubilized in 1 ml of 2% Na₂CO₃, 0.1 M NaOH. The acid-insoluble radioactivity was determined by scintillation counting in 6 ml of Beckman Readysafe.

**Flow Cytometric/Cell Cycle Analysis**

The proportion of cells in the G₀/G₁, S, G₂, and M phases of the cell cycle were determined by flow cytometric analysis. Confluent and serum-starved cultures of IEC-18 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1, v/v) containing various additions as described in the figure legends. After 19 h of incubation at 37 °C, cultures were washed twice with PBS. Cells were then detached by treatment with trypsin (0.025%), suspended in DMEM containing 10% FBS, and centrifuged at 1000g for 5 min. Cells (10⁶) were then resuspended and stained by adding 1 ml of a hypotonic DNA staining buffer containing propidium iodide (0.1 mg/ml), sodium citrate (1 mg/ml), Ribonuclease A (20 µg/ml), and Triton X-100 (0.3%). Samples were kept at 4°C protected from light for 30 min and analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ), using the software CELLQuest version 3.3 and Modfit 3.1 (Verity Software House, Topsham, ME).

**Akt siRNA**

To inhibit Akt protein expression, subconfluent cultures of IEC-18 cells were transfected with small interfering RNA (siRNA). The synthetic 21-mer oligonucleotides RNA duplex corresponding to the consensus sequence of Akt1 and Akt2 that is identical in both human and rat was synthesized by Cell Signaling (Beverly, MA) (27). A fluorescein-labeled non-targeted negative control duplex purchased from Cell Signaling
was used as a control. Transfection of duplex siRNAs at 100 nM were performed using TransIT-TKO® Reagent (Mirus, Madison, WI) according to the manufacturer’s protocol. Forty-eight hours after transfection, cells were used for experiments and subsequent Western blot analysis.

**Materials**

[^3]H]-thymidine was from Amersham Pharmacia Biotech (Piscataway, NJ). Tyrphostin AG1478, compound 56, PD98059, U0126, LY 294002, wortmannin, and heparin were from Calbiochem. Protein A-agarose was from Boehringer Mannheim (Indianapolis, IN). Angiotensin II, EGF, HB-EGF, and trypsin-EDTA solution (1X) were obtained from Sigma (St. Louis, MO). Anti-ERK2 polyclonal antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, California). 4G10 anti-Tyr(P) mAb and anti-EGFR antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-ERK-1/ERK-2 mAb, phospho-Ser473 Akt mAb, polyclonal Akt antibody, phospho-Ser2448 mTor mAb, phospho-Thr389 p70S6 Kinase1 mAb, polyclonal p70S6 Kinase1 antibody, phospho-S6 Ribosomal Protein (Ser235/236) antibody, and SignalSilence™ Akt siRNA Kit were obtained from Cell Signaling (Beverly, MA). Other items were from standard suppliers or as indicated in the text.
RESULTS

ANG II-stimulated EGFR transactivation is dependent on matrix metalloprotease activity in IEC-18 cells

To determine whether ANG II stimulates EGFR tyrosine phosphorylation in IEC-18 cells, serum-starved cultures of these cells were stimulated with 50 nM ANG II for various times and then lysed. The extracts were immunoprecipitated with anti-EGFR polyclonal antibody (Fig. 1A, upper panel), separated by SDS-PAGE, and further analyzed by Western blotting using anti-Tyr(P) mAb. As shown in Fig. 1A, ANG II stimulated a rapid EGFR tyrosine phosphorylation in IEC-18 cells, reaching a maximum within 30 sec and remaining elevated for at least 60 min.

Pretreatment of IEC-18 cells with the selective EGFR tyrosine kinase inhibitors tyrphostin AG1478 or the structurally unrelated compound 56 (20), both at 250 nM, completely prevented ANG II-induced increase in tyrosine phosphorylation of EGFR induced by ANG II. Stripping the same blots and reprobing with anti-EGFR antibody confirmed that similar amounts of EGFR protein were recovered after ANG II stimulation (Fig. 1A and 1B, lower panel). The results presented in Figs. 1A and 1B demonstrate that ANG II markedly stimulates EGFR tyrosine kinase activity in IEC-18 cells.

To determine whether the cross-talk between EGFR and GPCRs is mediated by extracellular release of EGFR ligands at the cell surface in IEC-18 cells, cultures of these cells were preincubated with GM 6001 (also known as Ilomastat or galardin), a broad-spectrum matrix metalloproteinase inhibitor (1, 21, 49). Serum-starved cultures of IEC-18 cells were preincubated with either control medium or medium containing 10 µM GM 6001 for 1 h and then stimulated with ANG II. The concentration of GM 6001 used in
these experiments (10 µM) was shown to inhibit MMP-1, 2, and 3 (21). Pretreatment of cells with GM 6001 suppressed the increase in EGFR tyrosine phosphorylation induced by ANG II stimulation (Fig. 1B). This result indicates that ANG II-stimulated EGFR tyrosine phosphorylation depends on the activity of GM 6001-sensitive metalloprotease(s) to mediate extracellular release of EGFR ligands.

Exogenously added heparin has been shown to inhibit EGFR transactivation by competing with cell surface–associated heparan sulfate proteoglycans (HSPGs) for HB-EGF-binding (25, 41). Here we used heparin to determine whether ANG II stimulates EGFR tyrosine phosphorylation through HB-EGF release in IEC-18 cells. Serum-starved cultures of these cells were pretreated with 50 µg/ml heparin prior to stimulation with ANG II, HB-EGF, or EGF. We verified that heparin inhibited HB-EGF-induced EGFR tyrosine phosphorylation, whereas EGF-induced EGFR tyrosine phosphorylation was not affected in IEC-18 cells(Fig. 1C). Interestingly, pretreatment with heparin significantly reduced ANG II-induced EGFR tyrosine phosphorylation. This result combined with our results with GM 6001 suggests that HB-EGF is required for ANG II-stimulated EGFR transactivation in IEC-18 cells.

**ANG II-stimulated DNA synthesis is dependent on EGFR tyrosine kinase activity**

We recently demonstrated that ANG II acts as a potent growth factor for IEC-18 cells promoting exit from G₀/G₁ and entry into S phase in IEC-18 cells (8), but the precise mechanism(s) that mediate ANG II-induced mitogenesis remains incompletely understood. To examine if EGFR transactivation contributes to ANG II-induced DNA synthesis, serum-deprived cultures of IEC-18 cells were preincubated for 1h with the selective EGFR tyrosine kinase inhibitor tyrphostin AG1478 at various concentrations
(30-500 nM) prior to stimulation with ANG II. As shown in Fig. 2A, treatment with tyrphostin AG1478 markedly attenuated $[^3]$H-thymidine incorporation in response to ANG II stimulation in a concentration-dependent fashion. A maximal inhibitory effect (~50%) was achieved at 100 nM tyrphostin AG1478.

