Fibroblast Variants Nonresponsive to Fibroblast Growth Factor 1 Are Defective in Its Nuclear Translocation*

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Fibroblast growth factors (FGF) elicit biological effects by binding to high affinity cell-surface receptors and activation of receptor tyrosine kinase. We previously reported that two NIH/3T3 derivatives, NR31 and NR33 (NR cells), express high levels of full-length FGF-1 and exhibit a complete spectrum of transformed phenotype. In the present study, we report that NR cells respond to the mitogenic stimulation of truncated FGF-1 but not to the full-length FGF-1. Incubation of the NR cells with either form of FGF-1 resulted in its binding to cell-surface FGF receptors, activation of mitogen-activated protein (MAP) kinase, and induction of c-fos and c-myc. These data demonstrate that the FGF receptor-mediated, MAP kinase-dependent signaling pathway is not defective in the NR cells. Our data further suggest that the activation of MAP kinase in response to full-length FGF-1 is not sufficient for mitogenesis. Subcellular distribution of exogenously added FGF-1 demonstrated that full-length FGF-1 fails to translocate to the nuclei of NR31 cells. Although the full-length FGF-1 was detected in the nuclear fractions of both NIH/3T3 and NR33 cells, its half-life is much shortened in NR33 than in NIH/3T3 cells. These observations suggest that nonresponsiveness of the two NR cell lines may be due to defectiveness at different steps of nuclear translocation mechanism of FGF-1.

Fibroblast growth factors (FGF)1 comprise a family of 14 structurally related polypeptide mitogens. They modulate cell growth, proliferation, and differentiation. FGFs also regulate angiogenesis, embryogenesis, and neurite outgrowth (1, 2). FGFs elicit these functions mainly by binding to high affinity cell-surface receptors and activating a family of receptor tyrosine kinases (3, 4). FGF activation of FGFR1 triggers autophosphorylation on at least seven tyrosine residues (5). One of these sites, Tyr-766 of FGFR1, serves as a binding site for phospholipase C-γ (6). The mutation of this site abrogates association and activation of phospholipase C-γ and phosphatidylinositol hydrolysis but not FGF-1-mediated mitogenesis in L6 myoblast and BaF3 cells or differentiation of PC12 cells (7–9). However, autophosphorylation of Tyr-766 may be required for efficient endocytosis of FGF receptors (10). Autophosphorylation of Tyr-653 and Tyr-654 is required for intrinsic tyrosine kinase activity. The mutation of other autophosphorylation sites of FGFR1 did not affect FGF-1-dependent cell proliferation in L6 myoblasts or neuronal differentiation in PC12 cells. The mutation of these four autophosphorylation sites together or even including Tyr-766 of FGFR1 also did not affect FGF-1-induced phosphorylation of Shc and a Grb2-associated 90-kDa protein or activation of MAP kinase (MAPK/ERK; see Ref. 5). FGFR1 triggers activation of at least two pathways, a Ras-dependent and a phospholipase C-γ-dependent protein kinase C pathway. Both pathways converge on a common signaling molecule Raf-1 and then activate the downstream MAPK signaling cascade and mitogenesis (11).

Several reports have demonstrated that Ras-dependent MAPK signaling path-way is important for FGF-1-mediated biological responses (12–14). Ras-dependent activation of Raf-1 and MAPK is sufficient for FGFR-mediated mitogenesis in BaF3 cell line expressing FGFR1 (11). The use of interfering mutants of MAPK showed that MAPK is necessary for fibroblast proliferation (15). FGF-mediated activation of MAPK is necessary and sufficient for mesoderm induction in Xenopus (12). Activation of MAPK is also shown to be involved in cellular transformation and differentiation (16). Expression of the constitutively activated MAPK kinase (MEK) in NIH/3T3 cells results in transformation of NIH/3T3 cells that closely resemble Ras-transformed NIH/3T3 cells and results in differentiation of PC12 cells (16). However, other reports showed that the activation of MAPK pathway is insufficient to promote FGF-17 or PDGF (18)-mediated differentiation of PC12 cells.

MAPK has been implicated as a critical component of the mitogenic signal transduction cascade and is likely to play a role in cellular pathways that control growth and differentiation (19–21). MAPK belongs to a family of serine/threonine protein kinases that are activated in response to a variety of stimuli and growth factors. MAPK is a potent pleiotropic regulator of biological responses, and it requires dual phosphorylation at tyrosine and threonine residues for full catalytic activity (22, 23). MAPK can be activated by both Ras-dependent and Ras-independent pathways (24). Phosphorylation and activation of MAPK is mediated by MEK. The activation of MEK is regulated by either Raf or MEK kinase (25); activated MEK in turn phosphorylates and activates MAPK (26). Activated MAPK translocates to the nucleus and is shown to activate...
and/or phosphorylate several important signaling elements such as p90 56k, p62 erk, c-jun, and c-myc (20, 27, 28). Thus, MAPK provides a link between the receptor tyrosine kinase cascade and the serine/threonine protein kinase cascade and also between cytoplasmic and nuclear signaling processes.

