Transforming Growth Factor-α and Epidermal Growth Factor Activate Mitogen-Activated Protein Kinase and Its Substrates in Intestinal Epithelial Cells

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Abstract. **The signal transduction pathways of mitogenic stimuli in intestinal epithelial cells are not clearly understood. We report here a possible signaling pathway of two closely related agonists, transforming growth factor-α (TGFα) and epidermal growth factor (EGF). Both increase thymidine incorporation in the intestinal epithelial cell (IEC) line IEC-6. This increase is dose dependent and inhibited by the tyrosine kinase inhibitors genistein and tyrphostin. The addition of either TGFα or EGF to IEC-6 cells also stimulates the activities of the two forms of mitogen-activated protein kinase, $p42^{erk2}$ MAPK and $p44^{erk1}$ MAPK, as evidenced by increased incorporation of radiolabeled phosphate in myelin basic protein. The main difference between the MAPK activity levels induced by the two agonists is in the intensity of the response. Maximum TGFα-induced stimulation of $p42^{erk2}$ MAPK activity is 9-fold at 2 ng/ml, while maximum EGF stimulation is only 4.5-fold at 25 ng/ml. These doses correlated closely with the dose required for maximum thymidine incorporation. The activity of the 90-kDa ribosomal S6 kinase, a downstream substrate for activated MAPK, is also enhanced as evidenced by increased incorporation of radiolabeled phosphate in the rsk kinase substrate peptide in IEC-6 cells following stimulation with either TGFα or EGF. This increase correlates closely with the stimulus-induced increase in MAPK activity with respect to dose, but the time of increased activity is more prolonged, especially after EGF stimulation. TGFα induced the synthesis of both c-Fos and c-Myc, two nuclear substrates for MAPK, and increased c-*fos* and c-*myc* message levels as well. However, c-Jun protein and c-*jun* mRNA were not induced. The increase in IEC-6 cell proliferation in response to TGFα and EGF stimulation may then be due, in part, to an increase in immediate early gene expression as a direct result of MAPK and RSK activation.** [P.S.E.B.M. 1995, Vol 210]

The epithelium of the small intestine is the site of continual proliferation and differentiation which is tightly regulated in the crypt to villus region where approximately 60% of crypt cells pass through the cell cycle every 12 hr (1). The growth factors and/or hormones that may be involved in regulating growth in this region, such as epidermal growth factor (EGF) (2), transforming growth factor-α (TGFα) (3, 4), transforming growth factor-β (TGFβ) (5), gastrin (6, 7), and fibroblast growth factor (8) are currently being studied. TGFα is of special interest because it is expressed by normal villus cells (9) and is also present in the mucosa of a high percentage (84%) of human colorectal cancers (10). Therefore, it appears to be an important paracrine growth factor in the intestinal crypts. Although EGF is structurally and functionally related to TGFα and binds the same receptor, certain biological activities of the two growth factors

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are different (11). The activation of the EGF receptor tyrosine kinase by either ligand results in an increase in proliferation of many cell types and an increase in the tyrosine phosphorylation levels of cellular proteins as well (4, 12). Downstream cellular responses to either TGFα or EGF stimulation have not been studied in intestinal epithelial cells.

The microtubule-associated protein kinase or mitogen-activated protein kinase (MAPK) signal transduction pathway is activated by mitogenic growth factors and involves a phosphorylation cascade that eventually links cell surface and nuclear events due to MAPK translocating to the nucleus (13–15). Downstream substrates for activated MAPK include ribosomal S6 kinase (RSK), a 90-kDa cytosolic protein which also translocates to the nucleus (16), and transcription factors such as c-Fos (17, 18), c-Myc (14), and c-Jun (19). The phosphorylation and activation of these transcription factors induces early gene expression and subsequent entry of the cell into the cell cycle (2, 20). The ability of TGFα or EGF to activate MAPK as well as other downstream substrates of MAPK in intestinal epithelial cells would, therefore, indirectly suggest a signaling pathway for these agonists.

We have shown previously that the 42- and 44-kDa isoforms of MAPK ($p42^{erk2}$ and $p44^{erk1}$) are tyrosine phosphorylated in response to TGFα stimulation of the rat intestinal epithelial cell (IEC) line IEC-6, an undifferentiated and nontransformed isolate from the crypt of the small intestine (4). The present studies are a continuation of our previous work and are designed to address three questions in order to further clarify the signaling pathway of TGFα and EGF. First, does MAPK activity increase following stimulation of IEC-6 cells with TGFα and EGF? Are the kinetics similar? Second, does the activity of RSK, a downstream substrate for MAPK, increase following stimulation by these two agonists? Third, does agonist stimulation of IEC-6 cells lead to an increase in expression of transcription factors which are nuclear substrates for MAPK?

Materials and Methods

Cell Culture. Rat small intestinal epithelial cells (IEC-6) were obtained from American Type Culture Collection (Rockville, MD) and were maintained at 37°C and 10% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 m*M* glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 1.0 μg/ml fungizone, and 5 μg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO) as previously described (4). Cells were used between Passage 15 and 25. Cells were grown in 100 mm^2 tissue culture plates until approximately 75% confluent and starved for 18 hr in DMEM containing 0.5% FBS prior to the experiment.

Thymidine Incorporation. Thymidine incorporation was measured as described previously (4). Briefly, cells were seeded at 1×10^4 cells/well in a 96-well tissue culture plate in DMEM + 10% FBS for 24 hr followed by 18 hr in DMEM + 0.5% FBS. The appropriate growth factor was then added for 24 hr. During the last 4 hr, 0.5 μCi [methyl-^{3}H]thymidine (specific activity: 70–85 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to each well. Cells were harvested onto filtermats after trypsinization using a Skatron cell harvester (Skatron Inc., Sterling, VA). Filtermats were dried and radioactivity of each disk was counted in a Delta 300 liquid scintillation counter (Searle Analytic Inc.).