To substantiate that the effects on $[^3]$H-thymidine incorporation reflects an increase in DNA replication through S phase of the cell cycle rather than alterations in transport and/or phosphorylation of $[^3]$H-thymidine, we used flow cytometric analysis to determine the proportion of cells in the various phases (G₀/G₁, S, and G₂/M) of the cell cycle. Confluent and serum-starved cultures of IEC-18 cells were subjected to identical conditions as those for the $[^3]$H-thymidine incorporation experiments and were preincubated for 1h with AG1478 prior to stimulation with ANG II. As shown in Fig. 2B, serum-starvation of IEC-18 cells resulted in 85.4 ± 2 % of cells in G₀/G₁, comparable to previous published results – confirming that serum-starvation induced the accumulation of IEC-18 cells into quiescence. Addition of 50 nM ANG II induced marked increase (from 9 to 20%) of cells into S phase of the cell cycle. Consistent with $[^3]$H-thymidine incorporation data, treatment with tyrphostin AG1478 reduced the shift towards S phase in response to ANG II stimulation by ~50%(Fig. 2B).

Since our results showed that the broad spectrum metalloprotease inhibitor GM 6001 also prevented EGFR transactivation in response to ANG II, we tested whether ANG II-stimulated DNA synthesis would be similarly inhibited by cell exposure to this antagonist. As shown in Fig. 2C, pretreatment with GM 6001 reduced $[^3]$H-thymidine incorporation in response to ANG II stimulation by ~50%, an inhibitory effect identical to that induced by the EGFR kinase inhibitor tyrphostin AG1478. The results presented
in fig. 2 indicate that a substantial component (~50%) of the mitogenic stimulus induced by ANG II in intestinal epithelial IEC-18 cells is mediated by EGFR transactivation.

**ANG II stimulates EGFR-dependent Akt activation**

Next, we attempted to identify the mechanism(s) by which EGFR contributes to ANG II-induced DNA synthesis in IEC-18 cells. In a variety of cell types, EGFR transactivation mediates mitogenic stimulation by GPCRs through ERK-1/2 activation via a well-defined mechanism involving SOS-Grb2/Ras/Raf and MEK (45, 54). However, our recent results with IEC-18 cells suggested that a different mechanism is operational downstream of EGFR in these intestinal epithelial cells. Specifically, ANG II-induced ERK activation in IEC-18 cells was mediated primarily by PKCs rather than EGFR and inhibition of the ERK pathway attenuated (but did not eliminate) ANG II-induced DNA synthesis (see also below). These findings suggested that a pathway other than ERK plays a substantial role in mitogenesis in response to ANG II in these epithelial cells.

In addition to the well recognized role of the ERKs in mitogenic signaling, the phosphatidylinositol (PI) 3-kinases (PI 3-kinase) pathway has also been implicated in signal transduction linked to cell division, differentiation, and tumorigenesis (4). Signaling by PI3-kinases is mediated by 3-phosphoinositide binding to the PH domains of both Akt and PDK1 thereby inducing their translocation to the plasma membrane where PDK1 phosphorylates and activates Akt. These findings prompted us to determine whether ANG II-induced EGFR tyrosine kinase acts via the PI 3-kinase/Akt pathway in IEC-18 cells.
To determine whether ANG II stimulates the PI 3-kinase pathway in IEC-18 cells, we first examined whether ANG II induces the activation of the key PI 3-kinase target Akt in these cells. Akt, translocated to the plasma membrane in response to products of PI3K, is activated by phosphorylation at Thr-308 by PDK1 (2) and by phosphorylation within the carboxy-terminus at Ser-473 by a less well-defined protein kinase referred to as PDK-2 (33). Recently, DNA-dependent protein kinase (DNA-PK) has been implicated as PDK-2 (15). Serum-starved cultures of IEC-18 cells were stimulated with 50 nM ANG II for various times (0-60 min), lysed, and Western blot analyses were performed using a monoclonal antibody that recognizes Akt phosphorylated at Ser-473, a major regulatory phosphorylation site commonly used as a marker for increased signaling via this pathway. As shown in Fig. 3A, ANG II stimulation induced phosphorylation at Ser-473 rapidly, reaching a maximum within 2.5 min.

Next, we investigated whether a functional EGFR is required for the ANG II-induced activation of Akt in IEC-18 cells. Serum-starved cultures of these cells were pretreated with the EGFR tyrosine kinase inhibitors tyrphostin AG1478 (250 nM) or compound 56 (500 nM) prior to stimulation with 50 nM ANG II. As shown in Fig. 3B, ANG II-induced Akt phosphorylation at Ser-473 was dramatically reduced by treatment with each of the selective EGFR tyrosine kinase inhibitors. Similarly, treatment with two structurally unrelated PI 3-kinase inhibitors, LY 294002 and wortmannin, completely blocked ANG II-induced Akt phosphorylation at Ser-473 (Fig. 3C). These results indicate that ANG II-stimulated activation of Akt is mediated via EGFR transactivation and PI 3-kinase in IEC-18 cells.
ANG II stimulates mTOR phosphorylation and EGFR-dependent p70S6K1 activation

Akt has been shown to mediate mTOR phosphorylation at Ser-2448 (34), a site of importance in the regulation of mTOR function (30, 42), via the TSC (tuberous sclerosis complex)/Rheb (Ras homolog enriched in brain) pathway (32). To determine whether ANG II can stimulate mTOR activation in IEC-18 cells, lysates of these cells stimulated with 50 nM ANG II for various times (0-60 min) were subjected to Western blot analyses using an antibody that detects mTOR phosphorylated at Ser-2448. As shown in Fig. 4A, ANG II stimulation rapidly induced mTOR Ser-2448 phosphorylation in IEC-18 cells. A half-maximal effect was achieved at 2.5 min, and a maximum was reached within 5 minutes.

p70S6K1 is a major downstream target of the PI3K/Akt/mTOR pathway which is implicated in cell growth and G1 cell cycle progression (39). Rapamycin, a macrolide immunosuppressant that complexes with FK506 binding protein (FKBP12), is a potent and selective inhibitor of TOR proteins and signaling to p70S6K1. The activity of p70S6K1 is controlled by multiple phosphorylation events located within the catalytic, linker and pseudosubstrate domains (39). Rapamycin-sensitive phosphorylation of Thr-389 by mTOR in the linker region is thought to be required for subsequent PDK1-mediated phosphorylation of Thr-229 in the catalytic domain (35, 38).