Three different forms of FGF-1 have been reported (29, 30). The two truncated forms have deletion of the first 14 or 20 amino acids (aa) from the full-length, 154-aa form based on the predicted amino acid sequence of the FGF-1 gene (31). There has not been any reported difference in the biological response between the full-length and truncated FGF-1 proteins (1, 32). Recent studies showed that translocation of FGF-1 to the nucleus is essential for FGF-induced mitogenesis. Deletion of nuclear localization sequence (NLS), from aa 21 to 27, abolished the mitogenic activity of FGF-1 without affecting receptor tyrosine phosphorylation and induction of c-fos expression (33). The synthetic peptide containing NLS of FGF-1 and cell membrane-permeable sequence is able to stimulate DNA synthesis in an FGF-R-independent manner (34). A dual mode of signaling pathway for FGF-1 has thus been proposed (35, 36). FGF-1 in fusion with diphtheria toxin is able to translocate, through diphtheria toxin receptor, into cells lacking functional FGF-1 receptors and induces DNA synthesis without detectable increase in tyrosine phosphorylation. The translocation of the fusion protein into cytosol and subsequently to the nucleus is essential for DNA synthesis. The receptor-mediated transduction pathway is necessary for other cellular processes including cell division and proliferation (35).

We have previously reported the characterization of various transfectants of NIH/3T3 expressing full-length human FGF-1. The expression of high levels of FGF-1 resulted in transformation of NIH/3T3 cells (37). In this report, we demonstrate that the transfectants, NR31 and NR33 (NR cells) which express high levels of full-length FGF-1 (154 aa), respond to the mitogenic stimulation of truncated FGF-1 (deletion of aa 1–14) but fail to respond to full-length FGF-1. Significantly, MAPK can be activated in NR cells with either full-length or truncated FGF-1. The subcellular distribution of exogenously added FGF-1 indicated that the full-length FGF-1 is unable to translocate to the nucleus of NR31 cells. Although the full-length FGF-1 was detected in the nuclear fractions of both NIH/3T3 and NR33 cells, its half-life is much shortened in NR33 than in NIH/3T3 cells. These observations suggest that non-responsiveness of the two NR cell lines may be due to defectiveness at different steps of nuclear translocation mechanism of FGF-1.

MATERIALS AND METHODS

Growth Factors—Full-length recombinant human (full-length rhFGF-1, aa 1–154) and truncated recombinant human FGF-1 (ΔNrhFGF-1, aa 15–154) were cloned in prokaryotic PET28a (+) expression system (Novagen, Madison, WI) and purified by heparin-Sepharose affinity chromatography as described (38). Full-length rhFGF-1 from NR31 (NhFGF-1; aa 1–154) was purified as described (37). PDGF was purchased from Upstate Biotechnology (Lake Placid, NY), native bovine brain FGF-1 from R & D Systems (Minneapolis, MN), and calf serum and heparin from Life Technologies, Inc.

Antibodies and Other Reagents—Monoclonal anti-phosphotyrosine antibody (4G10) was kindly provided by T. Roberts and B. Druker (Dana Farber Cancer Institute, Boston). Monoclonal anti-p42 4ERK/2 antobody, alkaline phosphatase-conjugated anti-mouse, or rabbit IgG and mouse IgG (MBP) were obtained from Upstate Biotechnology. Polyclonal anti-p44 4ERK antibody and monoclonal anti-p53 antibody (Pab 240) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-p53 and bovine FGF-1 antibody was from Promega (Madison, WI). Horseradish peroxidase-conjugated anti-rabbit IgG and the enhanced chemiluminescence (ECL) immunodetection system were from Amersham Corp. Na2125 I was obtained from ICN (Costa Mesa, CA), [γ-32P]ATP, [α-32P]Pi, and [3H]thymidine were from NEN Life Science Products. Prehybridization and hybridization reagents for Northern blot analysis were from 5 Prime to 3 Prime (Boulder, CO).

Cell Culture—The NIH/3T3-derived stable transfectants (NR and Tr cell lines) overexpressing full-length rhFGF-1 have been described (37). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The stable transfectants were maintained in medium supplemented with 400 μg/ml geneticin.

Mitogen Assay—Near-confluent monolayer cells were starved in media containing 0.5% calf serum for 48 h. The DNA synthesis was initiated by addition of 5 ng/ml various mitogens, and cells were incubated for an additional 20 h. Cells were then pulsed with 1 μCi of [3H]thymidine for 6 h. The level of DNA synthesis was determined by measuring the incorporation of [3H]thymidine into trichloroacetic acid-precipitable material as described previously (37).

Stimulation of Cells by Growth Factors and Preparation of Cell Lysates—Near-confluent monolayer NIH/3T3, NR31 and NR33, cells were starved in media containing 0.5% calf serum for 48 h. Cells were washed once with phosphate-buffered saline (PBS) and once with serum-free media containing 0.2% bovine serum albumin (BSA), 10 μg/ml heparin, and 25 mM HEPES (pH 7.4). Stimulation was performed by addition of 30 ng/ml full-length or ΔNrhFGF-1 at 37 °C for 10 min. Control and FGF-stimulated cells were washed three times with ice-cold PBS and harvested by scraping in PBS. The cells were pelleted and suspended in lysis buffer containing 10 mM Tris (pH 7.4), 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM sodium pyrophosphate, 10% glycerol, 0.1% sodium orthovanadate, 50 mM NaF, and protease inhibitors. Lysates were clarified by centrifugation at 14,000 rpm for 20 min at 4 °C to remove nuclei and cell debris.

