Inhibition of Cell Migration by 24-kDa Fibroblast Growth Factor-2 Is Dependent upon the Estrogen Receptor

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The single-copy gene for fibroblast growth factor-2 (FGF-2) encodes for multiple forms of the protein with molecular masses of 24, 22.5, 22, and 18 kDa. We reported previously that the 24–22-kDa FGF-2 forms inhibit the migration of endothelial and MCF-7 cells by 50% and 70%, respectively. Here we show that this inhibition of migration is mediated by the estrogen receptor (ER). We have found that depletion of the receptor in either cell line abrogates the inhibitory activity of 24-kDa FGF-2 while re-introduction of the ER into deficient cells once again promotes the inhibitory response. To determine whether exposure to 24-kDa FGF-2 resulted in the activation of the estrogen receptor, 3T3 cells were cotransfected with estrogen receptor cDNA and an estrogen regulatory element-luciferase gene reporter construct and treated with 24- and 18-kDa FGF-2. The high molecular weight form stimulated luciferase activity 5-fold while 18-kDa FGF-2 at the same concentration had no effect. Treatment of ER-positive MCF-7 cells transfected with the reporter construct only showed the same results. Inclusion of the pure estrogen antagonist ICI 182,780 blocked the increase in luciferase activity by 24-kDa FGF-2, further indicating that the response was estrogen receptor dependent. Expression of dominant negative FGF receptor 1 inhibited ER activation, indicating that this was the cell surface receptor mediating the effect. Although growth factor-dependent activation of the ER was reported to require mitogen-activated protein kinase-induced phosphorylation at Ser118 in COS and HeLa cells, this mechanism is not involved with the activation by 24-kDa FGF-2. These results suggest that the addition of 55 amino acids to the amino-terminal end of 18-kDa FGF-2 by alternative translation alters FGF-2 function and allows for the activation of a second signaling pathway involving the estrogen receptor.

Multiple forms of FGF-2 are produced from a single-copy gene as a result of alternative translation initiation at an AUG codon or at three in-frame upstream CUG codons. This results in the synthesis of 4 FGF-2 isoforms of 24, 22.5, 22, and 18 kDa (1, 2). The cellular localization and apparent functions of 18 and the three higher molecular weight forms of FGF-2 (hmwFGF-2) differ. The 18-kDa FGF-2 is mostly cytoplasmic and is exported to the cell surface, where it is localized to the basement membrane or extracellular matrix in association with matrix heparins and heparans (3, 4). In contrast, undetectable or extremely low levels of hmwFGF-2 are present in the media of cultured cells, suggesting that hmwFGF-2 is not released from cells. Instead, the majority of the hmwFGF-2 is directly translocated into the nucleus (5, 6). The residues associated with nuclear translocation are RG repeats found at several sites within the amino-terminal region of hmwFGF-2 (7) that is absent from the 18-kDa FGF-2 form. Potential differences in function have also been reported for the 18-kDa form versus the hmwFGF-2 forms. Transfection and expression of 18-kDa FGF-2 in 3T3 cells results in increased growth, motility, and levels of surface β integrins. In contrast, 3T3 cells transfected with the cDNA for hmwFGF-2 show enhanced growth but no increase in migration nor integrin expression (8, 9). Additionally, PC12 cells overexpressing 18-kDa FGF-2 were found to differentiate toward the neuronal phenotype whereas overexpression of the higher molecular weight isoforms resulted in a stabilization of the endocrine phenotype (10). Thus, the family of FGF-2 growth factors demonstrate isoform-specific functions that differ depending on the cell type studied. Further control over the effect of the FGF-2 forms is provided by the extracellular environment which regulates the ratio of their synthesis (11).

In a recently published article (12), we demonstrated that endothelial cells could be stimulated to secrete hmwFGF-2 in a regulated manner to levels capable of affecting cell behavior in an autocrine and paracrine fashion. The effects were 2-fold: stimulation of cell proliferation and inhibition of migration. The increase in proliferation was comparable to that promoted by an equal amount of 18-kDa FGF-2, indicating that the stimulation was independent of the additional amino-terminal peptide. On the other hand, the effect on migration was opposite to that of 18-kDa FGF-2. Although 18-kDa FGF-2 promotes cell motility, the hmwFGF-2 forms inhibited migration of endothelial cells by 50% and mammary carcinoma MCF-7 cells by greater than 70% even in the presence of unrelated mitogens that promote cell migration such as VEGF and IGF-1. Thus, we showed that, in addition to cellular effects promoted by hmwFGF-2 transported into the nucleus, these forms could also dramatically alter cell function from outside the cells.