To determine whether ANG II activates p70S6K1 in IEC-18 cells, serum-starved cultures of these cells were stimulated with 50 nM ANG II for various times (0-60 min), lysed, and the extracts were subjected to Western blot analyses using an antibody that recognizes p70S6K1 phosphorylated at Thr-389, a major phosphorylation site that
correlates closely with kinase activity (53). As shown in Fig. 4B, ANG II stimulation rapidly induced p70S6K1 phosphorylation at Thr-389. A half-maximal effect was achieved at 2.5 min, and maximal phosphorylation was reached within 10 min. Thus, the kinetics of p70S6K1 phosphorylation at Thr-389 parallels that of mTOR phosphorylated at Ser-2448. To determine whether a functional mTOR was required for the ANG II-induced phosphorylation of p70S6K1 at Thr-389, serum-starved cultures of IEC-18 cells were pretreated with rapamycin prior to stimulation with ANG II. As shown in Fig. 5A, rapamycin completely blocked ANG II-induced p70S6K1 phosphorylation at Thr-389.

In order to substantiate that p70S6K1 activation occurs downstream of PI 3-kinase in ANG II-stimulated IEC-18 cells, serum-starved cultures of IEC-18 cells were pretreated with either LY 294002 or wortmannin prior to stimulation with 50 nM ANG II. As shown in Fig. 4C, ANG II-induced p70S6K1 phosphorylation at Thr-389 was nearly abolished by cell exposure to the PI 3-kinase inhibitors. In contrast, treatment of parallel cultures with the MEK inhibitors U0126 or PD98059 exerted no discernible inhibitory effect on the phosphorylation of this site in response to ANG II. These results indicate that ANG II-induced p70S6K1 phosphorylation at Thr-389 is downstream of PI 3-kinase and is not dependent on MEK-mediated ERK activation.

Since our results demonstrated that the activation of the PI 3-kinase/Akt pathway by ANG II is mediated by EGFR transactivation, we next tested whether ANG II-induced p70S6K1 activation also requires a functional EGFR pathway. As shown in Fig. 5A, ANG II-induced p70S6K1 phosphorylation at Thr-389 was dramatically reduced by prior treatment of the cells with either tyrphostin AG1478 (250 nM) or compound 56 (500 nM). The Western blot was also probed for total p70S6K1 after stripping. Note
that p70S6K1 protein was present in all lanes, including those that did not show any phosphorylation.

Since our results demonstrated that the activation of the PI 3-kinase/Akt/p70S6K1 pathway by ANG II is mediated by EGFR transactivation, we next tested whether ANG II-induced p70S6K1 activation also requires HB-EGF activity. Serum-starved cultures of IEC-18 cells were pretreated with heparin prior to ANG II, HB-EGF, or EGF stimulation. As shown in Fig. 5B, heparin inhibited ANG II- and HB-EGF-induced p70S6K1 phosphorylation at Thr-389, whereas EGF-induced p70S6K1 phosphorylation was not affected. This result, combined with the experiments shown in Fig. 1, suggests that HB-EGF is required for ANG II-stimulated HB-EGF-mediated EGFR transactivation in IEC-18 cells.

**ANG II induces S6 ribosomal protein phosphorylation through EGFR, PI 3-kinase and p70S6K1 in IEC-18 cells**

In order to substantiate that phosphorylation of p70S6K1 at Thr-389 faithfully reflects p70S6K1 catalytic activity in IEC-18 cells stimulated with ANG II, we next examined the phosphorylation of the 40S ribosomal protein subunit S6, which is a well established downstream target of p70S6K1 in intact cells. Phosphorylation of S6 by p70S6K1 correlates with an increase in translation, particularly of mRNAs with an oligopyrimidine tract in their 5' untranslated regions (3). The main *in vivo* S6 ribosomal protein phosphorylation sites, including Ser-235, Ser-236, Ser-240 and Ser-244, have been identified and are located within a small 19 amino acid region (3).

To determine the kinetics of ANG II-stimulated S6 ribosomal protein phosphorylation, serum-starved cultures of IEC-18 cells were stimulated with 50 nM
ANG II for various times (0-30 min) and then lysed. The extracts were subjected to Western blot analysis using a polyclonal antibody that recognizes S6 ribosomal protein phosphorylated at Ser-235 and Ser-236. As shown in Fig. 6A, ANG II stimulation rapidly induced S6 ribosomal protein phosphorylation at Ser-235 and Ser-236. A half-maximal effect was achieved at 5 min and reached a maximum within 15 min. This kinetic profile of S6 ribosomal protein phosphorylation parallels that of p70S6K1 activation, as judged by phosphorylation at Thr-389. As shown in Fig. 6B, treatment with the EGFR tyrosine kinase inhibitors AG1478 or compound 56 completely abrogated ANG II-induced S6 ribosomal protein Ser-235 and Ser-236 phosphorylation. Furthermore, congruent with the notion that EGFR transactivation leads to the activation of the PI 3-kinase/Akt/mTOR/p70S6K1 pathway, treatment with either LY 294002 or rapamycin completely blocked ANG II-induced S6 ribosomal protein phosphorylation at Ser-235 and Ser-236 (Fig. 6B).

**Akt gene silencing inhibits ANG II-stimulated p70S6K1 and S6 ribosomal protein phosphorylation in IEC-18 cells**

To substantiate that Akt mediates p70S6K1 activation and S6 phosphorylation, we utilized gene silencing by RNA interference. Sub-confluent cultures of IEC-18 cells were transiently transfected with Akt siRNA that targets Akt1 and Akt2 (27). To test the specificity of the siRNA, subconfluent cultures of IEC-18 cells were transfected with a non-targeted negative control duplex. As shown in Fig. 6C, Akt protein level in IEC-18 cells transfected with Akt siRNA was dramatically reduced as compared to cells transfected with non-targeted negative control duplex. Akt gene silencing abrogated ANG II-induced p70S6K1 Thr389 phosphorylation (Fig. 6C) and dramatically reduced
ANG II-induced S6 ribosomal protein phosphorylation at Ser-235 and Ser-236, as compared with non-targeted negative control duplex transfected cells (Fig. 6C). In contrast, ANG II-induced ERK1/2 phosphorylation was not affected in IEC-18 cells transfected either with Akt siRNA or non-targeted negative control duplex (Fig. 6C). These results further demonstrate the selectivity of Akt gene silencing and indicate that ANG II induces ERK1/2 phosphorylation via a distinct signaling pathway, separate from the PI 3-kinase/Akt/mTOR/p70S6K1 pathway.

