Signaling pathways leading to transcription and translation cooperatively regulate the transient increase in expression of c-Fos protein

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Summary

The mechanisms by which growth factors trigger signal transduction pathways leading to the regulation of c-Fos expression are of great interest. In this study we investigated the effect of hepatocyte growth factor (HGF/SF) and epidermal growth factor (EGF) on the expression of c-fos and its product, c-Fos, in human epithelial cell line MKN74. The expression level of c-Fos protein in HGF/SF-stimulated cells was 5 to 10-fold higher than that in EGF-stimulated cells, whereas the level of c-fos mRNA induced by HGF/SF was similar to that by EGF.

The hyperphosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), indicative of an increased number of translation initiation complexes, was detected only in HGF/SF-induced MKN74 cells. Activation of phosphatidylinositol-3'-OH kinase (PI3-K) and FKBP-rapamycin-associated protein/ mammalian target of rapamycin (FRAP/mTOR) was observed after the treatment with HGF/SF. Pretreatment with an inhibitor of either one, i.e., LY294002 for PI3-K, or rapamycin for FRAP/mTOR, completely inhibited 4E-BP1 phosphorylation, and decreased the c-Fos synthesis induced by HGF/SF down to the level found in EGF-induced cells.

These results suggest that the phosphorylation of 4E-BP1 is stimulated by HGF/SF in a manner requiring both PI3-K-dependent and FRAP/mTOR-dependent pathways, thereby stimulating c-fos mRNA translation. Regulation of the translation process of c-fos mRNA, in addition to the immediate activation of c-fos transcription, is necessary for the transient increase in the level of c-Fos protein to stimulate cell proliferation.
**Introduction**

c-Fos, a transcription factor, is a product of a member of the immediate-early gene family and heterodimerizes with a transcription factor of the Jun family. The heterodimeric complex, termed activator protein-1 (AP-1), binds to the 12-O-tetradecanoylphorbol 13-acetate (TPA) response element (TRE) of certain genes crucial for cellular proliferation and differentiation and thereby activates transcription of such genes (1,2).

The regulatory mechanism of c-fos expression by extracellular signaling molecules has been studied in great detail at the step of transcription activation. Ligands such as growth factors bind to their specific receptors and activate the Ras-mitogen-activated protein (MAP)³ kinase cascade. MAP kinase phosphorylates ternary complex factors such as p62TCF or Elk-1 (3), which binds together with serum response factor (SRF) to the cis-acting regulatory element of the c-fos gene, termed SRE (serum response element), resulting in the induction of c-fos transcription.

The expression level of c-Fos is also regulated at post-translationally. c-Fos is an intrinsically unstable protein, and its degradation appears to occur via the ubiquitin-mediated proteolysis system (4,5). The phosphorylation state of c-Fos at serine residues, which are modified via the MAP-kinase pathway, is suggested to be involved in the stability of the c-Fos protein (6).

Recently, signaling pathways involved in translation control have become better-understood (7-9). Growth factor-regulated signaling pathways downstream of phosphatidylinositol-3'-OH kinase (PI3-K) and FKBP-rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR) are suggested to be involved in the
regulation of translation initiation. A signaling pathway that regulates the transcription of c-
fos, the Ras-MAP kinase pathway, has been well characterized (10-12). In contrast, it has not been determined whether c-Fos expression is regulated during the translation of c-fos mRNA.

Hepatocyte growth factor (HGF/SF) and epidermal growth factor (EGF) have been shown to stimulate the proliferation of epithelial cells. These factors bind to their intrinsic, membrane-spanning receptors, which are tyrosine kinases; and this binding is followed by the activation of the kinase via autophosphorylation of the receptor for individual factors. The receptor capable of transducing the signal of HGF/SF is the c-met proto-oncogene product (13,14). Two tyrosine residues of its β-chain are phosphorylated upon HGF/SF binding, which allows the receptor to transmit signals via association with p60^{src}, PI3-K, Grb2/SOS complex, and Gab1 (15-20). On the other hand, phosphotyrosine residues of the EGF receptor are associated with signaling molecules such as phospholipase Cγ, Shc, and Gab1 (21-25). Although both receptors for HGF/SF and EGF appear to be able to activate common signaling pathways, such as PI3-K and Ras-MAP kinase pathways (26-31), the set of pathways activated in response to HGF/SF or EGF depends on the individual systems used for experiments.