Among the various intracellular signaling pathways that are employed by peptide growth hormones to transmit signals into the nucleus is one that involves the activation of the ER. Although intuitively contrary to the characterization of these receptors as steroid hormone receptors, it has been shown repeatedly that they can be activated in a ligand independent...
manner. Several peptide growth hormones including EGF and IGF-1 activate the receptor in a variety of cell types and in vivo (13–16). In this study, we demonstrate that the ER mediates the inhibition of migration by 24-kDa FGF-2 in both endothelial cells and MCF-7 cells and that these forms of the growth factor can activate the ER leading to the transactivation of a reporter gene. In addition, we show that the mechanism by which 24-kDa FGF-2 activates the ER is different from that of other peptide growth hormones. This is the first report of an isoform-specific function of a growth factor being linked to an alternate intracellular signaling pathway.

MATERIALS AND METHODS

Synthesis of 24-kDa FGF-2—To generate pure recombinant 24-kDa FGF-2, full-length 24-kDa FGF-2 cDNA (obtained from Dr. M. Stachowiak, State University of New York, Buffalo, NY) was inserted in frame into a pPIC9K yeast expression vector (Invitrogen) between the SacI and AgeI restriction sites of the pPIC9K vector directly downstream of the DNA encoding the X-factor secretion signal region. The His6 mutant of P. pastoris GS 115, the methanol utilization-positive phenotype (Mut+), was transformed by electroporation with the pPIC9K construct vector linearized with SacI. The His+ yeast transformants were grown on plates and picked onto E. coli strain DH5α for further use. Multiple integrate copies were obtained by replating on plates containing 4.0 mg/ml G418 antibiotic. The multicopy transformant was grown in 50 ml of BMGY medium with the sole carbon source until the culture reached an A600 of 2.6 (~16–18 h). The cells were collected by centrifugation at 1500–3000 × g for 5 min at room temperature and the cell pellet resuspended in BMGY medium to induce expression. The cells were grown for 4 days, and 100% methanol was added every 24 h to a final concentration of 2% to maintain protein expression. The cells were grown for 4 days, and 100% methanol was added every 24 h to a final concentration of 2% to maintain protein expression. The cell pellet was harvested at 1500–3000 × g for 5 min at room temperature and the cell pellet resuspended in BMGY medium to induce expression. The cells were grown for 4 days, and 100% methanol was added every 24 h to a final concentration of 2% to maintain protein expression. The cell pellet was harvested at 1500–3000 × g for 5 min at room temperature and the cell pellet resuspended in BMGY medium to induce expression. The cells were grown for 4 days, and 100% methanol was added every 24 h to a final concentration of 2% to maintain protein expression. The cell pellet was harvested at 1500–3000 × g for 5 min at room temperature and the cell pellet resuspended in BMGY medium to induce expression. The cells were grown for 4 days, and 100% methanol was added every 24 h to a final concentration of 2% to maintain protein expression.
RESULTS

Depletion of the ER and Loss of Inhibition—A requirement for the presence of ER in 24-kDa FGF-2-mediated inhibition of migration was tested in MCF-7 and endothelial cells by depleting the receptor content and testing the effect on 24-kDa FGF-2-mediated migration. To reduce the level of the ER in endothelial cells, cultures were maintained in the presence of 500 nM ICI 182,780 which promotes changes in ER conformation which target the receptor for degradation (21). Immunoblot analysis for ER content demonstrates the reduction in the level of both the cytosolic (cyt) and nuclear (nuc) ER of the ICI-treated cells (Fig. 1A). ICI-treatment reduced the amount of the 67-kDa ER by greater than 70%, in the cytoplasm and to undetectable levels in the nucleus as compared with parallel cultures of the same cells cultured in the absence of the antiestrogen. Equivalent loading of cellular material was demonstrated by reprobing the blot with an anti-β actin peptide antibody. The loss of the ER had no significant effect on the base rate of endothelial cell migration (1.0 versus 0.86 ± 0.16, ER-positive versus ER-negative) nor VEGF-induced enhanced migration (1.8 ± 0.2 versus 2.0 ± 0.2, ER-positive versus ER-negative), indicating that basal motility and chemokinetic stimulation through the VEGF receptor were not impeded by negative), indicating that basal motility and chemokinetic transactivation ability was determined by measuring luciferase activity (20).

