The Nuclear Trafficking of Extracellular Fibroblast Growth Factor (FGF)-1 Correlates with the Perinuclear Association of the FGF Receptor-1α Isoforms but Not the FGF Receptor-1β Isoforms*

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The alternatively spliced fibroblast growth factor receptor (FGFR)-1 isoforms, FGFR-1α and FGFR-1β, are characterized by the presence of either three or two Ig-like loops in the extracellular domain and are differentially expressed during embryonic development and tumor progression. We have previously shown that in cells irreversibly committed to DNA synthesis by FGF-1, approximately 15% of cell surface FGFR-1 traffics to a perinuclear locale as a structurally intact and functionally active tyrosine kinase (Prudovsky, I., Savion, N., Zhan, X., Friesel, R., Xu, J., Hou, J., McKeehan, W. L., and Maciag, T. (1994) J. Biol. Chem. 269, 31720–31724). In order to identify the structural requirement for association of FGFR-1 with the nucleus, the expression and trafficking of FGFR-1 in FGFR-1α and FGFR-1β L6 myoblast transfectants was studied. Although FGFR-1α was expressed as p145 and p125 forms, FGFR-1β was expressed as p120 and p100 forms in the L6 myoblast transfectants. Tunicamycin and N-glycosidase experiments suggest that these forms of FGFR-1α and FGFR-1β are the result of differential glycosylation. However, only the p145 form of FGFR-1α and the p120 form of FGFR-1β were able to bind FGF-1 and activate tyrosine phosphorylation. Pulse-chase analysis of FGFR-1α biosynthesis suggests that the p125 and p100 proteins are the precursor forms of p145 FGFR-1α and p120 FGFR-1β, respectively. Because ligand-chase analysis demonstrated that FGFR-1β L6 myoblast transfectants exhibited a reduced efficiency of nuclear translocation of exogenous FGF-1 when compared with FGFR-1α transfectants, the intracellular trafficking of the FGFR-1α and FGFR-1β isoforms was studied using an in vitro kinase assay to amplify immunoprecipitated FGFR-1. Indeed, the appearance of the FGFR-1α but not FGFR-1β isoform in the nuclear fraction of L6 myoblast transfectants suggests that the distal Ig-like loop in FGFR-1α mediates the differential nuclear association of FGFR-1α as a structurally intact and functional tyrosine kinase. Further, the FGFR-1β L6 myoblast transfectants but not the FGFR-1α myoblast transfectants exhibited a pronounced morphologic change in response to exogenous FGF-1. Because this phenotype change involves the induction of a rounded cellular shape, it is possible that the FGFR-1α and FGFR-1β may ultimately exhibit differential trafficking to adhesion sites.

Members of the fibroblast growth factor (FGF)1 gene family encode nine proteins that play important roles in embryogenesis, wound repair, angiogenesis, tumor growth, and other biologic and pathologic processes (1–3). The biological activities of the FGFs, including the signal peptide-less prototypes, FGF-1 (acidic) and FGF-2 (basic), are mediated through high affinity cell surface-associated FGF receptors (FGFRs). Presently there are four FGF gene family members encoding proteins that contain a ligand-activated intrinsic tyrosine kinase domain (4). The ability of the FGF prototypes to initiate DNA synthesis requires the continual exposure of the target cell to exogenous FGF during the entire G1 phase of the cell cycle in vitro (5, 6). Throughout this period, receptor-mediated internalization of FGF-1 results in its partition between the nucleus and cytosol (5, 7) including the perinuclear trafficking of FGF-1 (8).

In addition to the exogenous signaling pathway, there also appears to be an endogenous pathway for mediating the intracellular traffic of FGF-1, FGF-2, and FGF-3 (9, 10). Indeed, sequences responsible for nuclear localization have been characterized in FGF-1 (7, 11, 12), FGF-2 (13–15) and FGF-3 (16). Interestingly, a nuclear localization sequence has recently been characterized in FGF-3 as an endogenous protein (17) and the intranuclear trafficking of FGF-1,2 FGF-2 (18), and FGF-3 (17) as endogenous intracellular proteins have been correlated with a decrease in proliferation potential in vitro. In addition, the nuclear trafficking of endogenous FGF-2 (19–22) and FGF-3 (23) but not FGF-1 appears to be further complicated by the presence of alternative 5’ -CUG translational start sites. In contrast, however, the biological significance of the nuclear trafficking properties of exogenous FGF is not clear, although the internalization of exogenous FGFs is receptor-dependent and correlates with an increased proliferative potential in vitro (1–3, 5, 9, 10). Indeed, the forced secretion of the FGF prototypes yields either a prominent transformed phenotype in vitro (24) or exaggerated hyperplasia in vivo (25, 26), and many of the signal peptide-containing FGF gene family members have been characterized as oncogenes (2, 3). It therefore appears that there may be distinct intracellular trafficking pathways for endogenous and exogenous FGFs that lead to different cellular phenotypes, which are dependent upon the ability of the ligand to interact with its receptor.