Northern Blotting Analysis—For analysis of FGFRI mRNA levels, total RNA was isolated from cells grown in the presence of 10% calf serum. For analysis of induction of immediate early genes, near-confluent cells were starved and stimulated with 30 ng/ml full-length or ΔNrhFGF-1 for 0–4 h as described above. Cells were lysed in 4 M guanidinium thiocyanate, and total RNA was isolated using 5.7 M CsCl density gradient essentially as described (31). Samples containing 10 μg of total RNA were electrophoresed on 1% formaldehyde gel and transferred onto nitrocellulose membrane (Schleicher & Schuell). The RNA was immobilized by baking the membrane at 80 °C for 2 h prior to hybridization with cDNA probes for human FGFR1 and mouse c-fos or c-myc. cDNA probe for c-fos gene was used as an internal control. The probes were generated with [α-32P]dCTP (~3000 Ci/mmoll) using redi prime random primer labeling kit as described by the manufacturer (Amersham Corp.). The hybridization was carried out in 50% formamide solution (5 Prime ~ 3 Prime, Inc.) containing 32P-labeled probes (1 × 106 cpm/ml) and 10% dextran sulfate at 42 °C for 20 h. At the end of the hybridization period, the filter was washed twice with 2 × SSC and 0.1% SDS at room temperature and once at 50 °C followed by an additional wash at room temperature for 15 min each and subjected to autoradiography.

Radioiodination and Binding Assay—FGF-1 was radioiodinated with Na125 I using the chloramine-T method as described (39). Labeled protein was purified on a heparin-Sepharose column. FGF-1 bound to heparin-Sepharose column was eluted from the column with 100 mM NaCl in media supplemented with 400 μg/ml geneticin.

The binding assay was performed as follows. Cells were seeded at a density of 4 × 104 cells/well in 24-well plates. Cells were washed twice with cold serum-free Dulbecco’s modified Eagle’s medium 48 h later and incubated in 1 ml of binding buffer (Dulbecco’s modified Eagle’s medium containing 25 mM HEPES (pH 7.3), 0.2% BSA, and 2.0 μg/ml heparin) at 4 °C for 30 min. Subsequently, the cells were incubated in 0.2 ml of binding buffer containing serial dilution of full-length ΔNrhFGF-1 at 4 °C for 3 h. Nonspecific binding was obtained in a parallel assay using the same serial dilution of full-length 125I-rhFGF-1 but in the presence of 100-fold excess of non-radioactive rhFGF-1. At the end of the incubation period, the cells were washed twice with cold binding buffer and once with cold PBS containing 1.0 mM NaCl and then solubilized in 0.25 mM NaOH. Bound radioactivity in duplicate samples was measured in a Beckman gamma counter, and data were subjected to Scatchard analysis (40).

Western Blot Analysis—To detect phosphorylated p42 4ERK and p44 4ERK in gel shift experiments, cell lysates containing 25 μg of protein were subjected to 7.5% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and subjected to immunoblot analysis. The nonspecific sites on the nitrocellulose membrane were blocked in Tris-
buffered saline (TBS; 100 mM Tris (pH 7.5), 0.9% NaCl) containing 3% BSA for 2 h. The blot was then incubated with either anti-p44<sub>ERK1</sub> or anti-p42<sub>ERK2</sub> antibody at a concentration of 1 μg/ml in TBS containing 1% BSA at room temperature for 6 h. Following two washes in TBS at room temperature for 10 min each, the blot was incubated in 1.100 dilution of alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG antibody at room temperature for 2 h. The blot was washed as above, and the immune complexes were detected by color reaction of alkaline phosphatase substrate reagents (Upstate Biotechnology). To detect p38 protein in the cytoplasmic and nuclear fractions, anti-p35 antibody was used as the primary antibody.

RESULTS

NR31 and NR33 Cells Are Non-responsive to Full-length FGF-1—We previously described that four NIH/3T3 derivatives (Tr31-5-1, Tr33-1-2, Tr34-4-1, and Tr34-1-1) stably transfected with full-length human FGF-1 cDNA in a retroviral expression vector are able to form foci, acquire anchorage independence, and can induce tumor formation in nude mice (37). We further showed that these four transfectants, unlike the parental NIH/3T3, produce an FGF-1-like mitogen, which was eluted off of a heparin-Sepharose column at 1.2 m NaCl and can stimulate serum-starved NIH/3T3 to synthesize DNA (37). Among the four transfectants, only Tr31-5-1 and Tr33-1-2 produced high levels of FGF-1 as determined by RNase protection analysis, Western blotting analysis, and [3H]thymidine incorporation assay (37). Initially, we examined the ability of various mitogens to stimulate DNA synthesis in various transfectants and NIH/3T3 cells. All transfectants displayed stronger mitogenic response to calf serum and PDGF than parental NIH/3T3 cells did. Surprisingly, Tr31-5-1 and Tr33-1-2 responded to the FGF-1 purified from Tr31-5-1 (NhFGF-1) in stimulating DNA synthesis only at 1.7 and 7.9%, respectively, the level of NIH/3T3 cells (Fig. 1). Tr31-5-1 and Tr33-1-2 transfectants are hence designated as NR31 and NR33 cells, respectively. The lack of response of NR cells to NhFGF-1 cannot be attributed to the trivial explanation of a defective mitogen as NhFGF-1 is mitogenic for Swiss/3T3, NIH/3T3, Tr34-4-1, and Tr34-1-1, and an FGF-1 antisense transfectant Tr31-11-1 (Fig. 1). The lack of response of NR31 and NR33 was specific to NhFGF-1 as these cells can still be mitogenically stimulated by calf serum and PDGF (Fig. 1). We noted that these cells are also responsive to native bovine FGF-1 (Fig. 1), an FGF-1 with deletion of the first 14 amino acids from the full-length protein (30). We have repeated this experiment at least 10 times with triplicate plates and obtained the same results.

