Transforming growth factor- α increases tyrosine phosphorylation of microtubule-associated protein kinase in a small intestinal crypt cell line (IEC-6)

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The small intestinal crypt cell line (IEC-6) is an undifferentiated, untransformed, mitotically active cell used in this study to determine the effect of transforming growth factor- α (TGF- α) on tyrosine phosphorylation levels of cellular proteins. Thymidine incorporation increased maximally after addition of 2 ng/ml TGF- α for 24 h. At the same dose, TGF- α induced the tyrosine phosphorylation of proteins with approximate molecular masses of 42, 44, 52, 80, 150 and 175 kDa as shown by Western blots treated with anti-phosphotyrosine antibody. The most intense phosphorylation was seen in the 42 kDa (p42) and 44 kDa (p44)

proteins, which were identified as two isoforms of microtubule-associated protein kinase (MAPK). This phosphorylation was seen as early as 5 min post stimulation and was dose dependent. Both p42 and p44 were found in the nucleus after stimulation, although a basal level of unphosphorylated protein was present before stimulation. The observed tyrosine phosphorylation of p42 and p44 was inhibited by genistein, a tyrosine kinase inhibitor, and tyrphostin 23, an epidermal growth factor receptor tyrosine kinase inhibitor. We conclude that MAPK is tyrosine phosphorylated in response to TGF- α stimulation of IEC-6 cells.

INTRODUCTION

The epithelial cells lining the lumen of the small intestine provide an excellent model of cells at different stages of proliferation and differentiation and are therefore ideal for studying the signal transduction pathways involved in cell growth. The crypt cells, in particular, are rapidly dividing, undifferentiated cells which migrate to the villus region as they differentiate into mature cells and are eventually extruded from the tip of the villus after 2 to 3 days [1]. The cultured cell line, IEC-6, isolated from the crypt of the rat jejunum, was used in the present study because it retains many of the antigenic and physical properties of intact crypt cells of the small intestine [2] and provides a mitotically active, untransformed cell for study.

The mechanism by which crypt cells are induced to divide and/or differentiate is unknown, but it is most likely mediated by a variety of growth factors and hormones. It has been shown that protein tyrosine kinases may be partially responsible for the proliferative response of crypt cells to gastrin, a gastric hormone with trophic properties [3,4]. Other studies have dealt with the effects of epidermal growth factor (EGF) on the proliferation of IEC-6 cells as well as other intestinal cell types [5–9]. The proliferative response of cells to EGF has been complicated by the synergistic effects of other growth factors [6,7] and seems to require doses of EGF that are higher than physiological levels [6,7,9].

Transforming growth factor- α (TGF- α) is a 50 amino acid, 5600 Da protein in its mature form that shares 35% sequence similarity with EGF [10] and also competes with EGF for binding to the EGF receptor [11]. It has been suggested that the two peptides bind differently to the EGF receptor [12] because

there are slight differences in the biological effects of these two peptides. TGF- α has been shown to induce a calcium release in rat long bone while EGF does not [13]. TGF- α is more effective than EGF in inducing neovascularization during angiogenesis [14], and it seems to be generally more potent than EGF (reviewed in [15]). Pertinent to this study, urogastrone EGF levels in gastric tissue are very low compared with TGF- α levels [16].

The expression and secretion of TGF- α have been examined in the small intestine and are extremely low in the crypt cells with progressively higher amounts being both expressed and secreted by more mature cells toward the villus [17–20]. This suggests a possible paracrine role for TGF- α in the growth and/or differentiation of intestinal crypt cells. It therefore seems likely that TGF- α is an important growth factor in the life cycle of intestinal cells.

Microtubule-associated protein kinases (MAPKs) are a family of serine/threonine kinases which are activated in response to several growth factors including EGF and platelet-derived growth factor (PDGF) [21], and also the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) [22,23]. MAPK requires both threonine and tyrosine phosphorylation for activation and is an important intracellular link between receptor tyrosine kinases and downstream transcriptional events [24].