In-Gel Kinase Assay. MAPK and RSK activities were determined as previously described (21). Cell lysates were prepared by scraping cells into hot SDS buffer (50 m*M* Tris-base, 1.0% SDS) and boiling for 5 min. Proteins were resolved in 10% SDS/polyacrylamide containing either 0.5 mg/ml myelin basic protein (Sigma) for MAPK activity analysis or 0.2 mg/ml rsk kinase substrate peptide (Upstate Biotechnology Inc, Lake Placid, NY) for RSK activity analysis. The gel was first washed for 1 hr in 50 m*M* Tris, pH 8.0 (Buffer A) containing 20% isopropanol, then Buffer A containing 6 *M* guanidine-HCl and 5 m*M* 2-mercaptoethanol for 1 hr. The proteins were then renatured by washing the gel in Buffer A containing 5 m*M* 2-mercaptoethanol and 0.04% Tween 40 for approximately 18 hr at 4°C. The gel was then washed for 30 min in kinase buffer (40 m*M* Hepes, 2 m*M* DTT, 0.1 m*M* EGTA, 5 m*M* magnesium acetate, pH 8.0) followed by a 1-hr incubation in kinase buffer containing 20 μ*M* ATP and 50 μCi [γ-^{32}P]ATP (specific activity: 3000 Ci/mmol; Amersham). The gel was then washed in 5% trichloroacetic acid containing 1% sodium pyrophosphate until background levels of radioactivity were obtained. The gel was dried and exposed to film (Kodak X-Omat; Eastman Kodak Co., Rochester, NY) overnight at −70°C. Autoradiographs were analyzed on a scanning laser densitometer (LKB Ultroscan XL, Bromma, Sweden).

Metabolic Labeling and Immunoprecipitations. Metabolic labeling, lysate preparation, and immunoprecipitations were performed as previously described (22) with several modifications. Cells were grown to 75% confluence, starved in DMEM containing 0.5% FCS for 18 hr, and either left as control or stimulated for 30 min with TGFα. Cells were washed twice with HBSS and incubated for 30 min in DMEM without methionine (Gibco-BRL). Media was removed and DMEM without methionine containing 0.5% dialysed FCS (Gibco-BRL), 2 m*M* glutamine and 0.5 mCi ^{35}S-

Protein Labeling Mix (specific activity: 1175 Ci/mmol; NEN Research Products, Boston, MA) was added to each plate. After 3-hr incubation at 37°C, cells were scraped into lysis buffer (50 m*M* Tris, pH 8, 200 m*M* NaCl, 0.5% NP-40, 2 m*M* sodium orthovanadate, 1 m*M* phenylmethanesulphonyl fluoride, 5 m*M* sodium pyrophosphate, 10 μg aprotinin, 10 μg leupeptin) while on ice. Lysates were centrifuged at 14,000*g* for 5 min and added to anti-rabbit IgG-agarose beads (Sigma) that had been linked to the appropriate antibody (rabbit anti-*fos* protein or rabbit anti-c-*myc* protein; Upstate Biotechnology Inc., Lake Placid, NY). After 4 hr at 4°C, the beads were washed twice with lysis buffer, twice with 0.5 *M* LiCl, 0.1 *M* Tris, pH 7.4, and twice with 100 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris, pH 7.4. Reprecipitations were then performed as follows: after the first immunoprecipitation, immune complexes were resuspended in 50 m*M* Tris, pH 7.5, 0.5% SDS, 5 m*M* dithiothreitol, and boiled for 10 min. To this was added 1 ml lysis buffer. Beads were spun out and supernatant was added to agarose beads linked to the appropriate antibody as above. After reprecipitation for 18 hr, immune complexes were dissociated by boiling in 5 m*M* Na_2HPO_4, 3% SDS, 10% glycerol, 0.05% bromphenol blue, 10% 2-mercaptoethanol and proteins were resolved on 12% SDS/polyacrylamide gel. The gel was destained for 5 min in dH_2O:ethanol: acetic acid (1:4.5:4.5), washed in Enhance (NEN Research Products) for 1 hr, washed in ice-cold dH_2O for 30 min, dried, and exposed to film at −70°C.

Tyrosine Kinase Inhibitors. Genistein (Upstate Biotechnology) was used at a concentration of 50 μg/ml. Cells were preincubated with the inhibitor for 1 hr (23). Cells were preincubated with 100 μ*M* Tyrphostin 23 (RG 50810) (Toronto Research Chemicals, Inc., Ontario, Canada) for 24 hr, which is required for inactivation of the EGF receptor tyrosine kinase (24). Cells were then washed free of inhibitor before the addition of agonists.

Northern Blot Analysis. Total cellular RNA was extracted from subconfluent monolayers of IEC-6 cells using RNAzol (Cinna/Biotecx Laboratories, Inc., Houston, Texas) as previously described (25). Adherent cultures were first washed twice with cold HBSS (JRH Biosciences, Lenexa, KS). One milliliter of cold RNAzol was added to each plate, and cells were scraped into an eppendorf tube. After vortexing each tube for 30 sec, 100 μl chloroform was added, followed by an additional 15 sec vortex. The tubes were then incubated on ice for 15 min. The tubes were centrifuged for 15 min at 12,000 rpm which resulted in the separation of the aqueous phase from the organic phase. The upper aqueous phase was then removed to another tube and an equal volume of ice-cold isopropanol was added. The tubes were incubated at −20°C for 45 min in order to precipitate the RNA, followed by a 15 min spin at 12,000 rpm. The RNA pellet was then washed twice with ice-cold 75% ethanol by centrifuging 8 min at 7500 rpm. RNA was resuspended in 40 μl diethylpyrocarbonate (DEPC)-treated water, and an aliquot was removed for quantitation at 260 nm.