To determine if the lack of response to NhFGF-1 was due to the source (NR31 versus brain), species (human versus bovine), or size of protein (full-length versus truncated), we cloned the full-length (rhFGF-1) and truncated (∆rhFGF-1) human FGF-1 cDNA in pET20b (+) expression vector. The recombinant FGF-1 proteins were purified using heparin-Sepharose affinity column.

Western blot analysis of the purified full-length rhFGF-1 and ∆rhFGF-1 proteins showed a single cross-reactive band of expected size (17.3 and 15.8 kDa, respectively) (data not shown). We then tested different forms of FGF-1 for their ability to stimulate DNA synthesis in NIH/3T3 and NR cells. Consistent with the above observations (Fig. 1) full-length rhFGF-1, similar to NhFGF-1, stimulated DNA synthesis in NIH/3T3 cells but failed to stimulate DNA synthesis in NR
cells (Fig. 2; rhFGF-1, NhFGF-1). Higher concentrations (up to 20 ng/ml) of full-length rhFGF-1 also failed to stimulate DNA synthesis in NR cells (data not shown). A slight reduction of mitogenic response to full-length rhFGF-1 and 

\[ \text{D} \text{rhFGF-1} \]

in parental NIH/3T3 when compared with that of bovine FGF-1 was also observed. In addition, there was some loss of mitogenic response to bovine and \n
\[ \text{D} \text{rhFGF-1} \]

in NR cells compared with NIH/3T3 cells. Significantly, the response of NR cells to full-length rhFGF-1 was completely abolished. In contrast, \n
\[ \text{D} \text{rhFGF-1} \]

could support DNA synthesis in parental NIH/3T3 as well as in NR31 and NR33 cells. Thus, the NR cells responded to the mitogenic stimulation of \n
\[ \text{D} \text{rhFGF-1} \]

but not to the full-length rhFGF-1.

Expression of FGFR1 mRNA in NR Cells—NIH/3T3 cells predominantly expressed FGFR1 as the FGF receptor. We compared mRNA levels of FGFR1 in NR cells with those in parental NIH/3T3 cells using Northern blot analysis. The results demonstrated that the levels of FGFR1 transcripts in NR31 and NR33 cells, after normalization against the levels of cyclophilin mRNA, were approximately 80% compared with NIH/3T3 (data not shown). Thus, the data show that NR cells express normal levels of FGFR1 mRNA.

Ligand Binding Properties of NR Cells—It is possible then that the failure of full-length rhFGF-1 to stimulate DNA synthesis in NR cells could be due to the inability of full-length rhFGF-1 to bind its receptors. We examined binding properties of iodinated full-length rhFGF-1 to NR cells and compared with those of the parental NIH/3T3 cells. Iodinated full-length rhFGF-1 was biologically active as determined by \[^{3}H\]thymidine incorporation in responsive cells. Binding of full-length rhFGF-1 was specific and saturable (Fig. 3). Scatchard analysis of saturable binding studies with full-length rhFGF-1 revealed that both NR31 and NR33 cells bound rhFGF-1 with high affinity and as efficiently as NIH/3T3 cells. The dissociation constant (\(K_d\)) for NIH/3T3 as well as NR cells was in the range of 350–610 pM. The numbers of FGF-1 receptors expressed in NR31 and NR33 were 21,000–23,000 sites per cell and were comparable with 28,000 sites/cell in NIH/3T3 cells (Fig. 3). These results demonstrate that the NR cells retained the binding activity of the parental NIH/3T3 cells toward full-length rhFGF-1 and that the expression of full-length rhFGF-1 in NR cells did not impair the function of receptors or down-modulate the expression of the receptors. Therefore, the difference in the mitogenic potential of NIH/3T3 and NR cells to full-length rhFGF-1 could be due to a difference in downstream signaling.

Phosphorylation of MAPK in NR Cells—It has been reported that externally supplied FGF-1 relays its mitogenic signal to the nucleus via activation of the MAPK/ERK cascade. We therefore, examined the effects of full-length and \n
\[ \text{D} \text{rhFGF-1} \]

on the activation of MAPK. Phosphorylation of two isoforms of MAPK, p44ERK1 and p42ERK2, causes a shift in the electrophoretic mobility of these proteins. In serum-starved NR31 and NR33, a low level (20% of total p42ERK2 protein) of constitutive phosphorylation of p42ERK2 was detected (Fig. 4, B and C, lanes...
The stimulation of cells with either form of rhFGF-1 resulted in increased phosphorylation of both p44ERK1 and p42ERK2 in NIH/3T3 as well as in NR31 and NR33 cells. Furthermore, in all three cell types, the levels of phosphorylation of p44ERK1 in response to full-length and truncated rhFGF-1 were similar (Fig. 4, A and B). The quantitative analysis of the immunoblot showed that both full-length rhFGF-1 and ΔrhFGF-1 caused mobility shift of a significant amount of p44ERK1 and p42ERK2 with p44ERK1 phosphorylated at higher levels than p42ERK2 in all three cell lines (Fig. 4C, lanes 4–9).