The present study was conducted to determine the tyrosine phosphorylation levels of proteins after stimulation of IEC-6 cells with TGF- α . It was found that several proteins with molecular masses of 42, 44, 52, 80, 150 and 175 kDa did show an increase in tyrosine phosphorylation levels, especially evident in a 42 kDa protein (p42) and a 44 kDa protein (p44). We conclude that p42 and p44 are members of the MAPK family.

Abbreviations used: TGF- α , transforming growth factor α ; MAPK, microtubule-associated protein kinase; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks Balanced Salt Solution; NP-40, Nonidet P-40; GM-CSF, granulocyte-macrophage colony-stimulating factor; GH, growth hormone; TBS-T, Tris-buffered saline/Tween 20.

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MATERIALS AND METHODS

Reagents

All tissue culture reagents were purchased from Gibco (Grand Island, NY, U.S.A.). Recombinant TGF- α was from R & D Systems (Minneapolis, MN, U.S.A.). Genistein was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and tyrphostin 23 from Toronto Research Chemicals (Downsview, Ontario, Canada). Hanks' Balanced Salt Solution (HBSS) was obtained from JRH Biosciences (Lenexa, KS, U.S.A.).

Antibodies

Monoclonal anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). This antibody is a mouse-mouse hybridoma propagated *in vitro*, and is specific for phosphotyrosine-containing proteins of all species. Mouse anti-MAPK antibody (clone Z033) was obtained from Zymed Laboratories (San Francisco, CA, U.S.A.) and is directed against a 21-residue synthetic peptide corresponding to the MAPK amino acid sequence 325-345. It recognizes only the p42 and p44 isoforms of MAPK. The secondary antibody used for immunoblotting, anti-(mouse IgG) conjugated to horseradish peroxidase, was from Amersham (Arlington Heights, IL, U.S.A.). Anti-(mouse IgG) (whole) agarose used for immunoprecipitation was obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Rat small intestinal epithelial cells (IEC-6) were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 4 mM glutamine, 50 units/ml penicillin, 50 μ g/ml streptomycin, 1.0 μ g/ml fungizone and 5 μ g/ml bovine insulin at 37 °C and 10% CO₂. All experiments were performed on cells between passages 15 and 25. Cells were grown in 100-mm² tissue culture plates until approx. 70% confluent and then were starved for 18 h in DMEM containing 0.5% FBS.

Thymidine incorporation

[³H]Thymidine incorporation was performed as described previously [25] with several modifications. Cells were seeded at 1×10^4 cells/well in a 96-well tissue culture plate. After 18 h in DMEM + 0.5% FBS, the appropriate growth factor was added for 24 h. During the last 4 h, 0.5 μ Ci [methyl-³H]thymidine (specific radioactivity: 82 Ci/mmol; Amersham Life Science, Arlington Heights, IL, U.S.A.) was added to each well. Cells were incubated with trypsin-EDTA for 10 min and then harvested on to filtermats using a Skatron cell harvester (Skatron, Sterling, VA, U.S.A.). Filtermats were dried and radioactivity of each disk was counted in a Delta 300 liquid-scintillation counter (Searle Analytic).

Cell lysates and immunoblotting

IEC-6 cells were stimulated with TGF- α for the appropriate time. Lysates were prepared as follows: cells were washed with ice-cold HBSS and scraped into 0.7 ml of ice-cold lysis buffer [50 mM Hepes, 150 mM NaCl, 10% (v/v) glycerol, 10 mM EDTA, 1 mM MgCl₂, 1% Nonidet P-40 (NP-40) and protease

inhibitors: 2 mM sodium orthovanadate, 1 mM phenylmethanesulphonyl fluoride, 5 mM sodium pyrophosphate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin]. Lysates were clarified by a quick spin in an Eppendorf Microfuge and protein determinations were made using the Bio-Rad DC protein assay (Bio-Rad, Richmond, CA, U.S.A.).