The migration rate of receptor positive endothelial cells was reduced to 48 ± 5% of control values but declined only 10% to 90 ± 16% with ER-negative cells. The same was true in the presence of VEGF. The migration rate was reduced dramatically by 24-kDa FGF-2 in VEGF-treated ER-positive cells (to 40 ± 12% of ER-positive control cells), while the ER-negative cells showed no such decline. Further evidence for the involvement of the ER in the inhibition of migration in endothelial cells was obtained with short term exposure of the cells to 10 nM ICI 182,780, which results in the inactivation of ER function without the loss of ER antigen (21). Simultaneous exposure of endothelial cells to ICI and 24-kDa FGF-2 resulted in a loss of the inhibitory potential of the 24-kDa FGF-2; motility was inhibited only 25% in cultures treated with ICI versus a 60% decline of migration in its absence (data not shown). Thus, ER function as well as ER protein is required for the inhibition of migration by the 24-kDa FGF-2.

Depletion of the ER had the same effect on 24-kDa FGF-2-mediated inhibition of MCF-7 migration (Fig. 1, C and D). MCF-7 cells were cultured and maintained in SDM, which results in a nonclonal population of cells that no longer exhibit a mitogenic response to β-estradiol (12). This lack of estrogen responsiveness is accompanied by a decrease in ER levels as assessed by immunoblot analysis (Fig. 1C). Cytoplasmic ER was undetectable, while nuclear ER levels were substantially reduced. 24-kDa FGF-2 inhibited the migration of these ER-positive cells by 77 ± 18%, while in ER-negative cells the migration rates actually increased to 1.3 ± 0.22 times those of control, ER-positive cells (Fig. 1D).

Reestablishing the 24-kDa FGF-2 inhibition of migration in ER-negative MCF-7 cells could be achieved by re-introducing the estrogen receptor to its normal levels. MCF-7 cells propagated in SDM (ER-negative; Fig. 2A, lane 2) were placed in serum-free Opti-MEM I for 4 h and then returned to SDM for 24 h. This short term exposure to Opti-MEMI resulted in the appearance of endogenous ER at levels approximating those of the MCF-7 cells retained in normal medium (Fig. 2A, compare lanes 1 and 3). Fig. 2B shows that the migration rate of the cells remaining in SDM (corresponding to lane 2, panel A) is not affected by 24-kDa FGF-2 but replicate cultures stimulated to produce the ER (lane 3, panel A) only migrated 37.1 ± 10.7% as fast as the control cells. Although 24-kDa FGF-2 inhibits the migration of MCF-7 cells, it acts as a stimulus for cell proliferation equally as well as 18-kDa FGF-2 (17). Therefore, the effect of ER-depletion on the growth promoting activities of 24-kDa FGF-2 was examined using MCF-7 cells cultured in SDM or regular medium (Fig. 2C). Cells were incubated with 24-kDa FGF-2 or 18-kDa FGF-2 and the rate of proliferation determined by thymidine incorporation studies. No change in the rate of cell proliferation was observed in either case regardless of the presence or absence of the ER (3.7 ± 0.3 versus 3.3 ±
stimulation. ER-positive or negative MCF-7 cells (6 presented as the mean ± S.D. for 4 h and then placed back into steroid-deficient complete DMEM for 24 h. This treatment resulted in the appearance of ER to levels equal that in control cells grown continuously in normal medium as demonstrated by immunoblot analysis with anti-ERs. Lane 1, control cells maintained in complete medium. Lane 2, cells maintained in steroid-deficient medium. Lane 3, cells shown in lane 2 treated with Opti-MEM I medium. B, relative migration of ER-negative MCF-7 cells and ER-negative MCF-7 cells stimulated to produce ER by treatment with Opti-MEM I. Cells represented by lanes 2 and 3 in panel A were treated with 5 ng/ml 24-kDa FGF-2 and the rates of migration in response to 10 ng/ml IGF-1 measured. The migration rates are presented as a ratio of the number of migrating cells in the presence of 24-kDa FGF-2 plus IGF-1 to those treated with IGF-1 alone (open bar). The data are presented as the mean ± S.D. C, the ER is not necessary for growth stimulation. ER-positive or negative MCF-7 cells (6 × 10^4) were plated in their respective growth medium for 48 h and then incubated in serum-deficient medium supplemented with 5 ng/ml 24-kDa FGF-2 or 18-kDa FGF-2 for 24 h. Two hours prior to the termination of the experiment 100 μCi/ml [3H]thymidine was added to the cultures. At the end of the experiment, the DNA was extracted with 0.3 N NaOH. The results are presented as the increase in the number of H counts/min compared with cultures not treated with growth factor. The data are presented as the mean ± S.D.