We then examined the tyrosine phosphorylation of p44ERK1 and p42ERK2 following the stimulation of cells with different forms of rhFGF-1. Cell lysates were immunoprecipitated with either anti-p44ERK1 or anti-p42ERK2 antibodies and blotted with 4G10. Fig. 5 shows that p44ERK1 and p42ERK2 were constitutively phosphorylated at tyrosine residues to a low level in serum-starved NR cells. Upon stimulation with either form of rhFGF-1, the levels of tyrosine phosphorylation were significantly increased with the levels slightly higher in NR cells than in NIH/3T3 cells. In response to full-length rhFGF-1, the extent of tyrosine phosphorylation of MAPK in NR cells was approximately 5–7-fold above the basal level and was comparable with that in NIH/3T3 cells. In response to ΔrhFGF-1, the level of MAPK phosphorylation was 3–5-fold higher than basal level in NIH/3T3 and NR33 cells and 7–9-fold above basal level in NR31 cells (Fig. 5D). Thus, the potential of full-length rhFGF-1 to activate MAPK appears to be similar among the three cell lines.

Stimulation of MAPK Activity in NR Cells—We then determined if the phosphorylated MAPK in NR cells are catalytically active by measuring their ability to phosphorylate MBP using in-gel kinase assay. Phosphorylation at both tyrosine and threonine residues is essential for full catalytic activity of MAPK (42). Fig. 6 shows that both full-length rhFGF-1 and ΔrhFGF-1 activated 42- and 44-kDa kinases in NR31, NR33, and NIH/3T3 cells. The size of these activated kinases corresponds to ERK2 and ERK1, respectively. The kinase activity of both forms of ERK was higher in the NR cells than in NIH/3T3 cells regardless of whether the full-length rhFGF-1 or ΔrhFGF-1 was the source of mitogen (Fig. 6, A and D). Also stimulation of p44ERK1 activity was much greater (4–6-fold) than p42ERK2 activity in all three cell types, perhaps due to high basal levels of p42ERK2 activity in serum-starved cells.

Induction of Immediate Early Genes in NR Cells—The immediate early genes c-fos, c-jun, and c-myc have been identified as downstream substrates of MAPK (21). Stimulation of three cell types with either full-length or ΔrhFGF-1 caused a rapid and transient induction of c-fos and c-myc early response genes, with maximal expression of c-fos within 30 min and c-myc within 1 and 2 h (Fig. 7, A and B). Expression of the c-myc gene was much more stable over a 4-h period compared with the expression of c-fos. Low levels of c-myc transcript but not c-fos were detected in serum-starved cells (Fig. 7, A and B, lanes 1–9). Thus, NR31 and NR33 cells also were able to respond to the stimulation by full-length rhFGF-1 with respect to receptor binding, activation of MAPK, and induction of immediate early genes but are unable to complete the mitogenesis.

Full-length rhFGF-1 Fails to Translocate to the Nuclei of NR Cells—It has been shown that the transport of exogenous FGF-1 to the nucleus is essential for DNA synthesis and cell growth (33–36). Since the receptor-mediated signaling of full-length FGF-1 in NR cells appears to be intact, we decided to look...
at the nuclear localization of FGF-1 in the NR cells. Iodinated full-length and ΔrhFGF-1 were added to serum-starved cells, and the subcellular distribution of 125I-FGF-1 was examined using SDS-PAGE. At 4 °C, nearly equal amounts of full-length and ΔrhFGF-1 were detected in the cytosolic fractions of NIH/3T3 and NR cells. As expected, the majority of the rhFGF-1 was found in cytosolic fractions (Fig. 8, lanes 1, 3, and 5) with molecular mass of 17.3 kDa for full-length (Fig. 8A) and 15.8 kDa for ΔrhFGF-1 (Fig. 8B). When cells were incubated with either form of 125I-rhFGF-1 for 3 or 20 h at 37 °C before lysis, significant amounts of full-length as well as ΔrhFGF-1 were found in the nuclear fractions of NIH/3T3 and NR33 cells (>60%). In contrast, very little full-length 125I-rhFGF-1 (3–14%) was detected in the nuclear fractions of NR31 under the same conditions even though a considerable amount of 125I-ΔrhFGF-1 was still detected in the nuclear fractions of NR31 (33–60%). The cytosolic fractions of internalized iodinated FGF-1 are degraded and appear to be equal in all three cell types (Fig. 8, lanes 7, 9, and 11).

When cells were incubated with either form of 125I-rhFGF-1, they were washed after 3 h and then incubated for an additional 20 h at 37 °C in the absence of rhFGF-1 (post-washing condition). 125I-labeled full-length rhFGF-1 were not detected in either NR cells (<1%), although significant amounts (>76%) could still be found in nuclear fractions of NIH/3T3 cells. In contrast, ΔrhFGF-1 could be found in both NIH/3T3 and NR cells although the levels detected in NR cells (6–12%) are significantly less than those detected in NIH/3T3 cells (42%) (Table I). Thus, nuclearly associated full-length rhFGF-1 may be degraded in NR cells during the post-washing conditions. Alternatively, nuclearly localized rhFGF-1 may be exported back to the cytoplasm where it is degraded. As an internal control, we used the nuclear protein p53. Tumor suppressor p53 was chosen because a large fraction of breast cancers are defective in translocating p53 to the nucleus (43). Using Western blotting analysis, we showed that the levels of p53 in the nuclear fractions of the three cell lines are within 10% of one another (Fig. 9). Additionally, p53 was not detected in any of the three cytoplasmic fractions (Fig. 9). The data not only reflected the purity of subcellular fractionation but also demonstrated that the nuclear translocation mechanism for FGF-1 differs from that of p53.