Western blots were performed as described previously [22]. Lysate samples containing equivalent protein were mixed with 2 $\times$ sample buffer [8% (w/v) SDS, 10% (v/v) glycerol, 86 mM Tris, pH 6.8, 0.5% Bromphenol Blue, 10% (v/v) 2-mercaptoethanol] and boiled for 5 min. Proteins were resolved on SDS/8% (w/v) polyacrylamide gels, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, U.S.A.), and probed with the appropriate antibody. Western blots were developed using the enhanced chemiluminescent method (Amersham, Arlington Heights, IL, U.S.A.). Briefly, blots were blocked with 5% (w/v) BSA in Tris-buffered saline/Tween-20 (TBS-T) (20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween-20), then incubated for 1.5 h with the appropriate primary antibody dissolved in TBS-T (anti-phosphotyrosine at 1:1000 and anti-MAPK at 1:4000), followed by incubation with the horseradish peroxidase-conjugated anti-(mouse IgG) (1:10000) for 25 min. Blots were developed with Kodak X-Omat film for 15 s to 2 min.

Blot stripping and reprobing

Immunoblots were stripped of primary and secondary antibody complexes by incubating them in stripping buffer [100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris/HCl, pH 6.7] for 30 min at 50 °C. Blots were subjected to autoradiography to confirm that the antibody signal had been removed. The blots were then blocked with 5% (w/v) BSA in TBS-T, and reprobed with a different antibody as described above.

Immunoprecipitation

Immunoprecipitation was performed as described previously [26,27] with modifications. The anti-MAPK antibody used required the protein to be denatured prior to immunoprecipitation [28]. Therefore, cells were lysed with 0.5 ml of boiling 1% SDS in 10 mM Tris, pH 7.4. The lysate was boiled for 5 min, then 100 μ l was diluted with 400 μ l of cold water. To this was added 500 μ l of 2 $\times$ immunoprecipitation (IP) buffer (1 $\times$ = 10 mM Tris, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, plus the same protease inhibitors used in the lysis buffer). Anti-(mouse IgG)-agarose beads had been linked overnight with 4 μ g of anti-MAPK and then were added to the lysate. After 2 h at 4 °C, the beads were washed twice with 1 $\times$ IP buffer, twice with LiCl buffer (0.5 M LiCl, 0.1 M Tris, pH 7.4) and twice with NaCl buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris). The beads were then resuspended in a dissociation buffer (5 mM Na₂HPO₄, 3% SDS, 10% glycerol, 0.05% Bromphenol Blue, 10% 2-mercaptoethanol) and resolved on SDS/8% (w/v) polyacrylamide gel. Western blots were processed as described above.

Immunoprecipitation with anti-phosphotyrosine antibody was performed in a similar manner except cells were scraped into 700 μ l of lysis buffer, clarified and then added to the agarose bead-antibody mixture. The samples were then processed as above.

Cell fractionation

Membrane and cytosol fractions were prepared according to Dusi et al. [29] with slight modification. Briefly, after stimulating

adherent IEC-6 cells as described above, cells were washed once with cold HBSS. Cells were then scraped into cold HBSS, centrifuged and resuspended in 400 μ l of sucrose buffer (10 mM Hepes, 0.1 M sucrose, 1 mM EGTA, pH 7.3) which contained protease inhibitors. Cells were sonicated with a Kontes Micro-ultrasonic cell disrupter on maximum speed for three 5-s bursts. After centrifuging at 10000 g for 10 min to remove cellular debris, supernatants were spun in a Beckman TL-100 ultracentrifuge at 110000 g and 4 $^{\circ}$ C for 20 min. The pellet (membrane) was resuspended in 400 μ l of sucrose buffer in order to ensure a cellular equivalent to the supernatant (cytosol). Equal volumes of cytosol and membrane were run on SDS/8% (w/v) polyacrylamide gel and immunoblotted as described above. Purity of fractions was monitored by the presence of Na^+/K^+ ATPase activity in the membranes [30].