For Northern analysis, 17.5 μg total RNA was denatured in formaldehyde sample buffer (1X MEA, 44% formamide, 16% formaldehyde, 0.3% ficoll, 0.1% bromphenol blue), size fractionated on 1% agarose containing 2.2 *M* formaldehyde, and transferred to nylon filters. Nylon filters (Gibco-BRL) were used for c-*fos*, c-*myc*, and c-*jun* hybridization and were hybridized in 50% formamide, 5X SSC, 5X Denhardt's solution, 1% SDS, 2% 1 *M* $NaPO_4$, pH 6.5, 1.5 mg salmon sperm DNA, and 8% dextran sulfate at 42°C for 24 hr. Filters were washed once in 1X SSC + 1% SDS at room temperature, once in 0.1X SSC + 0.1% SDS at 60°C, and twice in 0.1XSSC + 0.1% SDS at room temperature. Autoradiography was performed by exposing filters to Kodak X-Omat film at −70°C. Densitometry was performed on a LKB Ultroscan XL scanning laser densitometer (Bromma, Sweden). Equivalent loading was verified by photography of ethidium-bromide stained gels and hybridization to a GAPDH probe.

The probes for c-*fos* and c-*jun* were prepared using synthetic antisense oligonucleotides from R & D Systems (Minneapolis, MN). Human c-*myc* cDNA (Exon 3, 1.4-kb fragment) was obtained from Oncor (Gaithersburg, MD). The chicken GAPDH cDNA was the gift of Dr. B. Kream, University of Connecticut Health Center, Farmington, CT. All probes were prepared using the RadPrime DNA Labeling System (Gibco-BRL), and labeled with [^{32}P]dCTP (specific activity: 3000 Ci/mmol, NEN Research Products).

Results

Thymidine Incorporation. In order to compare the effect of TGFα and EGF on IEC-6 cell proliferation *in vitro*, thymidine incorporation was measured. The addition of either agonist resulted in an increase in thymidine incorporation in a dose-dependent manner (Fig. 1). TGFα increased thymidine incorporation by approximately 2-fold at a concentration of 2 ng/ml to 5 ng/ml (Fig. 1, closed squares). EGF increased thymidine incorporation maximally (approximately 2.3-fold) at a concentration of 5 to 25 ng/ml (Fig. 1, open circles). It is interesting to note that EGF could sustain the level of thymidine incorporation at higher doses whereas the addition of greater than 5 ng/ml of TGFα resulted in lower levels of incorporation.

Two tyrosine kinase inhibitors were used to determine the effect of tyrosine kinase activity on cell proliferation (Table I). Genistein is a general inhibitor of tyrosine kinases (23), which has been shown previ-

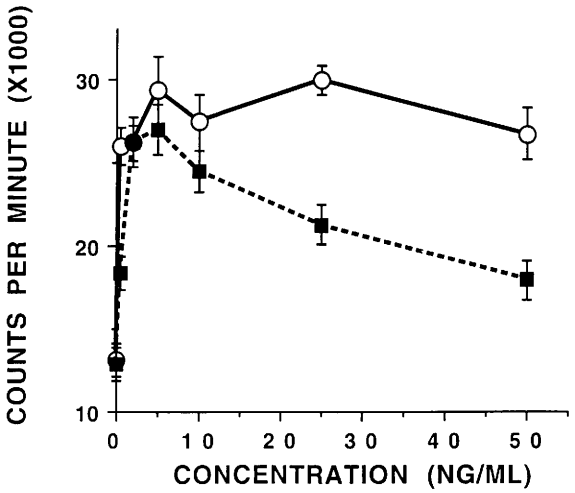

Figure 1. [^{3}H]thymidine incorporation in IEC-6 cells after 24-hr incubation with either TGFα or EGF at various concentrations. Closed squares, TGFα; open circles, EGF. Both growth factors increase thymidine incorporation in a dose-dependent manner. The results are expressed as mean counts per minute (×1000) ± SE of three experiments.

Table I. Effect of Tyrosine Kinase Inhibitors on Thymidine Incorporation

	Control	TGFα (2 ng/ml)	EGF (25 ng/ml)
No inhibitor	16.7 ± 1.4[a]	27.7 ± 1.0	29.2 ± 2.4
Genistein[b]	12.8 ± 0.6	14.7 ± 0.9	20.5 ± 1.4
Tyrphostin 23[c]	12.4 ± 0.03	14.0 ± 0.8	15.0 ± 0.6

[a] Mean counts per minute (×1000) ± SE of three experiments.
[b] Genistein (50 μg/ml) was added for 1 hr, removed, and growth factors were added for 24 hr.
[c] Tyrphostin 23 (100 μ*M*) was added for 24 hr, removed, and growth factors were added for 24 hr.

ously to decrease the gastrin-induced increase in cell counts (6) and the TGFα-induced increase of tyrosine phosphorylation levels of cellular proteins in IEC-6 cells (4). Tyrphostin 23 is a specific inhibitor of the EGF receptor tyrosine kinase and inhibits the TGFα-induced increase of tyrosine phosphorylation levels of proteins as well (4, 26). When cells were preincubated with 50 μg/ml genistein for 1 hr and washed free of inhibitor prior to growth factor addition for 24 hr, thymidine incorporation was decreased by 47% in the case of 2 ng/ml TGFα stimulation and only 30% when 25 ng/ml EGF was added. When cells were preincubated with 100 μ*M* tyrphostin 23 for 24 hr (required for EGF receptor tyrosine kinase inactivation), washed free of inhibitor, then stimulated with 2 ng/ml TGFα or 25 ng/ml EGF for 24 hr, thymidine uptake was decreased to near prestimulated levels. These concentrations of agonists were used because we wanted to test inhibition at maximum thymidine incorporation. Cell viability was verified as >90% using trypan blue dye exclusion; thus, the cells were not dead after incubation with these inhibitors.