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0.2, 18-kDa FGF-2; 3.4 ± 0.3 versus 3.7 ± 0.4, 24-kDa FGF-2). Thus, the involvement of the ER in 24-kDa FGF-2 function is specific to the inhibition of migration and is not relevant to its effect on cell division.

Activation of the ER by 24-kDa FGF-2—To assess the effect of 24-kDa FGF-2 on ER activity, we measured the ability of the growth factor to modulate the transcriptional activity of ER in transient transfection assays. 3T3 cells were cotransfected with a plasmid containing a triple estrogen regulatory element (ERE) proximal to the thymidine kinase promoter driving luciferase reporter gene expression and a plasmid containing the cDNA for the full-length human ER-α (22). Fig. 3A shows the low level of endogenous ER in the 3T3 cells and the increase in ER levels 24 h after transfection of the ER cDNA. Treatment with estradiol for 24 h had no effect on the intracellular content of the recombinant ER (Fig. 3B). Treatment of these cells with 10 nM estradiol increased the luciferase activity in the cell extracts 3.6 ± 0.1-fold. Dose-dependent increases in luciferase activity also were observed with increasing doses of 24-kDa FGF-2 from 5 to 20 ng/ml. As the concentration of the growth factor was raised, there was a corresponding increase in luciferase activity up to 2.5 times control levels. Higher doses (30 ng/ml) had no additional effect. When the cells were transfected with the reporter plasmid only (no ER expression), no increase in luciferase activity was observed, even at 20 ng/ml, demonstrating that the ER was necessary for the stimulation of luciferase gene transcription. In contrast to the higher molecular weight FGF-2, 18-kDa FGF-2 at 20 ng/ml concentration had little effect on luciferase activity, establishing the specificity of ER activation for the higher molecular weight forms of
FGF-2. Mammalian cell-derived recombinant hmwFGF-2 produced in COS cells (17) gave identical results (data not shown). The ability of 24-kDa FGF-2 to activate the ER also was tested in MCF-7 cells using the natural endogenous ER and the luciferase reporter construct. Cells were transfected with the reporter construct only and treated with estradiol or 24-kDa FGF-2. Treatment with 10 nm estradiol induced an increase in luciferase activity to 2.6 ± 0.4 times control (Fig. 3C). Addition of 24-kDa FGF-2 created a dose-dependent increase in luciferase activity to 2.3 ± 0.3 times control at 10 ng/ml. Further evidence for ER-dependent 24-kDa FGF-2 activation of luciferase expression was acquired with the pure anti-estrogen ICI 182,780 (Fig. 3D). This anti-estrogen inhibited the level of luciferase activity achieved with 10 ng/ml 24-kDa FGF-2 in a dose-dependent manner. Thus, consistent results were observed in 3T3 cells expressing an exogenous source of ER and in MCF-7 cells using the endogenous receptor.

18-kDa FGF-2 binds to and activates four different high affinity tyrosine kinase receptors, FGFR1/IIg to FGFR4, although not all are found in a single cell type (23–26). To determine which of the four receptors were present in the MCF-7 cells, aortic endothelial cells, and 3T3 cells employed in these studies, cells extracts were analyzed by cross-linking 125I-18-kDa FGF-2 to the cells and performing immunoprecipitation with antibodies against each of the four receptors (Fig. 4A). FGFR1 was the only receptor detected in each cell type and was the only one present in the 3T3 and endothelial cells. The doublet observed in 3T3 cells represents the two and three I-like domain isoforms of FGFR1 (data not shown). The possibility that FGFR1 mediates the activation of the ER by 24-kDa FGF-2 was tested in 3T3 cells by expressing a dominant negative FGFR1 in which the tyrosine kinase domain had been deleted. Expression of this receptor and the wild type receptor following transient transfection is shown in Fig. 4B (right panel). In cells expressing the mutant FGFR1, no increase in ER activation occurred with 10 ng/ml 24-kDa FGF-2 treatment while the activation by estradiol was not affected. When the experiments were repeated with cells transfected with a plasmid containing wild type FGFR1 cDNA, no difference in ER activation was found when compared with control cells.