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1 The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.
2 J. Shi, S. Friedman, and T. Maciag, unpublished observation.
The expression of the FGFR-1 gene is regulated by alternative splicing (27–31), and the two isoforms of FGFR-1 serve as an example. FGFR-1a and FGFR-1b have been characterized as containing three and two Ig-like structures in the amino-terminal ectodomain of their translation products (29–31). The differential expression of the FGFR-1a and FGFR-1b isoforms may be biologically significant because it has recently been shown that malignant astrocytomas express both forms of FGFR-1, whereas normal fetal and adult brain express only FGFR-1a (32). Further, similar FGFR-2a and β isoforms are differentially expressed during amphibian development (33), and it has been demonstrated that the FGFR-1a and FGFR-1b isoforms exhibit different affinities for ligand and heparin (34, 35). We have shown that FGFR-1 is translocated to a perinuclear locale as a structurally intact and functional tyrosine kinase during the G1 transition period in NIH 3T3 cells in vitro (8). Because ligand-induced FGFR-1 trafficking may be a component of the FGFR-1 signaling pathway, we compared the intracellular trafficking of the FGFR-1a and FGFR-1b isoforms using stable L6 myoblast transfectants and report that unlike the perinuclear association of FGFR-1a, FGFR-1b does not traffic to a perinuclear locale.

### EXPERIMENTAL PROCEDURES

#### Cell Culture and Transfection—L6 myoblasts (a gift from Dr. Lewis Williams, University of California-San Francisco) were grown in DMEM supplemented with either 10% (v/v) bovine serum or 10% (v/v) Williams, University of California-San Francisco) were grown in DMEM containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM EDTA, and 0.5% (v/v) fetal bovine serum and were density arrested in DMEM containing 0.5% (v/v) fetal bovine serum for 18 h. Quiescent cells were stimulated with 40 ng/ml of recombinant human FGF-1 expressed and purified as described previously (11) in the presence of heparin (10 μg/ml). L6 myoblasts were transfected with expression constructs encoding FGFR-1a and FGFR-3. The DNA encoding human FGFR-1 with the two amino-terminal Ig-like domains and FGFR-3 was digested with BamHI and inserted into the PMEX-neoplasms under the control of the murine sarcoma virus LTR (36). The DNA encoding the human three Ig-like disulfide loop form of FGFR-1 in a pBluescript SK plasmid was digested with BamHI and inserted into the PMEX-neoplasms. Cell transfection was performed using calcium phosphate according to the recommended protocol from Stratagene.

#### Antibodies—Rabbit antiserum recognizing a carboxy-terminal sequence in FGFR-1 was prepared against a synthetic peptide based on residues 764–776 of the deduced amino acid sequence of Xenopus FGFR-1 (FR-1) as described (37). The FGFR-1a-specific monoclonal antibody M2F12 was raised against the bacterially expressed extracellular domain of the 50-kDa FGFR-1 three Ig-like disulfide loop antigen with a recognition domain on the first Ig loop (residues 50–79) as described (38). Monoclonal anti-vinculin antibodies (Sigma, V-4505) were used for immunofluorescence microscopy.

#### Cell Fractionation—Cells were lysed in 1 ml of 20 mM Tris, pH 7.5, containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate (nuclear preparation buffer), and the cytosolic fraction and nuclear pellet were prepared as described (8). The purity of nuclear preparations was determined by the presence of less than 2% acid phosphatase activity in the nuclear fraction of the total cell lysate activity. Transmission electron microscopy analysis also demonstrated the absence of cytoplasmic membranes and organelles in the nuclear fraction. The nuclei were solubilized in 0.25 ml of nuclear preparation buffer containing 0.4% (w/v) SDS, 1 mM MgCl₂, and 10 units DNase 1 (Promega) for 3 min at 4°C followed by the addition of 0.75 ml of nuclear preparation buffer. The nuclear extract was incubated at 37°C for 5 min and at room temperature for an additional 10 min and centrifuged at 14,000 x g for 10 min. The pellets with the antibodies M2F12, the nuclear extract were solubilized by ultrasonication (10 pulses, 0.5 s each) in 1 ml of nuclear preparation buffer because SDS prevents the binding of M2F12 to FGFR-1a.