MAPK and RSK Activity. MAPK activity was measured using an in gel kinase assay with myelin basic protein as the substrate. The resulting autoradiographs were scanned with a laser densitometer, and the graphs represent the fold increase of the integrated optical density of experimental condition over control which was assigned an arbitrary value of one. The addition of TGFα to IEC-6 cells increased the kinase activity of both a 42- and 44-kDa protein in a dose-dependent manner (Fig. 2A). The identity of the two kinases that phosphorylated myelin basic protein in the experiments was verified as $p42^{erk2}$ and $p44^{erk1}$ MAPK by immunoprecipitation (data not shown). Maximum activity for both isoforms occurred with the addition of 2 ng/ml TGFα, although the 9-fold increase of $p42^{erk2}$ activity was two times that of $p44^{erk1}$ activity. There was a slightly different response of MAPK activities to increasing doses of EGF (Fig. 2B). The maximum activation was only a 4.5-fold increase of $p42^{erk2}$ seen at 25 ng/ml EGF, a much higher dose than the optimum TGFα dose. As with TGFα stimulation, $p42^{erk2}$ activity was 2-fold greater than $p44^{erk1}$ activity after EGF stimulation. A time course of MAPK activity was performed and the results indicate that the activities of both $p42^{erk2}$ and $p44^{erk1}$ were increased maximally by the addition of 2 ng/ml TGFα for 5 min (Fig. 3A), although an increase was seen in $p42^{erk2}$ activity as early as 1 min. Activity of $p42^{erk2}$ was only slightly elevated above control levels at the longer time points, while $p44^{erk1}$ activity, although lower than $p42^{erk2}$ activity, was sustained for 15 min. Similarly, the activity of $p42^{erk2}$ was maximally increased after 5 min stimulation by 25 ng/ml EGF, and decreased at 15 min (Fig. 3B). The activity of $p44^{erk1}$ was only slightly increased at 5 min stimulation with EGF.

RSK (p90) activity was measured using the same in gel assay, but using the rsk kinase substrate peptide as the substrate. A dose response was performed and doses of both agonists yielding maximum RSK activity were identical to those yielding maximum MAPK activity. Maximum RSK activity was 2-fold when stimulated with 2 ng/ml TGFα for 5 min (Fig. 4A). A 2-fold increase in RSK activity was seen with 25 to 50 ng/ml EGF stimulation for 5 min (Fig. 4B). The time course of RSK activity after stimulation with 2 ng/ml TGFα was more sustained than that of MAPK (Fig. 5A). Activity of RSK was increased at 5, 15, and 30 min and then decreased to near control levels at 45 min. The time course of RSK activity after stimulation by 25 ng/ml EGF was different from TGFα in that a 2-fold increase in activity was sustained from 5 to 60 min (Fig. 5B).

The TGFα-induced increases in the activities of $p42^{erk2}$ MAPK, $p44^{erk1}$ MAPK, and RSK were inhibited by more than 70% when cells were pretreated for 24 hr with 100 μ*M* tyrphostin 23, washed free of in-