**ANG II induces EGFR transactivation and ERK activation through parallel pathways**

If PI 3-kinase and p70S6K1 lie downstream of EGFR in ANG II-stimulated IEC-18 cells, inhibitors of these kinases should not interfere with EGFR transactivation. As shown in Fig. 7A, stimulation of IEC-18 cell cultures with ANG II induced a marked increase in the tyrosine phosphorylation of the EGFR, in agreement with the results presented in Fig. 1. Pretreatment of these cells with either LY 294002 or rapamycin did not produce any detectable effect on the increase in EGFR tyrosine phosphorylation induced by ANG II. These results substantiate that PI 3-kinase and p70S6K1 lie downstream of EGFR in ANG II-stimulated cells.

In many cellular systems, EGFR transactivation mediates activation of the ERK pathway in response to GPCR agonists. It is conceivable that ANG II-induced EGFR transactivation not only leads to the activation of the PI 3-kinase/Akt/TOR/p70S6K1 pathway but also mediates ERK activation. To directly test this possibility, we examined whether EGFR transactivation mediates ANG II-induced ERK1/2 phosphorylation. Serum-deprived cultures of IEC-18 cells were pretreated with either tyrphostin AG1478 or
compound 56 prior to addition of ANG II. The activated forms of ERK1/2 were monitored by using a specific anti-phospho-p44/p42\textsuperscript{mapk}-mAb that recognizes only the activated forms phosphorylated on Thr-202 and Tyr-204. As shown in Fig. 7B, ANG II-induced ERK1/2 activation was unaffected by either of the EGFR tyrosine kinase inhibitors, demonstrating that ERK is not rapidly activated by ANG II via EGFR transactivation. Furthermore, inhibition of PI 3-kinase or p70S6K1 had no effect on ANG II-induced ERK1/2 activation, supporting the notion that these pathways act in parallel (Fig. 7B). In addition, ANG II-induced EGFR transactivation was not prevented by cell treatment with the MEK inhibitors U0126 or PD98059, demonstrating that the ERKs are not upstream of EGFR transactivation (Fig. 7C). The results illustrated in Fig. 7 provide further support for the notion that the EGFR/PI 3-kinase/Akt/mTOR/p70S6K1 pathway and the MEK/ERK pathway act in parallel in ANG II-stimulated IEC-18 cells.

**Rapamycin attenuates ANG II-induced DNA synthesis in IEC-18 cells**

Given our results demonstrating that ANG II-induced p70S6K1 activation is mediated by EGFR transactivation, we next tested whether suppression of mTOR signaling by the potent and specific inhibitor rapamycin attenuates ANG II-induced DNA synthesis. Cultures of IEC-18 cells were treated with increasing concentrations of rapamycin, stimulated with ANG II and labeled with \[^3\text{H}\]-thymidine to determine the level of DNA synthesis. As shown in Fig. 8A, rapamycin markedly reduced ANG II-induced \[^3\text{H}\]-thymidine incorporation in IEC-18 cells in a concentration-dependent fashion. A maximal inhibitory effect (~50%) of rapamycin was achieved at a concentration of 1 nM.
The results presented above suggest that PI 3-kinase plays a critical role in mediating the downstream effects of EGFR transactivation induced by ANG II in IEC-18 cells. If EGFR and PI 3-kinase lie in the same pathway, concomitant inhibition of EGFR tyrosine kinase and PI 3-kinase should not produce any additional inhibitory effect on [³H]-thymidine incorporation in response to ANG II beyond that achieved by inhibition of EGFR alone. In agreement with this prediction, treatment with both the EGFR tyrosine kinase inhibitor tyrphostin AG1478 and the PI 3-kinase inhibitor LY 294002, did not inhibit ANG II-induced [³H]-thymidine incorporation to a greater extent than that induced by tyrphostin AG1478 alone (Fig. 8B). The results presented in Fig. 8 substantiate the notion that EGFR transactivation induced by ANG II in IEC-18 cells contributes to the mitogenic stimulation promoted by this GPCR agonist through the PI 3-kinase/Akt/TOR/p70S6K1 pathway.

**ANG II induces DNA synthesis through EGFR and ERK pathways in IEC-18 cells**

Our results in Fig. 1B demonstrate that selective EGFR tyrosine kinase inhibitor tyrphostin AG1478 could reduce [³H]-thymidine incorporation in response to ANG II stimulation in a concentration-dependent fashion, with maximal inhibitory effect at ~50%. The results presented in Fig. 8C show that inhibition of the ERK pathway by treatment of IEC-18 cells with the MEK inhibitor U0126 also attenuated [³H]-thymidine incorporation in response to ANG II stimulation by ~50%. Given these results, we hypothesized that inhibition of both EGFR tyrosine kinase and ERK would lead to an additive inhibitory effect, suggesting that these distinct pathways contribute to ANG II-induced mitogenesis in IEC-18 cells.
As shown in Fig. 8D, pretreatment with the combination of selective EGFR tyrosine kinase inhibitor tyrphostin AG1478 and selective MEK inhibitor U0126 gave an additive inhibitory effect that nearly abolished ANG II-induced DNA synthesis. Taken together, these results strongly suggest that ANG II-induced DNA synthesis requires two distinct pathways, involving the MEK/ERK and the EGFR/PI 3-kinase/Akt/mTOR/p70S6K1, as shown schematically in Fig. 9.
Discussion

The proliferation of epithelial cells of the intestinal mucosa is regulated by a broad spectrum of regulatory peptides acting through both tyrosine kinase receptors and heptahelical GPCRs (5, 26, 50). The understanding of the mechanisms involved requires the identification of the signal transduction pathways that mediate intestinal epithelial cell proliferation.