Nuclear fractions were prepared from IEC-6 cells as described previously [31]. Briefly, cells were harvested from 100-mm² plates by scraping into hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM 2-glycerophosphate, 2 mM MgCl_2 , 10 mM KCl, 1 mM dithiothreitol, 10 μ g/ml aprotinin, 1 mM sodium orthovanadate, 40 μ g/ml phenylmethanesulphonyl fluoride, 10 μ g/ml leupeptin, pH 7.2) and incubated on ice for 30 min. The cells were then Dounce-homogenized (30 strokes) and the homogenate was loaded on to sucrose buffer (1 M sucrose in hypotonic lysis buffer). After centrifuging for 10 min at 1600 g , the supernatant above the sucrose cushion was removed and saved as the postnuclear fraction. The sucrose cushion-purified nuclear pellet was washed in sucrose buffer and centrifuged at 1600 g for 5 min. The nuclear pellet was then resuspended in lysis buffer containing 0.5% NP-40, 0.1% deoxycholate and 0.1% Brij-35. Equal volumes of nuclear fractions were used for SDS/PAGE analysis of proteins. Purity of fractions was determined by measuring the cytosolic marker, lactate dehydrogenase [32].

RESULTS

Thymidine incorporation

The addition of TGF- α to IEC-6 cells *in vitro* causes an increase in [³H]thymidine incorporation in a dose-dependent manner (Figure 1). Thymidine incorporation increased significantly at 0.1 ng/ml TGF- α and reached a maximum at 2 ng/ml. Therefore, 2 ng/ml was chosen as the optimum dose for the remaining studies.

Tyrosine phosphorylation in IEC-6 cells stimulated with TGF- α : time course and dose response

IEC-6 cells were incubated with 2 ng/ml TGF- α for 0.5 min to 45 min and Western blots show that several proteins with approximate molecular masses of 42, 44, 52, 80, 150 and 175 kDa were rapidly phosphorylated on tyrosine (Figure 2a, closed arrows). The 175 kDa band corresponds to the EGF receptor (as identified by immunoblotting), which is autophosphorylated within 0.5 min of stimulation, proving activation of the receptor tyrosine kinase. The proteins corresponding to the 52 kDa, 80 kDa and 150 kDa bands are transiently phosphorylated starting at 0.5 min. These three bands have shown the same pattern of phosphorylation in each experiment. The most prominent and most sustained phosphorylation is seen on the proteins corresponding to the 42 kDa and 44 kDa bands. These proteins show an increase in phosphorylation levels beginning at 5 min after stimulation, which is sustained until 45 min. When immunoblots were stripped and reprobed with anti-MAPK, the 42 and 44 kDa isoforms align precisely with p42 and p44 (Figure 2b).

There is an increase in the molecular mass of the two proteins as they become phosphorylated at 5 min causing a mobility shift (Figure 2b, lane 5), but the relative amounts of the proteins remain the same in each lane. A doublet forms in p42 at 30 min post stimulation (lane 7) due to the presence of both unphosphorylated and phosphorylated forms of the protein.

The TGF- α -induced tyrosine phosphorylation of p42 and p44 is dose-dependent (Figure 3a). Significant tyrosine phosphoryla-

Figure 1 [³H]Thymidine incorporation of IEC-6 cells in response to various concentrations of TGF- α

Cells were stimulated for 24 h, pulsed with 0.5 μ Ci/well of [³H]thymidine for the final 4 h and harvested. Results are expressed as mean counts per minute (c.p.m.) $\pm$ S.E.M. of three experiments.