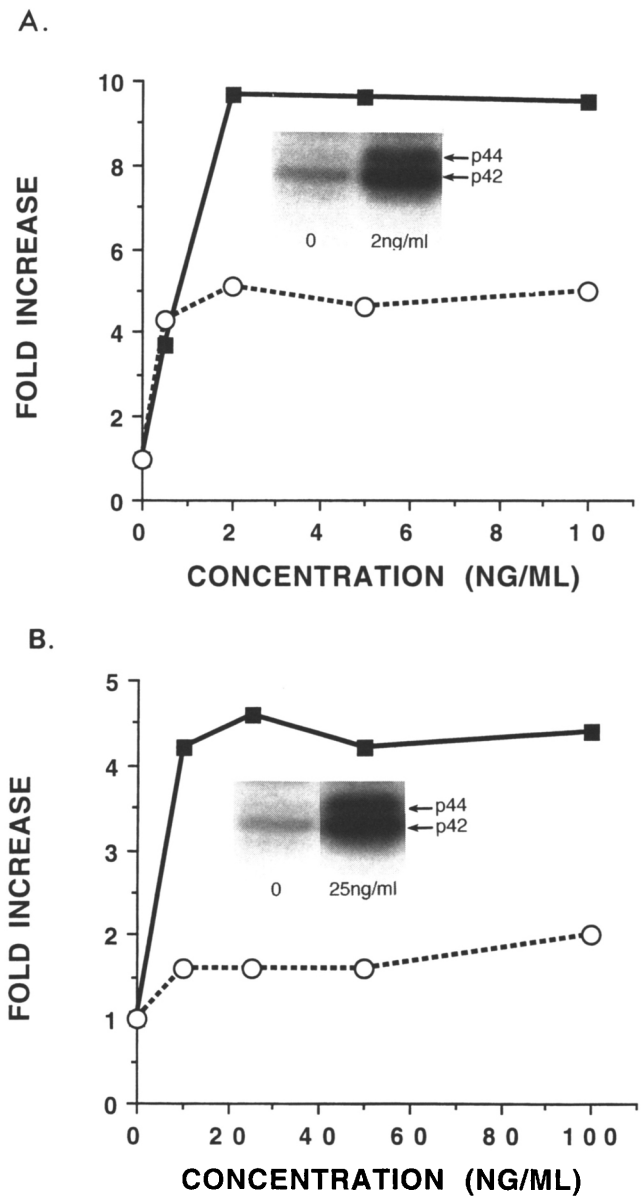

Figure 2. MAPK activity in response to increasing doses of agonists as evidenced by incorporation of radiolabelled phosphate in myelin basic protein. (A) MAPK activity in IEC-6 cells in response to stimulation by increasing concentrations of TGFα for 5 min. Activity of $p42^{erk2}$ and $p44^{erk1}$ increased in a dose-dependent manner. (B) MAPK activity in response to increasing concentrations of EGF for 5 min. Activity of $p42^{erk2}$ and $p44^{erk1}$ increase in a dose-dependent manner. Closed squares, $p42^{erk2}$; open circles, $p44^{erk1}$. Results are laser densitometer readings of an autoradiograph expressed as fold increase of experimental compared with control, which is given an arbitrary unit of one. Inset is photograph of representative autoradiograph. Results are representative of three separate experiments.

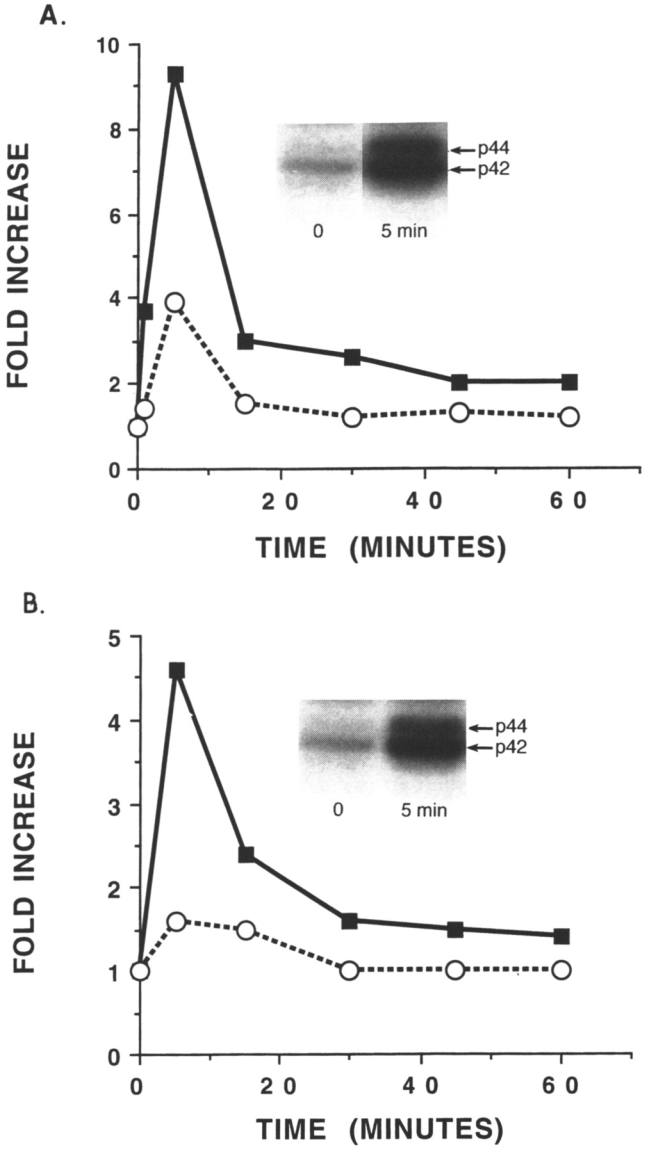

Figure 3. MAPK activity in IEC-6 cells in response to agonist stimulation over time as evidenced by incorporation of radiolabelled phosphate in myelin basic protein. (A) MAPK activity in IEC-6 cells in response to stimulation by 2 ng/ml TGFα for increasing times. Activity of $p42^{erk2}$ and $p44^{erk1}$ increased maximally at 5 min. (B) MAPK activity in IEC-6 cells in response to stimulation by 25 ng/ml EGF for increasing times. Activity of $p42^{erk2}$ increased at 5 min until 15 min. Activity of $p44^{erk1}$ increased slightly at 5 min. Closed squares, $p42^{erk2}$; open circles, $p44^{erk1}$. Results are laser densitometer readings of an autoradiograph expressed as fold increase of experimental compared with control, which is given an arbitrary unit of one. Inset is photograph of a representative autoradiograph. Results are representative of two separate experiments.

hibitor, and then stimulated with 2 ng/ml TGFα for 5 min (data not shown). The sensitivities of these enzymes to the tyrosine kinase inhibitor are similar to the responses obtained with thymidine incorporation (Table I).