Immunoprecipitation and in Vitro Kinase Assay—Aliquots of the nuclear and cytosolic fractions (0.5 ml each) were immunoprecipitated with the appropriate anti-FGFR-1 antibody as described previously (5). The various FGFR-1 antibody complexes were precipitated using Protein A-Sepharose beads and washed, and the in vitro kinase reaction was initiated by the incubation of the precipitates with 10 μCi of [32P]ATP as described previously (8).

Cross-linking of [125I]FGF-1 and Immunoblot Analysis—[125I]FGF-1 was prepared as described previously (8). Transfected L6 cells in 100-mm diameter dishes were incubated for 20 min at 22°C with 4 ml of binding buffer containing 10 ng of [125I]FGF-1/ml in the presence or the absence of 1 μg of unlabelled FGF-1/ml as described (39). Cells were transferred to 37°C for 5 min and then rapidly washed at 37°C in phosphate-buffered saline and disuccinimidyl suberate added to a final concentration of 0.3 mM. Dishes were incubated for an additional 15 min at 37°C and then rapidly washed as described (39). Cell lysates were analyzed by 7.5% (w/v) SDS-PAGE and prepared for autoradiography.

Cell lysates from L6 myoblast FGFR-1 transfectants were resolved by 7.5% (w/v) SDS-PAGE and transferred to a nitrocellulose filter. The filters were processed with either the affinity-purified anti-FGFR-1 carboxy-terminal antibody (FR-1) or a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology) as described previously (5).

Pulse-Chase and Ligand-Chase Analysis—Transfected L6 myoblasts were washed with phosphate-buffered saline and incubated in methionine-free DMEM for 20 min, after which [35S]methionine was added for 30 min followed by the addition of an excess of cold methionine (in DMEM) for the specified times. Cells were lysed and immunoprecipitation performed as described above.

Ligand-chase analysis was used to assess the kinetics of [125I]FGF-1 nuclear trafficking and was performed as described previously (5, 12). Briefly, serum-starved (48 h) L6 myoblasts (data not shown) were washed with phosphate-buffered saline and incubated at 37°C for an additional 15 min and then rapidly washed at 37°C in phosphate-buffered saline and 0.5% (v/v) Triton X-100 (Buffer A). The coverslips were washed three times with phosphate-buffered saline, incubated for 1 h with fluorescein-conjugated goat-anti-mouse Ig, washed three times with phosphate-buffered saline, and mounted in 50% (w/v) glycerol on microscope slides. Microscopy was performed with an Olympus fluorescence microscope.

### RESULTS

The expression of FGFR-1a and FGFR-1b exhibit multiple molecular weight forms, but only the high M₈ forms are able to bind exogenous FGF-1. Stable L6 myoblast FGFR-1a and FGFR-1b transfectants were obtained, and all exhibited a significant increase in DNA synthesis in response to either 10% (v/v) fetal bovine serum and insulin (0.3 μg/ml of transferrin) FGFR-1a and FGFR-1b L6 myoblast transfectants were exposed to 10 ng/ml of cold FGF-1 and 10 units/ml of heparin for various periods of time; the cells were washed with 10 μg/ml of heparin and exposed to [125I]FGF-1 (5 ng/ml) for 2 h after which cytosolic and nuclear fractions were obtained. The intracellular content of [125I]FGF-1 was analyzed by 15% (w/v) SDS-PAGE, and cell's maintained at 4°C served as a negative control (5).

Immunofluorescence Microscopy—Serum-starved and FGF-1-stimulated FGFR-1a and FGFR-1b L6 myoblast transfectants were grown on coverslips, fixed for 3 min in acetone (–80°C), air-dried, incubated for 1 h in phosphate-buffered saline containing 1% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 (Buffer A), and incubated with anti-vinculin monoclonal antibody (Sigma) for 1 h in Buffer A. The coverslips were washed three times with phosphate-buffered saline, incubated for 1 h with fluorescein-conjugated goat-anti-mouse Ig, washed three times with phosphate-buffered saline, and mounted in 50% (w/v) glycerol on microscope slides. Microscopy was performed with an Olympus fluorescence microscope.