In our ongoing studies on the effects of HGF/SF and EGF on the growth of several gastric carcinoma cell lines, we have found that the growth of MKN74 cells was significantly stimulated by HGF/SF, but was not changed by EGF. Moreover, the expression level of c-Fos in EGF-induced cells was far less than that in HGF/SF-induced cells, whereas the c-fos mRNA level was similar in cells treated with either factor. In the present study, we analyzed this differential expression of c-Fos in response to HGF/SF and
EGF, and found that the expression level of c-Fos was regulated not only at the transcription step but also at the translation step. Our results strongly suggest that the c-fos translation is regulated signaling pathways leading to the phosphorylation of 4E-BP1, the eukaryotic initiation factor 4E-binding protein 1, which phosphorylation leads to the increase in translation necessary for the transient increase in c-Fos protein expression in response to growth factors.
Experimental procedures

Materials

Recombinant human HGF/SF was provided by the Research Center of Mitsubishi-Tokyo Pharmaceuticals, Inc. (32,33). Lactacystin was prepared as described previously (34,35). EGF (ultrapure) from mouse submaxillary glands was purchased from Toyobo Co., Ltd. (Osaka, Japan); and fetal calf serum (FCS), from JRH Biosciences (Lenexa, KS). Phenylmethylsulfonyl fluoride (PMSF), pepstatin A, p-toluenesulfonyl-L-arginine methyl ester (TAME), and leupeptin came from Sigma (St. Louis). RPMI1640 was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Anti-c-Fos antiserum (Ab-2) was purchased from Oncogene Science, Inc. (Cambridge, MA), anti-phosphotyrosine antibody (PY20) from Transduction Laboratories (Lexington, KY), anti-4E-BP1 antibody (R-113) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-mTOR antibody from Oncogene research products (Cambridge, MA), and anti-c-Myc antibody from PharMingen (San Diego, CA). Swine horseradish peroxidase (HRP)-linked anti-rabbit Ig antibody was obtained from DAKO (Denmark). Recombinant tumor necrosis factor was purchased from R&D (Minneapolis, MN).

Cell cultures

Human gastric adenocarcinoma cell line MKN74 was kindly provided by Dr. E. Tahara (Hiroshima University School of Medicine). MKN74 cells were cultured to subconfluence in RPMI1640 supplemented with 10 % FCS and used for all of the experiments. FS-4 cells derived from human foreskin fibroblasts were cultured in DMEM supplemented with 10 %
FCS and treated with recombinant tumor necrosis factor as described previously (36,37).

**Cell proliferation assay**

MKN74 cells were seeded at a density of $1 \times 10^4$ cells/well in 96-well plates and cultured for 2 days in RPMI1640 supplemented with 5% FCS. The cells were subsequently treated with HGF/SF or EGF for 2 days, and cell proliferation was quantified by a colorimetric assay using the WST-1 reagent according to the manufacturer’s instruction (Dojindo Laboratories).

**Preparation of cellular lysates and immunoblotting**

Cells were seeded at a density of $1.6 \times 10^5$ cell / 35-mm-diameter dish and cultured for 2 days. The cells were washed with buffer A (20 mM Hepes / NaOH, pH 7.4, 150 mM NaCl) containing a cocktail of protease inhibitors (0.1 mg/ml PMSF, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml TAME). Subsequently, the cells were lysed with Laemmli SDS sample buffer (38). Total cellular lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). The membranes were sequentially incubated first with primary antibody for 2 h and then with HRP-conjugated species-specific Ig for 1 h; samples were subsequently developed with Western Blot Chemiluminescence Reagent (Renaissance; NEN Life Science Products, Inc., Boston, MA) and exposed to autoradiography film (REFLECTION; NEN Life Science Products, Inc.). Tyrosine phosphorylation of HGF/SF receptor and EGF receptor was examined as described previously (29).
Northern blot analysis

Cells were treated with 10 ng/ml HGF/SF or EGF, and total RNA was obtained by use of Isogen (Nippon Gene). Total RNA was separated electrophoretically and transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech Inc.). The blots were hybridized with the full-length human c-fos cDNA supplied by the Japan Cancer Research Resources Bank, which had been labeled with [\(^{32}\)P]dCTP by use of a multiprime labeling system (Amersham Pharmacia Biotech Inc.). The intensity of each band on the autoradiogram was determined by densitometry (AE-9600; Atto Corp.).

Sequencing of c-fos mRNA in MKN74 cells

Polyadenylated (poly (A)+) RNA was isolated with QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc.) according to the manufacture’s instruction. Amplification of c-fos cDNA was performed from first-strand cDNA with primers, 5'-CAGCGAGCAACTGAGA-3' (position 12-27) and 5'-TTGACAATGTCTTGGAA-3' (position 2065-2080). The PCR products were directly sequenced with Dye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 377 DNA Sequencer (Perkin Elmer, Foster City, CA).