Figure 2 Time course of tyrosine phosphorylation of proteins following TGF- α stimulation

(a) IEC-6 cells were incubated with 2 ng/ml TGF- α for the following times: lanes 1–8: 0, 0.5, 1, 2, 5, 15, 30 and 45 min respectively. Lysates were electrophoresed as described in the Materials and methods section. Immunoblots were probed with anti-phosphotyrosine antibody and detected using enhanced chemiluminescence. Closed arrows on the right indicate proteins showing increased levels of tyrosine phosphorylation over time. Their approximate molecular masses are 42, 44, 52, 80, 150 and 175 kDa. (b) The same blot was stripped of antibody, reprobed with anti-MAPK and developed with enhanced chemiluminescence. The two isoforms of MAPK are indicated. Immunoblots are representative of three separate experiments.

Figure 3 Dose response of tyrosine phosphorylation of proteins following TGF- α stimulation

(a) IEC-6 cells were incubated for 5 min with the following doses of TGF- α : lanes 1–7: 0, 0.05, 0.5, 2, 20, 50 and 100 ng/ml respectively. Samples were processed as indicated in Figure 1. Arrows on right indicate p42 and p44. (b) The same blot was stripped and reprobed with anti-MAPK. Results are representative of three similar experiments.

tion of p42 and p44 was observed at concentrations as low as 0.05 ng/ml TGF- α . When blots were stripped and reprobed with anti-MAPK, the two isoforms of MAPK align with p42 and p44 and the relative amounts of the proteins are constant in each lane (Figure 3b).

Subcellular localization

The location in the cell of the phosphorylated p42 and p44 was then determined. MAPK proteins are not associated with the plasma membrane [23]. This was verified by first fractionating cells into membrane and cytosol. Western blots of the IEC-6 cell fractions showed that p42 and p44 were found exclusively in the cytosol at both 5 min and 30 min post stimulation with 2 ng/ml TGF- α (Figure 4a). Doublets are seen in lanes 2 and 3 because of the phosphorylated and unphosphorylated forms of the proteins. Faint bands can be seen in lanes 4 and 6 because the membrane pellet is not washed and there is slight cytosolic contamination.

Next, nuclear and post-nuclear fractions were isolated from cells at different times post stimulation with TGF- α in order to observe nuclear translocation. A representative Western blot of the nuclear fractions is shown in Figure 4(b). The immunoblot was probed with anti-MAPK antibody and shows that both p42 and p44 are present in the nucleus in control cells as well as stimulated cells. However, the phosphorylated band of both isoforms is only seen after stimulation by TGF- α . In addition, the amount of p42 and p44 in the nucleus increases with time of stimulation, reaching a maximum at 90 min. Post-nuclear fractions did contain MAPK as well, and there was no quantifiable difference between times (results not shown).

Immunoprecipitation of p42 and p44

In order to establish that the phosphorylated p42 and p44 were indeed MAPK, immunoprecipitation techniques were used. In these studies, cellular proteins were immunoprecipitated with anti-MAPK antibody and immunoblotted with anti-phosphotyrosine antibody (Figure 5a). A 42 kDa band was visualized showing a high level of phosphorylation at 5 min post stimulation with 2 ng/ml TGF- α . The 44 kDa band was faintly

Figure 4 Localization of p42 and p44

(a) IEC-6 cells were stimulated with 2 ng/ml TGF- α for 5 and 30 min and fractionated by sonication into cytosol (CYT) and membrane (MEM) fractions. Cellular equivalents were electrophoresed and blots were probed with anti-MAPK antibody. Both p42 and p44 were present in the CYT fraction only. Lanes 1 and 4, control; lanes 2 and 5, 5 min stimulation; lanes 3 and 6, 30 min stimulation. (b) Cells were stimulated with TGF- α for 0, 30, 60, 90 and 120 min, and nuclear and post-nuclear fractions were prepared as described in the Materials and methods section. Proteins were resolved by SDS/8% PAGE and immunoblotted with anti-MAPK antibody. Lane 1, 0; lane 2, 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 120 min. Results are representative of three separate experiments.