We have recently demonstrated that the addition of ANG II, at nanomolar concentrations, induces DNA synthesis and cell proliferation in intestinal epithelial IEC-18 cells via the AT-1 receptor in the absence of any other exogenously added growth factor. ANG II induces a dramatic increase in PKC-dependent PKD activation in these cells (7) and stimulates PKC-dependent tyrosine phosphorylation of Pyk2, a non-receptor tyrosine kinase that has been implicated as an upstream element in ERK-1/2 activation (56). Accordingly, ANG II stimulates PKC-dependent ERK activation, a major pathway leading to cell cycle activation and cell proliferation. Interestingly, inhibition of the ERK pathway in these cells reduces ANG II-induced DNA synthesis by 50-60% (8). These findings not only indicated that ANG II-induced DNA synthesis is partially dependent on ERK and PKC but also raise the intriguing possibility that another pathway, in addition to ERK and PKC, plays a substantial role in AT1-mediated mitogenesis of IEC-18 cells. The experiments presented in this study were designed to identify the pathway that together with ERK drives cell cycle progression in ANG II-stimulated IEC-18 cells.

Activation of a variety of GPCRs has been shown to stimulate the tyrosine kinase activity of the EGFR in several cell types (10-12, 29, 52), a process termed
transactivation (6, 36). As a first step to evaluate the role of EGFR transactivation in mediating ANG II-induced mitogenesis in IEC-18 cells, we demonstrated that stimulation with this agonist induced a striking increase in EGFR tyrosine phosphorylation. This response was prevented by cell exposure to the selective EGFR tyrosine kinase inhibitor AG1478 or by the broad-spectrum metalloprotease inhibitor GM6001, suggesting that proteolytic release of EGFR ligand(s) is involved. We have further shown that heparin pretreatment significantly reduced ANG II-induced EGFR tyrosine phosphorylation (Fig. 1C) and ANG II-induced EGFR signaling (p70S6K1 phosphorylation), whereas ANG II-induced ERK activation was not affected (Fig. 5B). These results combined with those obtained by using the broad-spectrum metalloproteinase inhibitor GM 6001 suggest that HB-EGF is required for ANG II-stimulated EGFR transactivation and EGFR-dependent downstream signaling in IEC-18 cells.

Furthermore, treatment with EGFR tyrosine kinase inhibitors or GM 6001 also attenuated (by ~50%) DNA synthesis induced by ANG II in IEC-18 cells, indicating that EGFR transactivation plays a role in mediating the proliferative response induced by ANG II in IEC-18 cells. These initial findings prompted us to identify the pathway(s) downstream of EGFR that promotes mitogenesis in IEC-18 cells.

In many cell types, the EGFR has been proposed to mediate Ras/ERK-1/2 activation in response to GPCR activation, and the ERK pathway has been implicated in mitogenic signal transduction by many stimuli (13, 14, 18, 19). For example, in vascular smooth muscle cells (13, 14), pancreatic stellate cells (19) and breast cancer cells (18), ANG II has been shown to stimulate cell cycle progression through EGFR-dependent ERK activation.
In contrast, our results with IEC-18 cells indicate that EGFR transactivation contributes to ANG II-induced mitogenesis primarily through activation of the PI 3-kinase/Akt/p70S6K1 pathway, whereas PKC (not EGFR tyrosine kinase) mediates ANG II-stimulated ERK pathway. Specifically, several lines of evidence support this conclusion: 1) Suppression of EGFR tyrosine kinase had no effect on ANG II-stimulated ERK-1/2 activation in IEC-18 cells, in agreement with our recent results indicating that ANG II-induced ERK activation is mediated primarily by PKCs in these cells; 2) Activation of PI 3-kinase results in production of lipid-derived second messengers that promote PDK1-mediated activation of Akt. We demonstrated here that stimulation of IEC-18 cells with ANG II induced Akt phosphorylation at Ser 473, a major regulatory phosphorylation site required for kinase activity. Akt activation in response to ANG II was completely inhibited by prior exposure of the cells to selective inhibitors of PI 3-kinase (wortmannin, LY 294002) or EGFR tyrosine kinase (tyrphostin AG1478); 3) Another downstream effector of PI 3-kinase implicated in cell cycle progression is p70S6K1. We showed that ANG II markedly stimulated p70S6K1 phosphorylation at Thr-389, a major phosphorylation site that correlates closely with kinase activity, through an EGFR-, PI 3-kinase- and mTOR- dependent pathway; 4) siRNAs targeting Akt1 and Akt2, dramatically inhibited ANG II-induced p70S6K1 phosphorylation at Thr-389 and the phosphorylation at Ser235/236 of its downstream target, the S6 ribosomal protein. In contrast, ANG II-induced ERK1/2 activation was not prevented by treatment with LY 294002, rapamycin or Akt siRNA. Reciprocally, blocking MEK-mediated ERK activation prevented neither EGFR transactivation nor p70S6K1. Taken together, these results demonstrate that PI 3-kinase, Akt, and p70S6K1 are downstream effectors of
EGFR tyrosine kinase and lie in a pathway distinct from the ERKs in ANG II-stimulated IEC-18 cells.

We also produced several lines of evidence indicating that ANG II-induced activation of the PI 3-kinase pathway contributes to cell cycle progression into DNA synthesis in IEC-18 cells. Specifically, we demonstrated that inhibition of PI 3-kinase or mTOR/p70S6K1 by the specific immunosuppressant rapamycin reduced $[^3H]$-thymidine incorporation by 50%, i.e. to levels comparable to those achieved by addition of either selective EGFR tyrosine kinase inhibitors or the broad-spectrum metalloprotease GM6001. If PKC-dependent ERK activation and EGFR-dependent PI 3-kinase signaling operate as independent pathways that contribute to ANG II-induced DNA synthesis in IEC-18 cells, we expect that simultaneous blockade of these pathways should produce additive inhibition of DNA synthesis. In line with this prediction, we show that treatment of IEC-18 cells with a combination of the EGFR tyrosine kinase inhibitor tyrphostin AG1478 and the selective MEK inhibitor U0126 produced an additive inhibitory effect that nearly abolished ANG II-induced DNA synthesis. Inhibition of either pathway alone reduced ANG II-stimulated DNA synthesis by ~50%. As depicted by Fig. 9, we conclude that ANG II-stimulated mitogenesis in intestinal epithelial IEC-18 cells is mediated by two distinct signaling pathways: PKC-dependent MEK/ERK and an EGFR-dependent PI 3-kinase/Akt/mTOR/p70S6K1, dissected in the present study.