Protein Synthesis. In order to determine the effect of growth factor treatment on the protein expression of transcription factors, the levels of newly synthesized c-Fos and c-Myc were measured. IEC-6 cells were either left as control or stimulated for 30 min with 2 ng/ml TGFα, labeled with [^{35}S]methionine, and immunoprecipitated with either anti-Fos or anti-Myc antibodies as described in Materials and Methods. After stimulation, the amount of c-Fos and c-Myc synthesized increased (Fig. 6, A and B, Lane 2). The level of

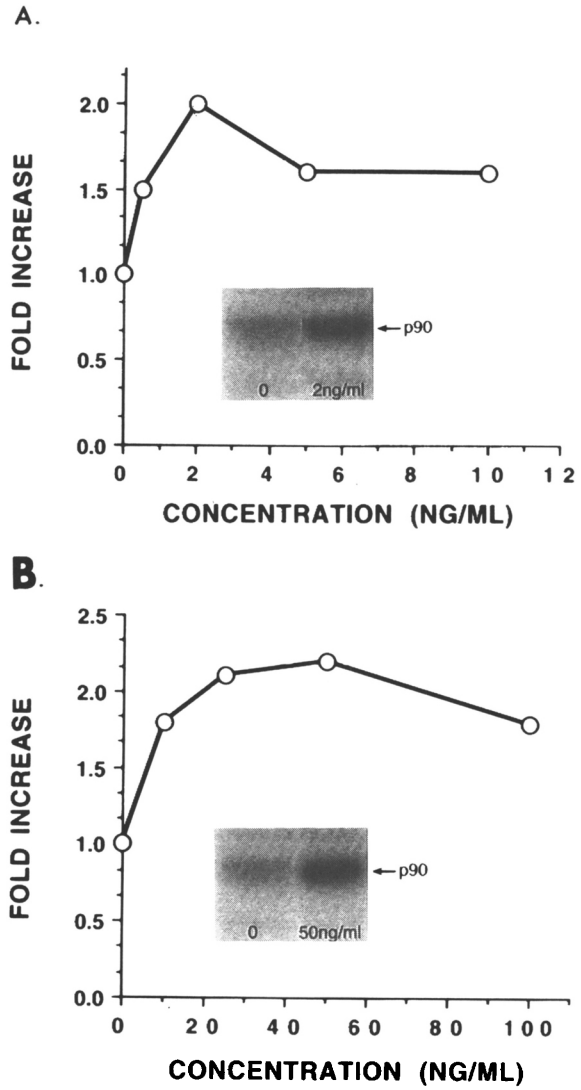

Figure 4. RSK activity in IEC-6 cells in response to increasing doses of agonists as evidenced by incorporation of radiolabeled phosphate in rsk kinase substrate peptide. (A) RSK activity in response to increasing doses of TGFα for 5 min. Activity of p90 increased 2-fold at 2 ng/ml. (B) RSK activity in response to increasing doses of EGF for 5 min. There was a 2-fold increase in activity with the addition of 25 ng/ml EGF. Results are laser densitometer readings of an autoradiograph expressed as fold increase of experimental compared with control, which is given an arbitrary unit of one. Inset is photograph of representative autoradiograph. Results are representative of two separate experiments.

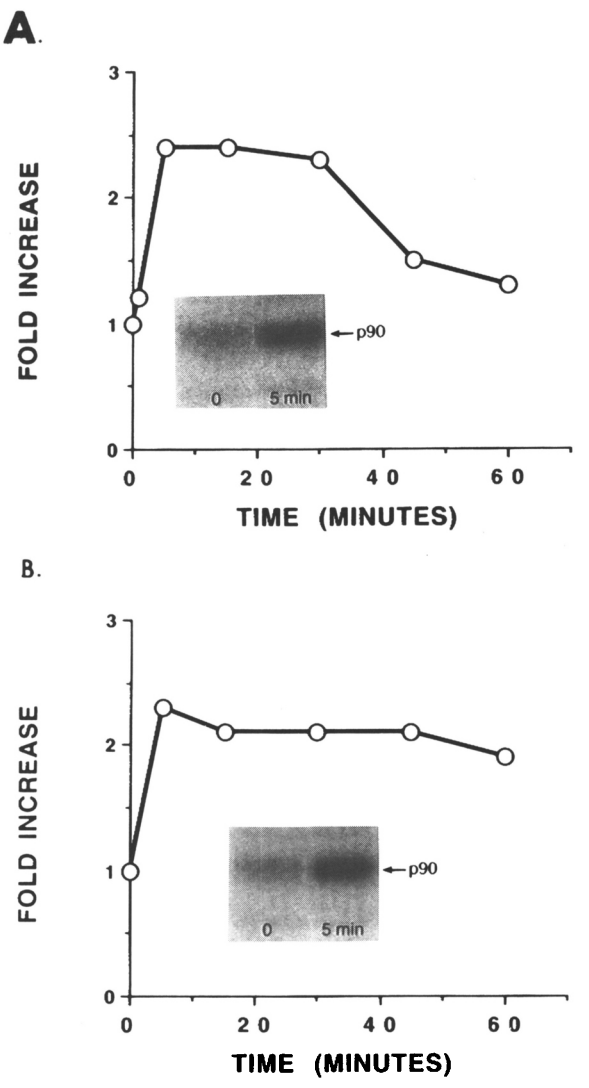

Figure 5. RSK activity in IEC-6 cells in response to agonist stimulation over time as evidenced by the incorporation of radiolabeled phosphate in rsk kinase substrate peptide. (A) RSK activity in IEC-6 cells in response to stimulation by 2 ng/ml TGFα for increasing times. A sustained increase in activity was seen from 5 to 30 min. (B) RSK activity in IEC-6 cells in response to stimulation by 25 ng/ml EGF for increasing times. There was a 2-fold increase in activity from 5 to 60 min. Results are laser densitometer readings of an autoradiograph expressed as fold increase of experimental compared with control, which is given an arbitrary unit of one. Inset is photograph of representative autoradiograph. Results are representative of two separate experiments.

c-Fos in stimulated cells increased over 2-fold compared with the control level when measured by densitometry. The level of c-Myc in stimulated cells increased 5-fold. The basal level of c-Fos synthesis in control cells was greater than the basal level of c-Myc synthesis, which was almost undetectable in control cells (Fig. 6, A and B, Lane 1). When the same experiment was performed using anti-c-Jun antibodies, no significant c-Jun synthesis was seen in either control or stimulated cells (data not shown).