Binding of FGF-2 to the extracellular domain of FGFR results in activation of the intrinsic kinase activity causing tyrosine phosphorylation of a number of signaling intermediates including MAPKs (27–33). In COS and HeLa cells, ER activation by EGF depends on the phosphorylation of the ER (34). The intracellular pathway responsible for this phosphorylation event is the MAPK signal transduction cascade and results in the phosphorylation of Ser118 of the ER, which is also the target of a phosphorylation event induced by estradiol (35–37). To determine whether 24-kDa FGF-2 can activate the MAPK signal transduction cascade, 3T3 cells were treated with 18- and 24-kDa FGF-2 and the activation of Erk1 and 2 examined by Western blot analysis with antibodies against the phospho-form of the enzymes (Fig. 5). Both growth factors were added to the cells at 10 ng/ml for increasing amounts of time, and the levels of phospho and total protein analyzed. MAPK phosphorylation was elevated by both 18- and 24-kDa FGF-2 within 10 min and remained elevated for at least 1 h after treatment. The constant total levels of Erk1/2 show that this increase reflected the phosphorylation of the protein and not differences in loading. Addition of antibodies to the 18-kDa COOH-terminal domain or the amino-terminal end of 24-kDa FGF-2 neutralized the effect on MAPK phosphorylation (lower panel). Direct evidence for the involvement of the MAPK pathway in the activation of the ER was sought with the MAPKK inhibitor PD98059. Dose titration analysis of the effect of this inhibitor on the phosphorylation of ERK1/2 in 3T3 cells showed that 1 μM or greater was capable of inhibiting the phosphorylation of MAPK induced by 24-kDa FGF-2 (Fig. 6). However, instead of reducing ER activation in parallel with the loss of MAPK phosphorylation, 1 μM PD98059 treatment increased the level of luciferase activity in response to 10 ng/ml 24-kDa FGF-2 by 1.5
ER activation, phosphorylation of the receptor at Ser118 is 2.0-fold). In addition to the domain specificity of EGF-induced the truncated HEG19 ER and the wild type (HEGO; 4.4 difference was observed between 24-kDa FGF-2 activation of 
[24-kDa FGF-2] also occurred through the AF2 domain (7.4 
[HEG19] was comparable to that observed with estradiol, which 
[activation (\(2.0\)-fold), although not as much as when 24-kDa FGF-2 was present. The same pattern of PD98059-enhanced FGF-2-dependent ER activation was observed with 5 and 10 ng/ml 24-kDa FGF-2 treatment (data not shown).

Specific activation factor domains of the ER have been related to the activation of the receptor by estradiol or peptide growth factors. In COS and HeLa cells, it is the AF-1 domain that mediates the growth factor-induced transactivation while the AF-2 domain is required for estradiol-induced activation (38–40). To determine whether 24-kDa FGF-2 activation of the ER can be localized to a specific domain of the receptor, 3T3 cells were transfected with truncated forms of the ER cDNA containing either the AF1 or AF2 domains plus the DNA binding domain (Fig. 7). In contrast to what has been found with other growth factors, increases of the reporter luciferase activity in response to 24-kDa FGF-2 occurred with the truncated ER containing the AF2 domain (HEG19; 5.4 ± 2.0-fold) but not the AF1 domain (HE15; 0.9 ± 0.1-fold). The increase with HEG19 was comparable to that observed with estradiol, which also occurred through the AF2 domain (7.4 ± 2.5-fold). Little difference was observed between 24-kDa FGF-2 activation of the truncated HEG19 ER and the wild type (HEGO; 4.4 ± 2.0-fold). In addition to the domain specificity of EGF-induced ER activation, phosphorylation of the receptor at Ser withdraw is required in COS1 and HeLa cells (39). Conversion of this serine to alanine eliminated activation of the receptor. To determine whether Ser phosphorylation was involved with 24-kDa FGF-2 activation of the ER, this residue was substituted with alanine by site directed mutagenesis. Comparison of the effect of 24-kDa FGF-2 on cells containing the mutant ER versus the wild type receptor showed a 5.6 ± 0.22-fold increase in luciferase activity in cells containing the mutant ER and 6.0 ± 0.29-fold with the wild type receptor.