Early studies demonstrated that AT1 is the predominant ANG II receptor in the gastrointestinal tract, as revealed by in situ autoradiography (22). ANG II has been shown to mediate epithelial sodium and water absorption in the jejunum, ileum, and distal colon (23, 24), but the role of ANG II-induced AT1 receptor signaling in intestinal
epithelial cell proliferation and differentiation is much less defined. Collectively, our previous and present results demonstrate that ANG II is a potent growth factor for intestinal epithelial cells and identify some of the critical molecular events that mediate the mitogenic effect of ANG II in these cells. These results assume an added significance in view of recent reports demonstrating that drugs blocking the biological activity of ANG II enhanced the antitumor effect of COX-2 inhibitors in colon carcinoma models, the prevalence of activating mutations in the PI 3-kinase, and the evidence suggesting that inhibitors of ANG II generation (i.e. angiotensin I-converting enzyme) protect against risk of multiple cancers (31, 48, 57)
FOOTNOTES

1The abbreviations used are:

AEBSF, 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride
ANG II, angiotensin II
\([\text{Ca}^{2+}]_i\), intracellular concentration of \(\text{Ca}^{2+}\)
DAG, diacylglycerol
DMEM, Dulbecco's modified Eagle's medium
ERK, extracellular signal-regulated kinase
G proteins, guanine nucleotide-binding regulatory proteins
GPCR, G-protein coupled receptor
HB-EGF, Heparin-binding epidermal growth factor
mAb, monoclonal antibody
p44/p42 MAPK, p44/p42 Mitogen Activated Protein Kinase
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate buffered saline
PDB, phorbol 12,13-dibutyrate
PDK1, 3’- phosphoinositides-dependent kinase-1
PKC, protein kinase C
PKD, protein kinase D
Pyk2, proline-rich tyrosine kinase 2
RAS, renin-angiotensin system
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LEGENDS

Fig. 1 ANG II-stimulated EGFR transactivation is dependent on matrix metalloprotease activity in IEC-18 cells.

A, Time course of ANG II-stimulated EGFR tyrosine phosphorylation. Confluent IEC-18 cells were washed and incubated at 37°C with 50 nM ANG II for various times as indicated. The EGF receptor was immunoprecipitated using a polyclonal EGFR antibody, and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-P membranes, then tyrosine phosphorylated EGFR was detected using 4G10 anti-Tyr(P) mAb. Membranes were then stripped and reprobed with anti-EGFR. B, Treatment with AG1478, compound 56, and GM 6001 prevents ANG II-induced EGFR tyrosine phosphorylation. Confluent and serum-starved cultures of IEC-18 cells were stimulated for 2 min with 50 nM ANG II after 1 h preincubation with 250 nM AG1478 (AG), 250 nM compound 56 (56) or 10 µM GM 6001 (GM), as indicated. C, Treatment with heparin differentially inhibits EGFR tyrosine phosphorylation induced by either ANG II or HB-EGF but not EGF. Serum-starved cultures of IEC-18 cells were incubated for 30 min with or without 50 µg/ml heparin. The cultures were subsequently left unstimulated (−) or stimulated (+) with 50 nM ANG II, 1 ng/ml HB-EGF, or 10 ng/ml EGF. EGFR immunoprecipitates were analyzed for tyrosine phosphorylation as describe above. The results presented above are representative of 3 independent experiments.
Fig. 2 ANG II-stimulated DNA synthesis is dependent on EGFR transactivation and matrix metalloprotease activity

A, Tyrphostin AG1478 inhibits ANG II-induced DNA synthesis in a concentration-dependent manner. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth’s medium containing 50 nM ANG II either without or with AG1478 at various concentrations, as indicated. [³H]-thymidine incorporation into acid-precipitable material was measured as described. Results shown are expressed as a percentage of the maximum increase ± S.E. (n=3) obtained with 50 nM ANG II after subtraction of the basal [³H]-thymidine incorporation from the respective controls. B, Treatment with the selective EGFR tyrosine kinase inhibitor attenuates the stimulation of cell cycle progression induced by ANG II. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth’s medium containing 50 nM ANG II either in the absence or in the presence of 250 nM AG1478. After 19 h of incubation at 37 °C, cultures were washed twice with PBS and cells detached by treatment with trypsin (0.025%). Cells (10⁶) were then resuspended and stained by 1 ml of a hypotonic DNA staining buffer containing propidium iodide (0.1 mg/ml), sodium citrate (1 mg/ml), Ribonuclease A (20 µg/ml), and Triton X-100 (0.3%). Samples were kept at 4°C protected from light for 30 min and analyzed on a FACScan (Becton Dickinson). The profiles shown represent the number of cells as a function of relative DNA content. In each case, the percentages of cells in G₀/G₁ (first peak), S (cross-hatched area well-defined in ANG II-treated cells), and G₂/M (second peak) are indicated. Results shown are representative of three independent experiments. C, Effect of AG1478 and GM 6001 on DNA synthesis in
response to ANG II. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth’s medium containing 50 nM ANG II either without or with 250 nM AG1478 and 10 µM GM 6001, as indicated. [³H]-thymidine incorporation into acid-precipitable material was measured as described. Results shown are expressed as a percentage of the maximum increase ± S.E. (n=3) obtained with 50 nM ANG II.

**Fig. 3 ANG II stimulates EGFR-dependent Akt activation in IEC-18 cells.**

A, ANG II induces Akt activation in a time-dependent manner. Confluent and quiescent cultures of IEC-18 cells were treated for various times with 50 nM ANG II at 37°C as indicated. Western blot analysis using a specific anti-phospho-Akt monoclonal antibody that recognizes only the activated form phosphorylated on Ser473 was performed following lysis of the cells with 2x sample buffer as described in “Material and Methods.” B, Tyrphostin AG1478 and compound 56 inhibit ANG II-induced Akt activation. **Upper panel,** confluent and quiescent IEC-18 cells were incubated for 1 h with 250 nM AG1478 (AG) and 500 nM compound 56 (CPD 56). The cultures were subsequently left unstimulated (−) or stimulated (+) for 2.5 min with 50 nM ANG II at 37°C. **B, middle panel,** the Western blot was also probed for total Akt, showing equal loading. C, PI 3-kinase inhibitors LY 294002 and wortmannin inhibits ANG II-induced Akt activation. **Upper panel,** confluent and quiescent IEC-18 cells were incubated for 1 h with either 10 µM LY 294002 or 100 nM wortmannin. The cultures were subsequently left unstimulated (−) or stimulated (+) for 2.5 min with 50 nM ANG II at 37°C. **C, middle panel,** the Western blot was also probed for total Akt, showing equal loading. B and C, **lower panels,** the results shown in the bar graphs are
the values (means ± S.E. n=3) of the level of Akt phosphorylation obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 50 nM ANG II. The Western blots shown above of the bar graphs are representative of 3 independent experiments.