Figure 5 Immunoprecipitation of IEC-6 cellular proteins after stimulation with 2 ng/ml TGF- α for 5 min

(a) Denatured proteins were immunoprecipitated with anti-MAPK antibody conjugated to agarose beads. After SDS/PAGE, immunoblots were probed with anti-phosphotyrosine antibody. Lane 1, control; lane 2, stimulated. (b) The same blot was stripped and reprobed with anti-MAPK. Lane 1, control; lane 2, stimulated. (c) Proteins were immunoprecipitated with anti-phosphotyrosine antibody and blotted with anti-MAPK. Lane 1, control; lane 2, stimulated. Immunoblots are representative of three similar experiments.

visible in both control and stimulated immunoprecipitates. Control cells always show a slight basal level of tyrosine phosphorylation of these proteins because the cells are never totally quiescent. The phosphorylated form of the 44 kDa MAPK seems less easily immunoprecipitated than the 42 kDa MAPK, possibly due to lower affinity to the antibodies. When blots were stripped and reprobed with anti-MAPK, there was an equal level of protein immunoprecipitated under each condition (Figure 5b). The increase in molecular mass of p42 and p44 after stimulation due to phosphorylation is quite evident (Figure 5b, lane 2). When anti-phosphotyrosine was used as the immunoprecipitating antibody and blots were then probed with anti-MAPK, a dark band was seen in stimulated cells at p42 (Figure 5c, lane 2). A very faint band was seen in both lanes corresponding to p44. As mentioned above, phosphorylated p44 seems to have a lower affinity to the antibody.

Effect of inhibitors on TGF- α stimulation of tyrosine phosphorylation

Two tyrosine kinase inhibitors, genistein and tyrphostin 23, were used to examine the effects of blocking the kinases involved in

Figure 6 The effect of inhibitors on tyrosine phosphorylation of cellular proteins after 2 ng/ml TGF- α stimulation for 5 min

Immunoblots were probed with anti-phosphotyrosine antibody, then stripped and reprobed with anti-MAPK (photo below each blot). (a) Cells were preincubated for 1 h with 50 μ g/ml genistein, then stimulated with TGF- α . (b) Cells were preincubated for 24 h with 100 μ M tyrphostin 23, then stimulated with TGF- α . For all figures: lane 1, control (cells preincubated with appropriate inhibitor but not stimulated); lane 2, cells not treated with inhibitor, then stimulated; lane 3, cells preincubated with appropriate inhibitor, then stimulated with TGF- α . Results are representative of three similar experiments.

this pathway. Genistein is a non-specific tyrosine kinase inhibitor [33] and tyrphostin 23 is a specific inhibitor for the EGF receptor tyrosine kinase [33–35]. Controls for the inhibition studies were incubated with the inhibitor and then were not stimulated. There was no difference between these controls and cells untreated with inhibitors and not stimulated (results not shown). Pre-incubating cells with genistein at 50 μ g/ml for 1 h caused a slight decrease in the TGF- α -induced tyrosine phosphorylation of most proteins and a significant decrease in the phosphorylation of p42 and p44 (Figure 6a). Genistein did not completely inhibit phosphorylation at 50 μ g/ml; however, higher concentrations were toxic to the cells. When 100 μ M tyrphostin 23 was added to the cells for 24 h before TGF- α stimulation, tyrosine phosphorylation of all proteins was completely inhibited (Figure 6b). Tyrphostin 23 requires at least 24 h incubation with the cells to inhibit the tyrosine kinase properties of the EGF receptor [35]. Stripping the blots and reprobing with anti-MAPK antibody showed that the 42 and 44 kDa isoforms were present in all lanes.