Northern Blot Analysis. In order to determine if protein synthesis was due to gene transcription, Northern blot analysis was performed on total cellular RNA. The blots were hybridized with either c-*fos*, c-*myc*, or c-*jun* probes and GAPDH probe as control for equal gel loading. All autoradiographs were analyzed on a laser densitometer as well in order to quantify the results. After 30-min incubation of IEC-6 cells with TGFα, there was a 5-fold increase of c-*fos* mRNA (Fig. 7). The level decreased to near the control level by 60 min poststimulation. The level of c-*myc* mRNA increased approximately 2-fold after 30-min stimulation with TGFα and returned to the control level by 60

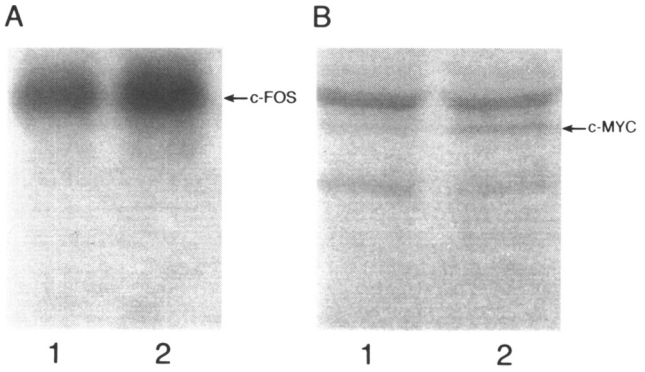

Figure 6. Newly synthesized protein after stimulation with TGFα. (A) Cells were either left as control (Lane 1) or stimulated for 30 min with 2 ng/ml TGFα (Lane 2), metabolically labeled with [^{35}S]methionine, and lysates immunoprecipitated twice with c-Fos antibody. The amount of c-Fos increases over 2-fold after stimulation when measured using densitometry. (B) Cells were either left as control (Lane 1) or stimulated for 30 min with 2 ng/ml TGFα (Lane 2), metabolically labeled with [^{35}S]methionine, and lysates immunoprecipitated twice with c-Myc antibody. The level of newly synthesized c-Myc increased 5-fold after TGFα stimulation when measured using densitometry.

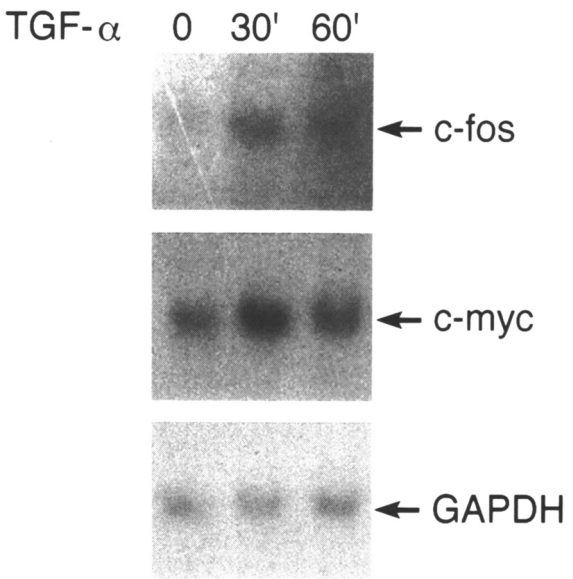

Figure 7. Northern blot analysis of the effect of TGFα stimulation on c-*fos* and c-*myc* expression. IEC-6 cells were stimulated for 30 or 60 min with 2 ng/ml TGFα or left as control. Total cellular RNA was isolated and subjected to Northern analysis with a c-*fos*, c-*myc*, or GAPDH probe. The levels of c-*fos* mRNA and c-*myc* mRNA increased at 30 min of stimulation and decreased to near control levels by 60 min of stimulation.

min. In contrast, c-*jun* mRNA was insensitive to TGFα stimulation and was undetectable in control cells (data not shown). When cells were pretreated with 50 μg/ml genistein for 1 hr prior to stimulation, the level of expression of c-*fos* and c-*myc* decreased to control (data not shown).

Discussion

The MAPK signal transduction pathway in proliferating cells links a plasma membrane event (receptor-ligand binding) to nuclear events (activation of transcription factors) through a series of sequential tyrosine and serine/threonine phosphorylations. Proliferation or differentiation may result depending on the type of receptor activation and cell type. The crypt cells of the small intestine respond to both proliferative and differentiating factors, although the exact mechanisms involved in these two responses have not been detailed. These studies were designed to further elucidate the possible signaling pathways in IEC-6 cells used by two similar mitogenic growth factors, TGFα and EGF, and to determine if there were significant differences in the responses to the two agonists.