To sequence 5'-region of c-fos mRNA, we used a 5'RACE System (Gibco Life Technologies, Inc.) according to the manufacturer’s instruction. Briefly, first-strand cDNA was reverse-transcribed with a specific primer for human c-fos cDNA (lower primer, 5'-AATGAACCCAATAGAT-3'; position 1617-1634). A homopolymeric tail was then added to the 3'-end of the cDNA by using terminal deoxynucleotidyl transferase (TdT) and dCTP, and amplified with a deoxyinosine-containing Abridged Anchor Primer as upper primer.
and a human c-fos cDNA specific primer (lower primer, 5'-GGTAGGTGAAGACGAA-3'; position 1161-1176). The primary PCR product was reamplified with an Abridged Universal Amplification Primer and a human c-fos cDNA specific primer (lower primer, 5'-GGAAGCCAGGTCACTAC-3'; position 762-777), and sequenced as described above.

For 3'RACE, first-strand cDNA was synthesized with an oligo (dT)-containing adapter primer (5'-GGCCACGCGTCGACTAGTAC TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'), and amplified with a primer for human c-fos cDNA (upper primer, 5'-GGTCACTGCCATCTCG-3'; position 344-359) and with the adapter (5'-GGCCACGCGTCGACTAGTAC-3') as lower primer. The primary PCR products were reamplified with a primer for human c-fos cDNA (upper primer, 5'-GCTGGAGGCCCTGTGC-3'; position 1112-1127) and with the adapter as lower primer, and sequenced as described above.

**Assay for PI3-kinase activity**

Activity of PI3-K was examined according to the method of Tanimura et al.(28). Briefly, MKN74 cells were stimulated with either HGF/SF or EGF for 3 min and 5 min. Immunoprecipitates prepared from cell lysates with anti-phosphotyrosine antibody (PY20) were incubated in an assay mixture containing 40 mM Tris/HCl, pH 7.4, 0.5 mM EGTA, 5 mM MgCl₂, 0.2 mM phosphatidylinositol 4, 5-diphosphate (PI4,5P₂, Sigma), 0.2 mM phosphatidylserine (Avanti Polar-Lipids, Inc.), 50 μM ATP, 25 μCi of [γ³²P] ATP (3000 Ci/mmol; NEN Life Science Products, Inc.), and resolved by thin layer chromatography (TLC) in CHCl₃/methanol/acetone/acetic acid/H₂O (7:5:2:2:2). Authentic phosphatidylinositol 3, 4, 5-triphosphate (PI3,4,5P₃) was purchased from Matreya Inc.,
Aliquots of each extract were analyzed by immunoblotting probed with mouse monoclonal anti-PI3-Kinase antibody (clone AB6; MBL, Nagoya, Japan) which recognizes p85 subunit of PI3-Kinase to determine the amount of the subunit.

**FRAP/mTOR kinase activity**

MKN74 cells were pretreated with or without rapamycin for 90 min, stimulated with either HGF/SF or EGF for 3 min, and washed with buffer A containing a cocktail of protease inhibitors. Subsequently, the cells were homogenized in lysis buffer B (20 mM Tris/HCl, pH 7.4, 100 mM KCl, 20 mM γ-glycerophosphate, 1 mM dithiothreitol (DTT), 0.25 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 10 nM okadaic acid, 1 mM PMSF, and 1 % Nonidet P-40). For immunoprecipitation of mTOR, the cell extracts were incubated with anti-mTOR antibody and protein G-sepharose (Amersham Pharmacia Biotech Inc.) for 2 h at 4 °C. The immune complexes were washed three times with lysis buffer B containing 0.4 M KCl and equilibrated with kinase buffer (40 mM Tris/HCl, pH 7.4, 50 mM NaCl, 20 % glycerol, 0.1 % Triton X-100, 10 mM MgSO₄, and 1 mM DTT); and then a kinase reaction mixture containing 100 µM ATP, 20 µCi of [γ-³²P] ATP (3000 Ci/ mmol; NEN Life Science Products, Inc.), and 2 µg of recombinant glutathione S-transferase (GST)-fused-HA-4E-BP was added to the equilibrated immune complexes. The reaction was carried out at 30 °C with rocking for 15 min and stopped by addition of Laemmli SDS sample buffer (38). Samples were reduced and resolved by SDS-PAGE. The radioactive bands were visualized with a Fujix Bioimaging analyzer BAS 1500.
Results and Discussion