**DISCUSSION**

The synthesis of various molecular weight isoforms of FGF depends upon the mechanism of alternative translation initiation (1, 2). This process employs three CUG initiation sites upstream from a traditional AUG start site. This results in three higher molecular weight forms containing an amino-terminal peptide of increasing length coupled to the 18-kDa G-2. The presence of this peptide alters the cellular localization of FGF-2 from the cytoplasm (18 kDa) to the nucleus due to the presence of nuclear localizing RG repeats within the amino-terminal extension (3–6). In addition to differences in localization, the hmwFGF-2 forms also have been found to modify cell behavior differently from the 18-kDa form. This difference was first suggested by experiments in which 18-kDa or hmwFGF-2 forms were overexpressed in 3T3 cells (8, 9). All forms stimulated proliferation, but, whereas the smaller form stimulated migration, the hmwFGF-2 had no effect. Overexpression of the different forms also had distinct effects on PC12 cell phenotype with the higher molecular weight FGF-2-expressing cells displaying neuronal properties and 18-kDa FGF-2-expressing cells being more endocrine-like (10). Thus, not only is localization of the proteins altered by the amino-termi-
nal extension, so is their function. Further control of cell behavior by this mechanism can occur since the different isoforms are expressed in response to changing environmental conditions (41), e.g. untransformed endothelial, epithelial, and fibroblastic cells express primarily the 18-kDa FGF-2 while stress conditions stimulate the synthesis of the CUG forms (11).

The difference in the function of 18-kDa and hmwFGF-2 forms was further demonstrated by studies in which recombinant hmwFGF-2 was added to endothelial cells and MCF-7 cells and their effects on cell migration evaluated (12). Cell migration was inhibited to greater than 50% by hmwFGF-2. This occurred even in the presence of saturating amounts of mitogens that stimulate cell motility (VEGF, IGF-1, 18-kDa FGF-2), suggesting that the effect of hmwFGF-2 can override the signals generated by these other factors. In this study we provide evidence that this effect on migration results from a change in the intracellular signaling pathway that the cell employs to transmit FGF-2-activated signals. With the synthesis of the amino-terminal end, FGF-2 can activate pathways that lead to the transactivation of the ER. An association between inhibition of migration and the activation of the ER is demonstrated by the parallel changes between the two with reduction in ER accompanied by loss of the inhibitory effect. This link was found in two different cell types (endothelial and MCF-7), which had been depleted of their ER by two different methods (maintenance in SDM and ICI 182,780 treatment, respectively). In addition, short term treatment with ICI 182,780 blocked the inhibition of migration indicating that the ER must not only be available but also needs to be functional. The latter premise is consistent with our observations that 24-kDa FGF-2 has the capacity to transactivate the ER as demonstrated by the expression of an estrogen regulatory element (ERE) located in the amino-terminal end. FGF-2 can activate pathways that lead to the transactivation of the ER. An association between inhibition of migration and the activation of the ER is demonstrated by the parallel changes between the two with reduction in ER accompanied by loss of the inhibitory effect. This link was found in two different cell types (endothelial and MCF-7), which had been depleted of their ER by two different methods (maintenance in SDM and ICI 182,780 treatment, respectively). In addition, short term treatment with ICI 182,780 blocked the inhibition of migration indicating that the ER must not only be available but also needs to be functional. The latter premise is consistent with our observations that 24-kDa FGF-2 has the capacity to transactivate the ER as demonstrated by the expression of an estrogen regulatory element controlled gene. Again, this occurred in two different cell types regardless of whether the ER was transfected into a transfected plasmid (3T3 cells) or was the natural product of the endogenous gene (MCF-7 cells).