FIG. 6. Activation of MAPK activity by FGF-1 in NR cells. Serum-starved cells were stimulated with FGF-1 as described in Fig. 4. A, cell lysates were resolved on 10% SDS-PAGE containing 0.4 mg/ml MBP. Activity of MAPK was determined by the in situ phosphorylation of MBP in the presence of [γ-32P]ATP. Western blot analysis of p44ERK1 (B) and p42ERK2 (C) in cell lysates to show the amounts of MAPK proteins. D, quantitative measurements of MAPK activity. Densitometric scan of autoradiogram (A) using LKB Laser Scanner. The values obtained from serum-starved NIH/3T3 were designated as one. NIH/3T3, lanes 1, 4, and 7; NR31, lanes 2, 5, and 8; and NR33, lanes 3, 6, and 9.

FIG. 7. Induction of immediate early gene expression in NR cells. Serum-starved NIH/3T3, NR31, and NR33 cells were stimulated with 10% calf serum at 37 °C for 4 h or with 30 ng/ml either full-length rhFGF-1 (lanes 4–15) or ΔrhFGF-1 (lanes 19–30) at 37 °C for times indicated. Total RNAs (10 µg) were analyzed on 1% formaldehyde gel and Northern blot-hybridized with 32P-labeled mouse c-fos (A) and c-myc (B) probes and subjected to autoradiography. Cyclophilin (CP) was used as a hybridization probe for an internal control. NIH/3T3, lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, and 28; NR31, lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, and 29; and NR33, lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30.
TABLE I

Translocation of exogenous FGF-1 to the nucleus

| Conditions       | Full-length rhFGF-1 | ΔrhFGF-1          |
|------------------|---------------------|-------------------|
|                  | NIH/3T3  | NR31   | NR33  | NIH/3T3  | NR31   | NR33  |
| 4 °C, 3 h        | 10.8    | 3.2    | 3.4    | 6.0    | 6.0    | 6.0    |
| 37 °C, 3 h       | 100.0   | 3.2    | 60.8   | 100.0  | 33.3   | 81.8   |
| 37 °C, 3 h; wash;| 76.0    | <1.0   | <1.0   | 42.4   | 6.0    | 12.1   |
| 37 °C, 20 h      | 108.7   | 14.0   | 132.6  | 133.3  | 60.0   | 100.0  |

DISCUSSION

In the present study, we have characterized the activation of receptor-mediated signaling pathway and mitogenesis mediated by full-length and ΔrhFGF-1 in NIH/3T3 and in non-responsive NR cells. We have also examined the nuclear translocation of exogenous rhFGF-1 in NR cells and compared with that in parental NIH/3T3 cells. Our results demonstrate that the "classical" receptor-mediated signaling pathway is not defective in NR cells and that the activation of MAPK is not sufficient for the full-length rhFGF-1-mediated mitogenic signaling cascade in NR cells. Furthermore, our data suggest that the non-responsiveness of NR cells to mitogenic stimulation of the full-length rhFGF-1 may be due to defective steps in the nuclear translocation pathway.

The comparison of mitogenic activities of various growth factors demonstrated that NR31 and NR33, unlike the parental NIH/3T3 cells, could not be activated to initiate DNA synthesis in response to full-length FGF-1 (Figs. 1 and 2). These results suggest that the NR cells may have lost a signaling component(s) responsive to full-length FGF-1, and the first 14 aa of full-length FGF-1 may have an inhibitory role in eliciting mitogenic signaling. These observations prompted us to evaluate the signaling cascade mediated by the full-length and ΔrhFGF-1. We initially speculated that overexpression of FGF-1 in NR cells may down-modulate the expression of surface receptors in NR cells. Northern blot analysis showed that all three cell lines expressed comparable levels of FGFR1 transcripts (data not shown). Scatchard analysis revealed that the...
binding properties of NR cells to full-length rhFGF-1 were indistinguishable from those of NIH/3T3 cells. Furthermore, NIH/3T3 as well as NR cells expressed similar number of receptors per cell (Fig. 3). Thus, cell-surface receptors for FGF-1 in NR cells are not down-modulated.

One of the FGF-1-stimulated mitogenic signaling pathways includes FGFR, Raf, MAPK, leading to transcriptional activation of immediate early genes and DNA synthesis. It has been demonstrated that the activation of MAPK cascade was essential for nerve growth factor- and FGF-induced differentiation of PC12 and for proliferation of NIH/3T3 (16). Ras-dependent activation of Raf and MAPK was sufficient and necessary for transduction of FGFR mitogenic signal in BaF3 cells (11). FGF also activated MAPK signaling cascade in cardiac myocytes (44). On the other hand, activation of Ras/Raf/MAPK pathway mediated by PDGF receptor β was not sufficient for PC12 differentiation (18). Our data demonstrated that NR31 and NR33 achieved full activation of MAPK catalytic activity in response to either form of rhFGF-1 (Figs. 4–6). Thus, the biochemical characterization of essential steps of FGF-1 signaling pathway revealed that the NR and NIH/3T3 cells respond to FGF-1 in a similar manner.

The constitutive activation of MAPK in NR cells (Figs. 4–6) may contribute to the transforming properties of these cells. The constitutive activation of MAPK has been found in cell lines expressing Ras or Raf oncogenes (45, 46). Overexpression of constitutively activated MEK resulted in the transformation of NIH/3T3 cells (16). Thus, it appears that activation of MAPK cascade is a critical step for the FGF-1-mediated induction of various cellular responses. Our data demonstrate that the activation of MAPK cascade is required but not sufficient for full-length rhFGF-1 to initiate DNA synthesis in NR31 and NR33 cells.