**Fig. 4 ANG II stimulates mTOR phosphorylation and EGFR-dependent p70S6K1 activation in IEC-18 cells.**

**A,** ANG II induces mTOR phosphorylation in a time-dependent manner. Confluent and quiescent cultures of IEC-18 cells were treated for various times with 50 nM ANG II at 37°C as indicated. Western blot analysis using a specific anti-phospho-mTOR polyclonal antibody that recognizes only the activated form phosphorylated on Ser-2448 was performed following lysis of the cells with 2x sample buffer as described in “Material and Methods.”

**B,** ANG II induces p70S6K1 activation in a time-dependent manner. Confluent and quiescent cultures of IEC-18 cells were treated for various times with 50 nM ANG II at 37°C as indicated. Western blot analysis using a specific anti-phospho-p70S6K1 monoclonal antibody that recognizes only the activated form phosphorylated on Thr-389 was performed following lysis of the cells with 2x sample buffer as described in “Material and Methods.” The Western blot was stripped and also probed for total p70S6K1, showing similar loading.

**C,** Effects of PI 3-kinase inhibitors (LY 294002 and wortmannin) and MEK inhibitors (U0126 and PD98059) on ANG II-induced p70S6K1 activation. **Upper panel,** confluent and quiescent IEC-18 cells were incubated for 1 h with 10 µM LY 294002, 100 nM wortmannin, 2.5µM U0126 or 10 µM PD98059. The cultures were subsequently left unstimulated (−) or stimulated (+) for 10 min with 50 nM ANG II at 37°C. Western blot analysis using a specific anti-
phospho-p70S6K1 monoclonal antibody that recognizes only the activated form phosphorylated on Thr-389 was performed following lysis of the cells with 2x sample buffer as described. The Western blot was also probed for total p70S6K1 after stripping. Note that p70S6K1 protein was present in all lanes, including those that did not show any phosphorylation. Lower panel, results shown in the bar graphs are the values (means ± S.E. n=3) of the level of p70S6K1 phosphorylation obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 50 nM ANG II. Above Western blots shown are representative of 3 independent experiments.

Fig. 5. Effects of inhibition of EGFR tyrosine kinase and HB-EGF binding on p70S6K1 phosphorylation

A, EGFR tyrosine kinase inhibitors (AG1478 and compound 56) and rapamycin inhibits ANG II-induced p70S6K1 activation. Upper panel, confluent and quiescent IEC-18 cells were incubated for 1 h with 250 nM AG1478, 500 nM compound 56, or 1 nM rapamycin. The cultures were subsequently left unstimulated (−) or stimulated (+) for 10 min with 50 nM ANG II at 37°C. Western blot analysis for phospho-Thr389 p70S6K1 was performed as described. After stripping the same blot, it was also reprobed for total p70S6K1. Lower panel, results shown in the bar graphs are the values (means ± S.E. n=3) of the level of p70S6K1 phosphorylation obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 50 nM ANG II. B, Heparin inhibits ANG II-induced p70S6K1 phosphorylation. Serum-starved cultures of IEC-18 cells were preincubated for 30 min with or without 50 µg/ml heparin. The cultures were subsequently left
unstimulated (−) or stimulated (+) with 50 nM ANG II, 1 ng/ml HB-EGF, or 10 ng/ml EGF, as indicated. Western blot analysis for phospho-Thr389 p70S6K1 and phospho-Thr202/Tyr204 ERK-1/ERK-2 were performed on the same blot as described. The Western blot was also probed for total ERK, showing equal loading. The results presented above are representative of 3 independent experiments.

**Fig. 6. Effects of inhibition of EGFR tyrosine kinase, Akt and mTOR on p70S6K1 and S6 phosphorylation**

**A.** ANG II induces S6 activation in a time-dependent manner. Confluent and quiescent cultures of IEC-18 cells were treated for various times with 50 nM ANG II at 37°C as indicated. Western blot analysis using a specific anti-phospho-S6 polyclonal antibody that recognizes only the activated form phosphorylated on Ser-235 and Ser-236 was performed following lysis of the cells with 2x sample buffer as described. **B,** AG1478, compound 56, LY 294002, and rapamycin inhibit ANG II-induced S6 phosphorylation. *Upper panel,* confluent and quiescent IEC-18 cells were incubated for 1 h with 250 nM AG1478, 500 nM compound 56, 1 nM rapamycin or 10 µM LY 294002. The cultures were subsequently left unstimulated (−) or stimulated (+) for 10 min with 50 nM ANG II at 37°C. Western blot analysis using a specific anti-phospho-S6 polyclonal antibody that recognizes only the activated form phosphorylated on Ser-235 and Ser-236 was performed following lysis of the cells with 2x sample buffer as described. Above Western blots shown are representative of 3 independent experiments. **C.** Effects of Akt knockdown on ANG II-induced signaling in IEC-18 cells. IEC-18 cells were transfected with Akt siRNA or non-targeted negative control duplex as described in “Material and Methods.” After 48 h, cells were incubated in the absence (−) or presence (+) of 50M
ANG II for 5 min. Then, the cells were lysed and subjected to Western blot analysis, using polyclonal anti-Akt antibody, anti-phospho-p70S6K1 monoclonal antibody, anti-phospho-S6 polyclonal antibody, or anti-phospho-ERK-1/ERK-2 mAb. The Western blot was also probed for total ERK, showing equal loading. The blots shown are representative of 3 independent experiments.

**Fig. 7. Effects of inhibition of EGFR tyrosine kinase, PI 3-kinase, mTOR and MEK on EGFR tyrosine phosphorylation and ERK activation in IEC-18 cells.**