MKN74 cells were treated with HGF/SF or EGF for 2 days, and the mitogenic response was then determined (Fig. 1A). HGF/SF significantly stimulated the cell growth, as we had previously reported (39). In contrast, EGF did not affect the growth of MKN74 cells, even at the concentration of 100 ng/ml. Both growth factors elicit their biological activities through the activation of their intrinsic receptors induced by autophosphorylation in response to these factors. Thus, it is likely that EGF did not induce autophosphorylation of its specific receptor in MKN74 cells. To explore this possibility, we treated cells with EGF or HGF/SF for 5 min, and then immunoprecipitated the receptors in the cell lysates with antibody against EGF- or HGF/SF-receptor. Immunoblot analysis for phosphotyrosine of their receptors showed, in fact, that both receptors were tyrosine phosphorylated in response to their specific ligands (Fig. 1B). This result suggests that the EGF receptor can transmit a signal to intracellular signaling molecules in MKN74 cells.

To address the mechanism by which HGF/SF, but not EGF, increased cell proliferation of MKN74 cells, signaling pathways downstream of the receptors were analyzed in HGF/SF- and EGF-treated cells. We first examined the induction of c-Fos protein by these growth factors. Cells were incubated in the presence of HGF/SF or EGF for 1, 2, or 3 h, and cell lysates were prepared from these cells to determine the c-Fos expression by immunoblotting (Fig. 1C). At the concentration of 10 ng/ml both growth factors induced c-Fos expression, and its level reached maximum 1 h after the start of treatment with either factor and subsequently decreased. However, EGF induced a significantly smaller amount of c-Fos than HGF/SF; i.e., densitometric analysis revealed
that the amount of c-Fos in the HGF/SF-treated cells was approximately 5 to 10-fold higher than that in the EGF-treated ones 1 h after the start of treatment. A higher concentration (100 ng/ml) of EGF did not increase the expression level of c-Fos protein above that obtained with 10 ng/ml of EGF (data not shown).

Next, we compared HGF/SF- and EGF-induced c-fos mRNA levels to examine the upstream response of MKN74 cells. Cells were incubated with 1, 10, or 100 ng/ml of HGF/SF or EGF for 40 min, and total RNA was then analyzed by Northern hybridization. The expression level of c-fos mRNA reached its maximum at 10 ng/ml of HGF/SF or EGF (data not shown). To examine the time course of c-fos mRNA expression, we treated MKN74 cells with 10 ng/ml HGF/SF or EGF for different time periods, and then examined their total RNA (Fig. 2, upper panel). The expression of c-fos mRNA increased in response to not only HGF/SF but also EGF: the mRNA level reached its maximum 40 min after the start of treatment with either growth factor and thereafter rapidly decreased. Densitometric determination showed that the level of c-fos mRNA induced by either factor was similar at all time points (Fig. 2, lower panel). Next, we compared the nucleotide sequences of c-fos mRNA induced by HGF/SF and EGF. c-fos mRNA was isolated from HGF/SF- and EGF-induced cells, and the sequence of c-fos cDNA from the 5' cap to the poly A signal was determined and found to be identical (data not shown). Therefore, a difference in the sequence of c-fos mRNA can not account for the difference in c-Fos expression level between HGF/SF- and EGF-stimulated MKN74 cells. Taken together, the data suggest the c-Fos expression level to be regulated by extracellular stimuli at the post-transcriptional level as well as at that of transcription activation.

The expression level of c-Fos protein in HGF/SF-stimulated cells was higher than
that in EGF-stimulated cells, whereas the level of c-fos mRNA induced by HGF/SF was similar to that by EGF. These results allowed us to postulate two possibilities: HGF/SF, but not EGF, may activate signals leading to an increase in the c-Fos protein level. Alternatively, EGF may activate signals to decrease the expression level of c-Fos. In the latter case, the c-Fos level in HGF/SF-treated cells should be decreased in the presence of EGF. To know if this is the case, we treated MKN74 cells with both HGF/SF and EGF for 1 h, and then prepared total cell lysates to determine the c-Fos expression. As shown in Fig. 3A, there was no difference in c-Fos expression between cells treated with HGF/SF alone or together with EGF. Also, Northern hybridization showed that c-fos mRNA level in the cells treated with a combination of HGF/SF and EGF was equal to that in cells treated with HGF/SF alone (data not shown). Therefore, HGF/SF seems to activate the signaling pathway leading to the regulation of the c-Fos expression level.