Thymidine incorporation in IEC-6 cells was increased following stimulation with either TGFα or EGF in a dose-dependent manner (Fig. 1). The concentrations of TGFα that induced the greatest response (2–5 ng/ml) were somewhat lower than the optimum EGF concentrations (10–25 ng/ml). Derynck (27) reported an increase in potency of TGFα compared with EGF in inducing a mitogenic response in hepatocytes, similar to our results. However, another study (28) reports that TGFα and EGF had very little effect on IEC-6 cell growth or thymidine incorporation. The reasons for the contradiction are unclear, although the complete medium we use contains glutamine, a necessary supplement for intestinal cells (29). Interestingly, increasing the concentration of TGFα decreased the level of thymidine incorporation. This type of response is not unusual and may be due to a change in the receptor recycling times in response to the two growth factors. Others have shown that high-density cultures of A431 cells recycle EGF receptor more rapidly than low-density cultures and consume more EGF (30). Perhaps, receptor binding by TGFα differs in this regard, and receptors are downregulated in high-density cultures.

Genistein, a general tyrosine kinase inhibitor, was less effective than the more specific EGF receptor tyrosine kinase inhibitor, tyrphostin 23, in blocking the thymidine incorporation increase by EGF compared to TGFα (Table I). The concentration of genistein (50 μg/ml) used in the present study should have been sufficient to inhibit proliferation, since Basson *et al.* (31) found a dose-dependent effect on EGF induced proliferation of Caco-2 cells by genistein which was maximum at 100 μ*M* (28 μg/ml). This raises the intriguing possibility that EGF may activate mitogenic pathways in IEC-6 cells which bypass nonreceptor tyrosine kinases completely.

The addition of TGFα or EGF to IEC-6 cells increased the activity of $p42^{erk2}$ and $p44^{erk1}$ in a time- and dose-dependent manner as evidenced by increased incorporation of radiolabeled phosphate in myelin basic protein (Fig. 2 and 3). The increase of the activity of $p42^{erk2}$ was much greater than that of

$p44^{erk1}$ following stimulation by either factor, which suggests a more critical role for $p42^{erk2}$ in the signaling path. A similarly rapid and transient activation of both isoforms of MAPK was seen in fibroblasts after stimulation by a variety of mitogens (32). EGF activated MAPK in both undifferentiated and differentiated neuronal cells as well (33). However, this is the first report using intestinal epithelial cells.

The main difference between the activity levels induced by the two agonists was in the intensity of the increase and the optimum dose. TGFα at 2 ng/ml induced a 9-fold increase in p42 activity, while EGF at 25 ng/ml induced only a 4.5-fold increase. Thus, TGFα is more potent than EGF in raising MAPK activity above control levels in IEC-6 cells.

The stimulus-induced increases in the activities of $p42^{erk2}$ and $p44^{erk1}$ correlate well with the enhanced tyrosine phosphorylation of these two proteins reported previously (4). TGFα at 2 ng/ml increased the tyrosine phosphorylation level of both $p42^{erk2}$ and $p44^{erk1}$ at 5 min, which is the same dose and time of maximum MAPK activation. Also, both growth factors increased MAPK activity several-fold at the same dose required for maximum thymidine uptake. Therefore, there is a strong correlation between cell proliferation, tyrosine phosphorylation of MAPK, and MAPK activity.

The activity of RSK increased after stimulation of IEC-6 cells by either TGFα or EGF (Fig. 4 and 5). The increase in activity correlates closely with the stimulus-induced increase in MAPK activity with respect to dose, but the time course differs. TGFα induced RSK activity until 30 min poststimulation, and EGF induced activity as long as 60 min poststimulation. The prolonged activation of RSK by both growth factors may be due to upstream activation of other MAPK isoforms such as p63, which is a putative activator of RSK (34). Initial activation of RSK may be due to MAPK and the later activity may be due to p63. There has been some controversy about whether RSK is an *in vivo* substrate for $p42^{erk2}$ and $p44^{erk1}$ (35). The present results suggest that stimulation of $p42^{erk2}$ and $p44^{erk1}$ activity does lead to an increase of RSK activity, but other activators may also be involved.

The product of the c-*fos* and c-*myc* genes are thought to be important in cell proliferation and the regulation of their expression is critical. The activation of $p42^{erk2}$, $p44^{erk1}$, and $p90^{rsk}$ is involved in regulation of the expression of transcription factors such as c-Fos and c-Myc, both *in vitro* (36, 37) and *in vivo* (38, 39). In IEC-6 cells stimulated with TGFα or EGF, these three kinases are activated within 5 min, thus suggesting that the transient expression of c-*fos* and c-*myc* in this study results from their activation. The fact that c-Jun protein levels and c-*jun* expression were unchanged is consistent with the finding that a different MAPK isoform (p54) termed c-Jun NH_2-terminal kinase (JNK) regulates c-*jun* expression (40, 41). Activation of JNK follows a different signal transduction pathway from the ERK1 and ERK2 activation paths (41) and therefore, may not be activated as a result of TGFα stimulation in IEC-6 cells. Davidson *et al.* (42) reported that TGFα increased c-*jun* expression, in contrast to our results; but these investigators were studying a breast cancer cell line which might respond differently than an untransformed cell like the IEC-6 cell line. The fact that genistein pretreatment inhibited the increase of expression of c-*fos* and c-*myc* further correlates tyrosine kinases with mitogenic stimulation.

In summary, this study has shown that both TGFα and EGF stimulation of IEC-6 cells increase the activities of $p42^{erk2}$, $p44^{erk1}$, and $p90^{rsk}$, although with slightly different kinetics. TGFα was more potent than EGF in stimulating enzyme activity, which has not been reported previously. The increase in activities of these enzymes leads to an increase in the synthesis of c-*fos* and c-*myc* protein levels as well as message levels, suggesting a link to nuclear events. Therefore, the increase in IEC-6 cell proliferation in response to TGFα and EGF stimulation may be due, in part, to an increase in immediate early gene expression as a direct result of MAPK and RSK activation.

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