In addition to the activation of MAPK pathway, we compared the pattern of tyrosine-phosphorylated proteins in the cytosols of NIH/3T3 and NR cells. In response to either full-length or ΔrhFGF-1, the pattern of tyrosine-phosphorylated proteins among the three cell lines was similar. Thus, FGF-1 induced tyrosine phosphorylation of FGFR1, a 90-kDa protein and MAPK (data not shown). These results suggest that the FGFR-mediated intracellular pathways involving the activation of tyrosine phosphorylation could not explain the lack of mitogenic response of NR cells to full-length rhFGF-1.

A nuclear event activated by growth factors is the induction of immediate early gene transcription. Transcription of c-fos is enhanced upon stimulation with FGF-1, but its activation by FGF-1 is not sufficient for DNA synthesis (33). We showed that the increased expression of c-myc and c-fos follows the stimulation of cells with either form of FGF-1 (Fig. 7, A and B). However, the mitogenic signal elicited by the full-length rhFGF-1 does not lead to DNA synthesis in NR cells. It has been demonstrated that the nuclear localization of externally supplied FGF-1 is essential for mitogenic response (33–35). Diphtheria toxin fused with FGF-1 can translocate through the toxin receptor and induce DNA synthesis without measurable increase in tyrosine phosphorylation (35). Synthetic peptides containing NLS of FGF-1 were able to stimulate DNA synthesis in an FGFR-independent manner (34). From our observations of subcellular distribution of exogenous full-length rhFGF-1 and ΔrhFGF-1 in nuclear and cytosolic fractions of three cell lines, it appears that the full-length 125I-rhFGF-1 fails to translocate to the nucleus of NR31. Although the full-length FGF-1 was found in the nuclear fraction of NR33, it has a much shorter half-life in NR33 than in NIH/3T3, correlating with the inability of NR33 cells to synthesize DNA in response to full-length FGF-1. These findings agree with those reported by others (33–35).

The shorter half-life of the mitogens in the nuclei of NR cells (Fig. 8, A and B, lanes 16 and 18) may also explain the reduced level of mitogenic response to ΔrhFGF-1 and possibly to bovine FGF-1 (Figs. 1 and 2). Moreover, both NR cells under post-washing condition did not respond not only to full-length rhFGF-1 but also to ΔrhFGF-1 in stimulating DNA synthesis, whereas NIH/3T3 cells responded to both forms of rhFGF-1 under the same post-washing condition (Fig. 10). These findings are consistent with the assumption that the continuous presence of FGF-1 in the nucleus (Fig. 8, A and B, lanes 14, 16, and 18) is essential for the mitogenic response. Together, our data suggest that, in addition to the activation of MAPK pathway, translocation of FGF-1 to the nucleus is required for DNA synthesis and mitogenesis in NIH/3T3 and in NR cells.

A number of recent reports suggest that the nuclear targeting of most nuclear proteins is initiated by binding of cytosolic receptor (termed importin) to NLS. Importin mediates docking of the NLS-importin complex at the nuclear pore complex (47, 48), whereas GTPase Ran/TC4 (49, 50) and NTF2 (51, 52) mediate the translocation of NLS-importin complex through the nuclear pore complex. It is possible that NR31 and NR33 cells may have defects in separate steps of the two-step process of receptor-mediated nuclear protein import pathway. In NR31, importin protein may fail to bind to the NLS of FGF-1 and thus cannot transport it to the nucleus. The binding of cytosolic receptor to NLS may have been inhibited by yet another cytosolic factor(s). In NR33, the defect could be in the second step of nuclear import. Thus, the NLS-receptor complex may dock at the nuclear pore complex but fail to enter the nucleus through the nuclear pore. Alternatively, full-length rhFGF-1 imported into the nucleus of NR33 cells may have degraded rapidly or being transported back to the cytoplasm.

In summary, our data demonstrate that the activation of MAPK is insufficient for FGF-1-mediated mitogenesis in NR cells. Our data further suggest that (i) dual signal transduction pathways, including both Ras-dependent signaling pathway and nuclear localization of FGF-1, may be required for the necessary integration of specific and distinct signals to induce mitogenesis, and (ii) the first 14 aa of FGF-1 may play an interfering role in its nuclear translocation. Availability of NR31 and NR33 cell lines should facilitate the characterization of the nature of nuclear signaling elicited by FGF-1 as well as the nuclear translocation mechanism in general.