C-Fos protein is unstable and subjected to degradation via the ubiquitin-proteasome pathway (4,5). This observation prompted us to investigate whether the degradation process is inhibited by HGF/SF, as this might be the reason that c-Fos expression level in HGF/SF-stimulated cells was higher than that in EGF-stimulated cells. To test this possibility, we determined the effect of a proteasome inhibitor, lactacystin (40), on c-Fos expression in HGF/SF- and EGF-treated cells (Fig. 3B). As is shown in Fig. 3C, the c-Fos level in these cells gradually decreased with time in the absence of lactacystin. On the other hand, this decrease in c-Fos level was not observed in lactacystin-treated cells. Even when the proteasome degradation system was inhibited by lactacystin, the c-Fos level induced by EGF was still low compared with that by HGF/SF. The fact that a proteasome inhibitor did not increase the expression level of c-Fos in EGF-induced cells to the level in
HGF/SF-treated cells strongly argues that the higher-level expression of c-Fos protein in HGF/SF-induced cells is not due to the prevention of c-Fos degradation via proteasome action, suggesting that HGF/SF may regulate the c-Fos expression at the level of translation of c-fos mRNA rather than at the post-translation step.

Next, we examined whether signal transduction pathways activated by HGF/SF might control the process of c-fos translation. Recently, pathways that transmit signals from extracellular stimuli to translation initiation machinery have been identified (7-9), such as phosphatidylinositol-3'-OH kinase (PI3-K) -dependent and FKBP-rapamycin-associated protein/ mammalian target of rapamycin (FRAP/mTOR) -dependent pathways (41-44). We tested whether PI3-K and FRAP/mTOR were activated in HGF/SF-treated cells. First, we performed an in vitro kinase assay of FRAP/mTOR isolated from cell lysates of HGF/SF- or EGF-treated cells. Since eIF4E-binding protein 1 (4E-BP1) is known to be phosphorylated by FRAP/mTOR in vitro, recombinant 4E-BP1 was used as a substrate for FRAP/mTOR. Incorporation of $^{32}$P was increased after 3 min of the stimulation, and the band after the stimulation with HGF/SF was stronger than that obtained with EGF (Fig. 4A). Pretreatment with rapamycin significantly decreased the incorporation of $^{32}$P into 4E-BP1(Fig. 4B), suggesting that FRAP/mTOR was activated and could phosphorylate 4E-BP1 in HGF/SF-treated MKN-74 cells.

Next, we determined the PI3-K activity in MKN74 cells. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and then used for an in vitro kinase assay of PI3-K, since it is known that the kinase is activated via phosphorylation of tyrosine residues in its regulatory subunit. As shown in Fig. 4C, when the cells were stimulated with HGF/SF for 3 or 5 min, increased incorporation of $^{32}$P into the PI3,4,5P$_3$
was observed. In contrast, no PI3-K activity was detectable in EGF-stimulated cells. Immunoblot analysis revealed that the regulatory subunit of PI3-K was not detected in phosphotyrosine immunoprecipitates of EGF-treated cells (Fig. 4D), also suggesting that PI3-K was not phosphorylated by EGF.

Translation initiation, which is regulated by a set of eukaryotic initiation factors (eIF’s), is the rate-limiting step and a major target of translational control. Among initiation factors, eIF4E directly binds to the 5' cap structure of mRNA and is obligatory for the start of cap-dependent translation initiation (45,46). 4E-BP1 is able to prevent binding of eIF4E to the 5' cap structure by binding with eIF4E (44,47). Binding of 4E-BP1 to eIF4E is reversible and dependent on the phosphorylation status of 4E-BP1; i.e., hypophosphorylated 4E-BP1 strongly interacts with eIF4E, whereas hyperphosphorylated 4E-BP1 does not. The phosphorylation of 4E-BP1 induced by extracellular stimuli is thus suggested to trigger the release of 4E-BP1 from eIF4E, resulting in the stimulation of translation initiation (48,49). Therefore, we examined the effect of HGF/SF and EGF on the phosphorylation of 4E-BP1. Cells were treated with HGF/SF or EGF for 20, 40, or 60 min, and thereafter 4E-BP1 in the cell lysates was detected by immunoblotting analysis (Fig. 5A). Among multiple bands of 4E-BP1 detected in each lane, the density of slower migrating bands, which represent hyperphosphorylated forms of 4E-BP1, were increased by HGF/SF, but not by EGF.

Recently, the involvement of PI3-K- and FRAP/mTOR-dependent pathways in 4E-BP1 phosphorylation has been established (41,50-52). FRAP/mTOR can directly phosphorylate 4E-BP1; however, a direct link between the PI3-K pathway and FRAP/mTOR remains to be proven. To examine the involvement of PI3-K and/or
FRAP/mTOR in the phosphorylation of 4E-BP1 induced by HGF/SF, we treated cells with a PI3-K inhibitor, LY294002 or with a FRAP/mTOR inhibitor, rapamycin, prior to the incubation with HGF/SF or EGF, and assayed the phosphorylation of 4E-BP1 (Fig. 5B,C). The HGF/SF-induced phosphorylation of 4E-BP1 was completely inhibited by LY294002 or rapamycin, indicating that this phosphorylation was dependent on the activities of PI3-K and FRAP/mTOR.