The activation of the ER by a peptide growth hormone is not unusual; direct evidence for peptide growth factor activation of the ER has been obtained in multiple cell types (13–16) and in ER knockout animals (42). Transforming growth factor-α or EGF treatment of HeLa, Ishikawa (endometrial), and BG-1 (ovarian) cells transiently transfected with ER-responsive target genes results in the dose-dependent activation of the ER in an estrogen-independent manner (38). In primary rat uterine cell cultures (43), GH3 pituitary cells (16), and the neuroblastoma cell line, SK-ER3 (44), ER-dependent gene expression is stimulated by IGF-1. Growth factors also mimic estrogen in their ability to increase the expression of ER target genes, such as the progesterone receptor and the iron-binding glycoprotein, lactoferrin (45). EGF and IGF-1 also promote cell growth through the ER both in vitro and in vivo (38, 43). What distinguishes hmwFGF-2 from these other growth factors is the fact that it affects motility, which has not yet been associated with peptide activation of ER. The mechanism by which EGF activates the ER has been shown to occur through the EGF plasma membrane receptor (38) and activation of specific MAPKs resulting in the phosphorylation of serine 118 of the ER. This occurs, however, in only certain cell types (e.g. COS and HeLa cells). Studies employing vascular smooth muscle cells failed to show involvement of either MAPKs or Ser118 phosphorylation, indicating that multiple pathways are available for ER activation by these peptide hormones (20). In fact, overexpression of the dominant positive MAPKK in endothelial cells inhibited basal ER activity, suggesting that in these cells there is a reverse relationship between MAPKK activity and ER activity.

Our results are more consistent with those reported for the smooth muscle cells than the studies performed with COS or HeLa cells. Although 24-kDa FGF-2 clearly stimulates the phosphorylation of MAPK, inhibition of that phosphorylation with the MAPKK inhibitor PD98059 did not reduce the level of ER activation. In fact, we observed an increase in the basal level of ER activity and an increase in the 24-kDa FGF-2 induced activation of the ER with the addition of the MAPKK inhibitor. Despite the independence from the MAPK pathway, it is apparent that a fully functional FGFR1 receptor must be present for 24-kDa FGF-2 to activate the ER. It is also likely that the tyrosine kinase activity of FGFR1 is responsible for the transduction of the 24-kDa FGF-2 generated signals for ER activation. The diversity of the different transducer proteins associating with FGFR1 and the number of signaling intermediates that are activated may reflect the capacity of the different forms of FGF-2 to exert the complex array of biological responses in the same cell, a characteristic shared by other tyrosine kinase receptor-binding growth factors (46–48).

In addition, the mechanism of ER activation by FGFR1 appears to involve distinct domains of the ER. Previously, the AF-1 domain was shown to be required for peptide growth factor ER activation while the AF-2 domain mediated the estradiol effect. The use of the same truncated forms of the ER transfected into 3T3 cells demonstrated the opposite effect with 24-kDa FGF-2. Thus, activation of the ER by 24-kDa FGF-2 requires the AF-2 and not the AF-1 domain. Consistent with these results is our observation that the elimination of the putative MAPK phosphorylation site Ser118 by conversion to alanine has no effect on the activation of the receptor, a result consistent with the effect of growth factors on the ER in smooth muscle cells but not in COS or HeLa cells.

Alternative translation site usage was first characterized as occurring during viral replication, particularly among the picornaviruses. It is a mechanism that allows for the expansion of gene diversity within a highly restricted genome by changing the localization and the functions of a single gene. Although much less common, alternative translation initiation using noncanonical codons also exists for mammalian proteins other than FGF-2. Among these are several whose function changes with the synthesis of their alternate forms. For example, there are two forms of GATA-1 that differ in their transactivation potential and developmental expression (49), the CAMP-responsive element modulator is converted from a transcriptional activator to a repressor (50), and Egr3 transcription factors differ in their ability to activate transcription (51). As transcription factors, the changes in these three proteins most likely involve how they interact directly with DNA or other DNA-binding proteins. In contrast, changes in 24-kDa FGF-2 function involve a more complex process involving multiple steps between cell surface receptor activation, ER activation, and the inhibition of migration. This represents the novelty of our observations on FGF-2 that, in addition to the functional change that occurs with the additional amino-terminal peptide, there is also an activation of a separate signaling pathway that can be directly linked to the change in function.

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