DISCUSSION

In the present study, we report, for the first time, that TGF- α increased the levels of phosphorylation on tyrosine residues of proteins in IEC-6 cells with approximate molecular masses of 42, 44, 52, 80, 150 and 175 kDa. Others have found proteins with similar molecular masses that are tyrosine phosphorylated in response to different growth factor stimulation; e.g. MAPK

(both 42 and 54 kDa isoforms), phospholipase C γ (150 kDa), and phosphatidylinositol-3-kinase (85 kDa) [36]. In addition, SH2-containing protein (46, 52 and 66 kDa isoforms), was found associated with the autophosphorylated EGF receptor after EGF stimulation of mammalian cells [37]. All of these proteins are important intracellular signalling proteins which play a role in the signal transduction pathway of other growth factors and therefore may also be critical to the TGF- α signalling path in IEC-6 cells.

Of special interest in this study was the intense tyrosine phosphorylation of p42 and p44. Both p42 and p44 were phosphorylated on tyrosine within 5 min of TGF- α stimulation (Figure 2a) and the phosphorylation was evident at 0.05 ng/ml TGF- α (Figure 3a). In a similar study, p42 and p44 were tyrosine phosphorylated when neutrophils were stimulated with GM-CSF, a cytokine which activates neutrophils, and were identical with two isoforms of MAPK [22]. Others have shown that growth hormone (GH) stimulates the tyrosine phosphorylation and activity of MAPK-related 42 and 45 kDa proteins in 3T3-F442A cells, a cell line which is induced to differentiate in response to GH [38]. We provide new evidence that MAPK may be involved in the signalling pathway of TGF- α as well.

Activated MAPK is translocated to the nucleus following stimulation of cells with growth factors [31,39,40]. The results in Figure 4 confirm that TGF- α also causes the translocation of tyrosine-phosphorylated MAPK to the nucleus. Of special interest is the presence of unphosphorylated MAPK in the nucleus of IEC-6 cells before stimulation by TGF- α . The presence of a phosphatase in the nucleus which dephosphorylates MAPK has not been determined, but would seem likely in view of these results.

The finding that the general tyrosine kinase inhibitor, genistein, decreased TGF- α -stimulated tyrosine phosphorylation levels of p42 and p44 was expected (Figure 6a). Gomez-Cambronero et al. found similar results with genistein and GM-CSF [22]. A more significant finding is that tyrphostin 23, a specific inhibitor of the EGF receptor tyrosine kinase, completely inhibited the TGF- α -induced tyrosine phosphorylation of p42 and p44 (Figure 6b). Tyrphostins inhibit EGF receptor tyrosine kinase activity, but not EGF binding or EGF receptor number [31]. There are no studies on the effect of tyrphostins on TGF- α binding, but it seems likely binding is not affected because a short incubation of the cells with tyrphostin 23 does not alter tyrosine phosphorylation of proteins in response to TGF- α (results not shown). The fact that tyrphostin 23 inhibits tyrosine phosphorylation of p42 and p44 supports the hypothesis that TGF- α stimulates the EGF receptor tyrosine kinase, known to be present in IEC-6 cells [18], and that the phosphorylation of p42 and p44 is downstream of that event.

We have previously shown a correlation between tyrosine kinases and cell growth of IEC-6 cells in response to gastrin stimulation [3]. The present study suggests a link between a mitogenic growth factor (TGF- α) and subsequent tyrosine phosphorylation of proteins in response to stimulation by that growth factor. We have determined that p42 and p44 are two isoforms of MAPK based on the following results: (1) p42 and p44 immunoreact with the two isoforms of MAPK recognized by the antibody used in this study (Figures 2b and 3b). (2) p42 and p44, as well as MAPK, are not associated with the membrane and are translocated to the nucleus following stimulation (Figure 4). (3) Immunoprecipitation studies of IEC-6 cells stimulated with TGF- α and then immunoprecipitated with anti-MAPK showed an increase in the tyrosine phosphorylation level of two anti-MAPK-reactive proteins (Figure 5). Future studies will be directed towards measuring the enzyme activity of MAPK in

response to TGF- α stimulation and examining cell proliferation in more detail.

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