We next examined the effect of these two inhibitors on c-Fos expression induced by HGF/SF and EGF. HGF/SF-induced expression of c-Fos was inhibited by either LY294002 or rapamycin, and its level was decreased to that induced by EGF (Fig. 6A,B upper panel). We confirmed that neither inhibitor changed the level of c-fos mRNA induced by HGF/SF or EGF by Northern blotting (Fig. 6A,B lower panel). These results suggest that both PI3-K-dependent and FRAP/mTOR-dependent pathways are involved in the translation of c-fos mRNA by HGF/SF in MKN74 cells.

In the present study, 4E-BP1 was significantly phosphorylated after HGF/SF treatment of MKN74 cells, and pretreatment with a PI3-K inhibitor, LY294002, or a FRAP/mTOR inhibitor, rapamycin, inhibited 4E-BP1 phosphorylation (Fig.5). Moreover, HGF/SF-induced c-Fos expression was decreased by LY294002 or rapamycin down to the level found in EGF-stimulated cells (Fig.6). These results suggest that the HGF/SF signal can induce phosphorylation of 4E-BP1 via the PI3-K- and FRAP/mTOR-dependent pathways and thereby stimulate the translation initiation rate of c-fos mRNA.

We further investigated whether c-fos mRNA was the only one molecule specifically regulated in the translation process by PI3-K- and FRAP/mTOR-dependent pathways in MKN74 cells. Recently, West et al. (53) reported that translation induction of
c-myc was activated by 4E-BP1 phosphorylation. Therefore, we examined the change in expression levels of c-myc mRNA and c-Myc protein after the treatment of MKN74 cells with HGF/SF or EGF (Fig 7). The mRNA level of c-myc was not changed irrespective of the stimulus used. The expression of c-Myc protein, however, was increased after the treatment with HGF/SF and EGF, although the expression level in HGF/SF-stimulated cells was twofold of that in EGF-stimulated cells. In addition, the inhibitor of PI3-K decreased the c-Myc level in HGF/SF-stimulated cells down to the level in EGF-stimulated cells. These results are consistent with the observation we showed in this report as to the regulation of c-Fos induction by HGF/SF.

In the case that the PI3-K- and FRAP/mTOR-dependent pathways are not activated, as in EGF-stimulated MKN74 cells, c-fos mRNA would not be translated effectively, resulting in less accumulation of c-Fos protein, no matter how great an amount of mRNA is transcribed. The present results suggest the notion that the activation of signaling pathways affecting the translation initiation is essential, as well as the activation of transcription, for the induction of c-Fos to an expression level sufficient for stimulating cell growth.

To test this notion, we examined the expression levels of c-fos mRNA and c-Fos protein in another type of human cell line, foreskin fibroblast FS-4 cells. It is known that proliferation of FS-4 cells is stimulated when the quiescent cells cultured under serum-starved condition are treated with a cytokine, e.g., tumor necrosis factor (TNF) (54). TNF increased the level of c-fos mRNA in quiescent FS-4 cells (Fig.8B), as Lin and Vilcek reported (36). In these cells, the expression level of c-Fos was also increased by TNF, and this increase was inhibited by LY294002 and rapamycin (Fig.8A). In contrast, the c-fos mRNA level was not changed by the inhibitors as in the case of HGF/SF-stimulated
MKN74 cells (Fig. 6). 4E-BP1 phosphorylation was significantly increased in TNF-stimulated FS-4 cells, and was inhibited by LY294002 or rapamycin (Fig. 8C). These results indicate that our observation regarding regulation of c-Fos translation in MKN74 cells was particular to neither the cell line or HGF/SF, and could support the notion that the translation initiation of c-Fos as well as the activation of the gene transcription is regulated when cell proliferation is stimulated. From all of the data taken together, we propose the concept that the signaling pathway leading to 4E-BP1 phosphorylation in concert with the Ras-MAP kinase pathway, which activates the transcription of c-fos, is necessary for the functional activation for c-Fos-mediated transcription of target genes.