A, ANG II-induced EGFR tyrosine phosphorylation is not affected by inhibition of PI 3-kinase or mTOR. Confluent and serum-starved cultures of IEC-18 cells were stimulated for 2 min with 50 nM ANG II after 1 h preincubation with 10 µM LY 294002 or 10 nM rapamycin, as indicated. The EGF receptor was immunoprecipitated using a polyclonal EGFR antibody, and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-P membranes, then tyrosine phosphorylated EGFR was detected using 4G10 anti-Tyr(P) mAb. Membranes were then stripped and reprobed with anti-EGFR. The results presented here are representative of 3 independent experiments. B, ANG II induces p42\(^{\text{mapk}}\) (ERK-2) and p44\(^{\text{mapk}}\) (ERK-1) phosphorylation in a time-dependent manner. Confluent and serum-starved cultures of IEC-18 cells were incubated for 1 h with 250 nM AG1478, 1 nM rapamycin, 10 µM LY 294002, or 2.5 µM U0126. Control cells (−) received equivalent amount of solvent (−). The cultures were subsequently left unstimulated (−) or stimulated (+) for 2.5 min with 50 nM ANG II at 37°C. Western blot analysis using a specific anti-phospho-ERK-1/ERK-2 mAb that recognizes only the activated forms phosphorylated on Thr202 and Tyr204 was performed following lysis of the cells with 2x sample buffer as described in “Material and Methods.” The Western
blot was also probed for total ERK, showing equal loading. C, ANG II-induced EGFR tyrosine phosphorylation is not affected by MEK inhibitors. Confluent and serum-starved cultures of IEC-18 cells were stimulated for 2 min with 50 nM ANG II after 1 h preincubation with 2.5 µM U0126 or 10 µM PD98059, as indicated. The EGF receptor was immunoprecipitated using a polyclonal EGFR antibody, and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-P membranes, then tyrosine phosphorylated EGFR was detected using 4G10 anti-Tyr(P) mAb. Membranes were then stripped and reprobed with anti-EGFR. The results presented here are representative of 3 independent experiments.

Fig. 8. Effects of inhibition of EGFR tyrosine kinase, PI 3-Kinase, p70S6K1, and MEK on DNA synthesis in IEC-18 cells.

A, Rapamycin inhibits ANG II-induced DNA synthesis in a concentration-dependent manner. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth’s medium containing 50 nM ANG II either without or with rapamycin at various concentrations, as indicated. [3H]-thymidine incorporation into acid-precipitable material was measured as described.

B, Inhibition of EGFR tyrosine kinase and PI 3-Kinase did not have an additive inhibitory effect on ANG II-induced DNA synthesis. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth’s medium containing 50 nM ANG II with 250 nM AG1478, 10 µM LY 294002, both inhibitors or vehicle (control). C, U0126 inhibits ANG II-induced DNA synthesis in a concentration-dependent manner. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml
DMEM/Waymouth’s medium containing 50 nM ANG II either without or with U0126 at various concentrations, as indicated. [\(^3\)H]-thymidine incorporation into acid-precipitable material was measured as described. Results shown are expressed as a percentage of the maximum increase ± S.E. (n=3) obtained with 50 nM ANG II after subtraction of the basal [\(^3\)H]-thymidine incorporation from the respective controls. D, Pretreatment with the combination of AG1478 and U0126 gave an additive inhibitory effect that nearly abolished ANG II-induced DNA synthesis. Confluent and serum-starved cultures of IEC-18 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth’s medium containing 50 nM ANG II with 250 nM AG1478, 2.5 µM U0126, both inhibitors or vehicle (control). [\(^3\)H]-thymidine incorporation was determined as described. Results shown are in CPM ± S.E. (n=3). (* p< 0.01)

Fig. 9. Signal transduction pathways involved in ANG II-induced mitogenesis in IEC-18 cells.

This scheme summarizes our results demonstrating that ANG II-induced mitogenesis is dependent on two independent, distinct pathways, involving the PKC/MEK/p44/p42 MAPK (ERK) and the EGFR/PI 3-Kinase/Akt/mTOR/p70S6K1 pathways. ANG II also stimulates calcium- and PKC-dependent tyrosine phosphorylation of Pyk2, a non-receptor tyrosine kinase that has been implicated as an upstream element in p44/p42 MAPK activation, in IEC-18 cells (56). The inhibitors used in this study to block (\(\square\)) specific pathways are shown as marked by boxes. The dotted lines imply the existence of intermediary steps leading to the subsequent event; solid lines represent direct consequence of the previous effector. GF-1 or GF 109203X (also known as bisindolylmaleimide I) is a PKC inhibitor.
Figure 1
Figure 2

Panel A: Graph showing the effect of AG 1478 concentration on 3H-Thymidine Incorporation (% Maximum).

Panel B: Flow cytometry analysis showing the cell cycle distribution:
- AG 1478:
  - Go/G1: 84
  - S: 9
  - G2/M: 7
- AG 1478/ANG II:
  - Go/G1: 85
  - S: 9
  - G2/M: 7
- ANG II:
  - Go/G1: 85
  - S: 20
  - G2/M: 15
- GM:
  - Go/G1: 15
  - S: 20
  - G2/M: 15

Panel C: Bar chart showing the effect of AG 1478, GM, and ANG II on 3H-Thymidine Incorporation (% Maximum)
- AG 1478:
  - − −
- GM:
  - − −
- ANG II:
  - − +
- AG 1478/ANG II:
  - − +
Figure 3

(A) Time course of pS473 Akt phosphorylation over 60 minutes.

(B) Dose response of pS473 Akt phosphorylation with different treatments.

(C) Effect of various inhibitors on AKT phosphorylation % Maximal Response.

Legend:
- AG1478: - - +  - - +  -
- Cpd 56:  - - +  - - +  -
- ANG II: - - -  + + +  +
- LY: - + - - - + -
- Wort: - - +  - - +  -
- ANG II: - - -  + + +  +
Figure 4

A

pS2448 mTOR

Time (min)

0 0.5 1 2.5 5 10 20 30

B

pThr389 p70 S6K

Total p70 S6K

Time (min)

0 0.5 1 2.5 5 10 15 30 60

C

pThr389 p70 S6K

Total p70 S6K

pThr389 p70 S6K phosphorylation % Maximal Response

U0126

ANG II

LY Wort U0126 PD

− − + − + − + − +
Figure 5

**A**

[Graph showing pThr389 p70 S6K phosphorylation response to different treatments.]

**B**

[Diagram showing pThr389 p70 S6K, pERK1, pERK2, and ERK1, ERK2 under different conditions.]
Figure 6

A

B

C

Control siRNA    +    +    −    −    −
Akt siRNA        −    −    +    +    +
ANG II           −    +    −    +    +
Figure 7
Figure 8

A

3H-Thymidine Incorporation (% Maximum)

Rapamycin [nM]

B

\[ p = 0.5 \]

AG1478
LY
ANG II

C

3H-Thymidine Incorporation (% Maximum)

U0126 [μM]

D

AG1478
U0126
ANG II
Figure 9

ANG II → G_{q} → PLC_{β} → DAG → PKC → [Ca^{2+}]_{i} → Pyk2

EGFR → PI3K → AKT → mTOR → p70 S6K → MEK → p42/p44 MAPK

LY294002 Wortmannin → PI3K

Akt siRNA

Rapamycin → mTOR → p70 S6K

AG1478 Cpd 56 → G_{q}

DG198095 U0126

G_{o} → S Transition