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REFERENCES

1. Basilico, C., and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115–165
2. Burgess, W. H., and Maciag, T. (1989) Annu. Rev. Biochem. 58, 575–606
3. Jaye, M., Schlessinger, J., and Dionne, C.A. (1992) Biochem. Biophys. Acta 1135, 185–199
4. Johnson, D. E., and Williams, L. T. (1993) Adv. Cancer Res. 60, 1–41
5. Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M., and Schlessinger, J. (1996) Mol. Cell. Biol. 16, 977–989
6. Mohammadi, M., Homagger, A. M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C., Jaye, M., Rubinstein, M., and Schlessinger, J. (1991) Mol. Cell. Biol. 11, 5068–5078
7. Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del Rosario, M., Mirda, D., and Williams, L. T. (1992) Nature 358, 676–681
8. Mohammadi, M., Dionne, C. A., Li, W., Li, N., Spivak, T., Honegger, A. M., Jaye, M., and Schlessinger, J. (1992) Nature 358, 681–684
9. Spivak-Kroizman, T., Mohammadi, M., Hu, P., Jaye, M., Schlessinger, J., and Lax, I. (1994) J. Biol. Chem. 269, 14419–14423
10. Sorokin, A., Mohammadi, M., Huang, J., and Schlessinger, J. (1994) J. Biol. Chem. 269, 17056–17061
11. Huang, J., Mohammadi, M., Rodrigues, G. A., and Schlessinger, J. (1995) J. Biol. Chem. 270, 5065–5072
12. Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W., and Smith, J. C. (1995) Nature 376, 58–62
13. MacNicol, A. M., Muslin, A. J., and Williams, L. T. (1993) Cell 73, 571–583
14. Whitman, M., and Melton, D. A. (1992) Nature 357, 252–254
15. Pagès, G., Lenormand, P., L’Allermand, G., Chambard, J. C., Meloche, S., and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8319–8323
16. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
17. Renaud, P., Dessein, S., Oliver, P., Gimenez-Gallego, G., Van Obberghen, E., Courtot, Y., and Laurent, M. (1996) J. Biol. Chem. 271, 2801–2811
18. Vaillancourt, R. R., Hesseley, L. E., Zamarrapita, J., Storey, B., Valius, M., Kallauskaus, A., and Johnson, G. L. (1995) Mol. Cell. Biol. 15, 3644–3653
19. Thomas G. (1992) Cell 68, 3–6
20. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) Nature 351, 670–674
21. Blenis, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5889–5892
22. Sturgill, T. W., Ray, L. B, Anderson, N. G., and Erickson, A. K. (1991) Methods Enzymol. 196, 508–531
23. Alesi, D. R., Smythe, C., and Keyse, S. M. (1993) Oncogene 8, 2015–2020
24. Burgering, B. M., DeVries-Smits, A. M., Medema, R. H., Van Weeren, P. C., Tertoolen, L. G., and Bos, J. L. (1993) Mol. Cell. Biol. 13, 7248–7256
25. Nishida, E., and Goto, M. (1989) Trends Biochem. Sci. 18, 124–131
26. Gomez, N., and Cohen, P. (1991) Nature 353, 170–173
27. Pelech, S. L. (1993) Curr. Biol. 3, 513–515
28. Seth, A., Alvarez, E., Gupta, S., and Davis, R. J. (1991) J. Biol. Chem. 266, 23521–23524
29. Burgess, W. H., Mehlmian, T., Marshak, D. R., Fraser, R. A., and Maciag, T. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7216–7220
30. Thomas, K. A., Rico-Candelore M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Reddy, J., Fitzpatrick, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6409–6413
31. Wang, W.-P., Lehtoma, K., Varban, M. L., Krishnan, L., and Chiu, I.-M. (1989) Mol. Cell. Biol. 9, 2387–2395
32. Mason, I. J. (1994) Cell 78, 547–552
33. Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeber, D., Jackson, A., Maier, J. A. M., Hla, T., and Maciag, T. (1990) Science 248, 1567–1570
34. Lin, Y.-Z., Yao, S. Y., and Hawiger, J. (1996) J. Biol. Chem. 271, 5305–5308
35. Wiedlocha, A., Falnes, P. O., Madshus, I. H., Sandvig, K., and Olins, S. (1994) Cell 76, 1039–1051
36. Wiedlocha, A., Falnes, P. O., Sapak, A., Munoz, R., Klingenbergen, O., and Olins, S. (1996) Mol. Cell. Biol. 16, 270–280
37. Bunnag, P., Waddell, K. S., Varban, M. L., and Chiu, I.-M. (1991) In Vitro Cell. Dev. Biol. 27, 89–96
38. Patric, K., Botelho, M. J., Ray, S. K., Mehta, V., and Chiu, I.-M. (1997) Growth Factors 14, 39–57
39. Patric, K. M., Kudla, A. J., Obwin, B. B., and Chiu, I.-M. (1995) J. Biol. Chem. 270, 29018–29024
40. Scotcher, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
41. Wang, H.-C., and Erickson, B. L. (1992) Mol. Cell. Biol. 3, 1329–1337
42. Avruch, J., Zhang, X. P., and Kyriakis, J. M. (1994) Trends Biochem. Sci. 19, 279–283
43. Moll, U. M., Riou, G., and Levine, A. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7262–7266
44. Bogoyevitch, M. A., Glenon, P. E., Andersson, M. B., Clerk, A., Lazzu, A., Marshall, C. J., Parker, P. J., and Sughden, P. H. (1994) J. Biol. Chem. 269, 1110–1119
45. Gupta, S. K., Gallego, C., Johnson, G. L., and Heasley, L. E. (1992) J. Biol. Chem. 267, 7987–7990
46. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Reng, S., Fukasawa, K., Wade, G. F., and Anh, N. G. (1994) Science 265, 966–970
47. Gorlich, D., and Mattaj, I. W. (1996) Science 271, 1513–1518
48. Pante, N., and Aebi, U. (1996) Curr. Opin. Cell Biol. 8, 397–406
49. Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993) J. Cell Biol. 123, 1649–1659
50. Moore, M. S., and Blobel, G. (1993) Nature 365, 661–663
51. Moore, M. S. and Blobel, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10212–10216
52. Paschal, B. M., and Gerace, L. (1995) J. Cell Biol. 129, 925–937
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