The effect of growth factors on cell proliferation generally depends on individual cell lines and factors. In the case of MKN74 cells, EGF was not able to activate PI3-K, although many reports have shown that the EGF receptor can transmit signals to activate PI3-K(55,56). Adapter proteins such as Gab1 (25), c-Cbl (55), and a member of the EGF receptor family, ErbB3 (56), are necessary for association between EGF receptor and PI3-K, suggesting a possibility that the activated EGF-receptor may not be able to recruit an adapter protein due to lack of the adapter or some mutation in the EGF receptor in these cells. As is the case for the EGF effect on MKN74 cells, the differential actions of a given factor on the proliferation of cells might reflect the susceptibility of a particular signaling molecule involved in translation stimulation in individual cell lines.
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Footnotes

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3Abbreviations used are the following: HGF/SF, hepatocyte growth factor/scatter factor; EGF, epidermal growth factor; eIF, eukaryotic initiation factor; 4E-BP1, eIF4E-binding protein 1; PI3-K, phosphatidylinositol-3'-OH kinase; FRAP/mTOR, FKBP-rapamycin-associated protein/mammalian target of rapamycin; MAP-K, mitogen-activated protein kinase.

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Figure legends

Figure 1

(A) Proliferation of MKN 74 cells in the presence of HGF/SF or EGF.
MKN74 cells were treated with each concentration of HGF/SF or EGF, as indicated, for 2
days. Cell proliferation of MKN74 treated with HGF/SF (open circle) and EGF (closed
circle) was determined by a colorimetric assay. Each point represents the mean of six
different samples. (B) Tyrosine phosphorylation of HGF/SF receptor and EGF receptor.
MKN74 cells were treated with 10 ng/ml HGF/SF (left panel) or 10 ng/ml EGF (right
panel). Immunoprecipitates of HGF/SF receptor and EGF receptor were probed with anti-
phosphotyrosine antibody. Similar results were obtained from three separate experiments.
Arrows indicate tyrosine-phosphorylated receptors. (C) Time course of c-Fos expression in
HGF/SF- and EGF-treated cells. MKN74 cells were treated with 10 ng/ml HGF/SF or 10
ng/ml EGF for the indicated times. Total cellular lysates were resolved by SDS-PAGE, and
the immunoblot was probed with anti-c-Fos antibody. Molecular size markers are indicated
at the right. Bracket indicates c-Fos protein.

Figure 2

Effect of HGF/SF or EGF on the expression of c-fos.
Upper panel shows results of Northern blot analysis of c-fos mRNA in MKN74 cells. Total
RNA was isolated at the indicated times after the addition of 10 ng/ml HGF/SF or 10 ng/ml
EGF. Lower panel shows results of densitometric analysis of the bands in the upper panel.
Figure 3

(A) c-Fos expression in MKN74 cells treated with a combination of HGF/SF and EGF. MKN74 cells were treated or not with 10 ng/ml HGF/SF or/and 10 ng/ml EGF for 60 min. Total cellular lysates were resolved by 10% SDS-PAGE and subjected to immunoblot analysis for the detection of c-Fos. Molecular size markers are indicated at the left. Arrow indicates c-Fos protein. (B) Effect of lactacystin on HGF/SF- and EGF-induced c-Fos expression. MKN74 cells were pretreated or not with 10 µM lactacystin for 2 hours. The cells were then treated with 10 ng/ml HGF/SF or 10 ng/ml EGF in the presence of lactacystin for the indicated times. Cell extracts were analyzed for c-Fos expression by immunoblotting. Bracket indicates c-Fos protein.

Figure 4

Activation of FRAP/mTOR and PI3-K in MKN74 cells.

(A,B) In vitro kinase assay for FRAP/mTOR. (A) MKN74 cells were stimulated with 10 ng/ml HGF/SF or 10 ng/ml EGF for 3 or 10 min. Immunoprecipitates of FRAP/mTOR from these cell lysates were incubated with GST-HA-4E-BP1 as described in the Experimental procedures. Radioactivity of $^{32}$P incorporated into GST-HA-4E-BP1 was shown by autoradiography. (B) MKN74 cells were preincubated with or without 100 ng/ml rapamycin (RM) for 90 min, and then stimulated with 10 ng/ml HGF/SF or 10 ng/ml EGF for 3 min. The FRAP/mTOR kinase assay was performed as described above.

(C,D) PI3-K activity in HGF/SF-stimulated cells. (C) MKN74 cells were treated with 10 ng/ml HGF/SF (H) or 10 ng/ml EGF (E) for the indicated periods of time. Cell lysates
were incubated with anti-phosphotyrosine (PY) antibody and used for the kinase reaction as described in the *Experimental procedures*. The position of authentic phosphatidylinositol 3, 4, 5-triphosphate (PI3,4,5P₃) is indicated. (D) PI3-K in PY immunoprecipitates and total cell lysate was analyzed by immunoblotting.

**Figure 5**

(A) 4E-BP1 phosphorylation in response to growth factor treatment.
MKN74 cells were treated with 10 ng/ml HGF/SF or EGF for the indicated time periods, and the resulting cell extracts were resolved by 13.5% SDS-PAGE and subjected to immunoblot analysis for the detection of 4E-BP1. Bracket indicates multiple bands of 4E-BP1 representing differently phosphorylated forms of the molecule. (B,C) Inhibition of 4E-BP1 phosphorylation by LY294002 (LY) and rapamycin (RM). MKN74 cells were pretreated for 90 min with 10 µM LY294002 (B) or 100 ng/ml rapamycin (C), and then incubated with 10 ng/ml HGF/SF or 10 ng/ml EGF in the presence of the inhibitor for 60 min. Bracket indicates multiple forms of 4E-BP1. – indicates incubation without inhibitor.

**Figure 6**

Inhibition of c-Fos expression by LY294002 and rapamycin.
MKN74 cells were pretreated with 10 µM LY294002 (A) or 100 ng/ml rapamycin (B) for 90 min, and then incubated with 10 ng/ml HGF/SF or EGF for 60 min for immunoblotting.
analysis of c-Fos (upper panel). Total RNA was isolated after a 40-min stimulation with either of the factors for Northern blotting analysis of c-fos (lower panel). C indicates no stimulation with the factors.

**Figure 7**

Effect of HGF/SF and EGF on the expression of c-Myc in MKN74 cells.

MKN74 cells were pretreated or not with 10 µM LY294002 (LY) for 90 min prior to the stimulation with 10 ng/ml HGF/SF or EGF, and analyzed for c-Myc protein and c-myc mRNA expression by immunoblotting (upper panel) and Northern blotting (lower panel), respectively. C indicates no stimulation with the factors. The results shown are representative of three independent experiments.

**Figure 8**

Inhibition of TNF-induced c-Fos expression by LY294002 and rapamycin in FS-4 cells.

FS-4 cells were pretreated with 10 µM LY294002 (LY) or 100 ng/ml rapamycin (RM) for 90 min and then incubated with 30 ng/ml TNF in the presence of the inhibitor for 60 min. Cell extracts were prepared for immunoblotting analysis of c-Fos (A) and 4E-BP1 (C) and Northern blotting analysis of c-fos (B). The cells were treated with TNF under the same condition as described above except that the cells were incubated with TNF for 40 min.
Fig. 1

A

Absorbance (A450)

growth factor (ng/ml)

0 0.1 0.3 1 3 10 30 100

- HGF/SF
- EGF

B

HGF/SF 0 10 EGF 0 10 (ng/ml)

IP : HGF/SF-R
blot : PY

IP : EGF-R
blot : PY

C

HGF/SF 0 1 2 3

EGF 0 1 2 3 (hr)

94 kDa

67

43
Fig. 3

A

|     | HGF/SF | EGF |
|-----|--------|-----|
|     | -      | -   |
|     | +      | +   |

kDa: 94, 67, 43

B

|     | HGF/SF | EGF |
|-----|--------|-----|
|     | 0      | 0   |
|     | 1      | 1   |
|     | 2      | 2   |
|     | 3      | 3   |

Lactacystin

|     | HGF/SF | EGF |
|-----|--------|-----|
|     | 0      | 0   |
|     | 1      | 1   |
|     | 2      | 2   |
|     | 3      | 3   |

kDa: 94, 67, 43
Fig. 6

A

B

kDa

LY294002

C H E C H E

c-Fos

c-fos mRNA

Rapamycin

C H E C H E

c-Fos

c-fos mRNA
Fig. 7

![Western blot analysis of c-Myc and c-myc mRNA expression with HGF/SF, EGF, and LY294002 treatments.](image)

- **HGF/SF** treatment conditions:
  - Control (C)
  - Treatment (+)

- **EGF** treatment conditions:
  - Control (C)
  - Treatment (+)

- **LY294002** treatment conditions:
  - Control (C)
  - Treatment (+)

- **c-Myc** protein levels:
  - Decreased with HGF/SF and EGF treatments
  - Increased with LY294002 treatment

- **c-myc mRNA** levels:
  - Increased with HGF/SF and EGF treatments
  - Decreased with LY294002 treatment
**Fig. 8**

A: c-Fos

B: c-fos mRNA

C: 4E-BP1
Signaling pathways leading to transcription and translation cooperatively regulate the transient increase in expression of c-Fos protein
Kenji Takeuchi, Sayumi Shibamoto, Kentaro Nagamine, Ichiro Shigemori, Satoshi Omura, Naomi Kitamura and Fumiaki Ito

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