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Excess of unlabeled FGF-1 containing 10 band with an apparent (p125) and FGFR-1 between 135 and 140 kDa and a minor band with a

Although attempts to covalently cross-link [125I]FGF-1 to non-

These data suggest that the high Mr Forms of FGFR-1 relates with a competitive ligand-receptor complex between [125I]FGF-1 and p120, we were unable to detect a band that

In contrast, competitive [125I]FGF-1 covalent cross-linking analysis of the FGFR-1

Because the data obtained from competitive

Fig. 1. Immunoblot analysis of FGFR-1α and FGFR-1β expression and covalent cross-linking of [125I]FGF-1 in control and transfected L6 myoblasts. A, Immunoblot analysis. FGFR-1α and FGFR-1β L6 myoblast transfectants and a nontransfected control were grown to confluence and harvested, and cytosolic lysates were prepared as described under “Experimental Procedures.” Lysates were analyzed by 7.5% (w/v) SDS-PAGE, transferred to nitrocellulose membrane, and probed with the FR-1 antibody, which recognizes carboxyl-terminal domain common to the FGFR-1α and FGFR-1β proteins. The positions of bands p145, p125, p120, and p100 are indicated with arrows. B, covalent cross-linking analysis. Control L6 myoblasts and FGFR-1α and FGFR-1β L6 myoblast transfectants were incubated with [125I]FGF-1 in the presence (+) or in the absence (−) of a 100 molar excess of unlabeled FGF-1 containing 10 μg/ml of heparin and ligand-receptor covalent cross-linking analysis performed as described under “Experimental Procedures.” Cell lysates were resolved by 7.5% (w/v) SDS-PAGE analysis, and the resultant autoradiogram is shown.

Quite low in contrast with the FGFR population present on the surface of other cell lines such as the NIH 3T3 cell (5, 39). Although attempts to covalently cross-link [125I]FGF-1 to non-transfected L6 myoblasts were not successful, multiple covalently cross-linked bands were readily visible in both FGFR-1α and FGFR-1β L6 myoblast transfectants (Fig. 1B).

The FGFR-1α L6 myoblast transfectants exhibited a major band with an apparent Mr between 160–165 kDa, which correlates with a competitive ligand-receptor complex between FGF-1 and the p145 form of FGFR-1α. Interestingly, we were not able to detect a cross-linked band that would correlate with a complex between [125I]FGF-1 and the p125 form of FGFR-1α.

In contrast, competitive [125I]FGF-1 covalent cross-linking analysis of the FGFR-1β L6 myoblast transfectants revealed the presence of two bands, a major band with an apparent Mr between 135 and 140 kDa and a minor band with a Mr of approximately 100 kDa (Fig. 1B). Although it is likely that the high Mr band represents a covalent complex between [125I]FGF-1 and p120, we were unable to detect a band that would correlate with a covalent complex between [125I]FGF-1 and p100 (approximate Mr of 120 kDa). Although we do not know the identity of the p100 band, it may represent a degradation product of the high Mr, FGFR-1β[125I]FGF-1 complex. These data suggest that the high Mr forms of FGFR-1α (p145) and FGFR-1β (p120) but not the low Mr forms of FGFR-1α (p125) and FGFR-1β (p100) are able to associate with exogenous FGF-1.

FGF-1 is able to induce the tyrosine phosphorylation of the High Mr Forms of FGFR-1α and FGFR-1β in L6 Myoblast Transfectants—Because the data obtained from competitive

[125I]FGF-1-FGF-1 covalent cross-linking analysis suggested that only the high Mr forms of FGFR-1α and FGFR-1β are able to bind FGF-1, we were curious whether this discriminatory pattern would also be evident in the ability of FGF-1 to induce FGFR-1 autophosphorylation of tyrosine residues. Therefore, FGFR-1α and FGFR-1β L6 myoblasts were treated with exogenous FGF-1, and the cells were subjected to immunoprecipitation with anti-FGFR-1 antisera followed by immunoblot analysis using an anti-phosphotyrosine antibody. As shown in Fig. 2, we were only able to detect the presence of p145 and p120 as phosphotyrosine-containing proteins in the FGFR-1α and FGFR-1β L6 myoblast transfectants, respectively. Thus, our failure to detect p125 and p100 bands as phosphotyrosine-containing proteins in the FGFR-1α and FGFR-1β L6 myoblast transfectants is consistent with the prior observation that only the high Mr forms of FGFR-1α and FGFR-1β are able to associate with exogenous FGF-1.

The Low Mr Forms of FGFR-1α and FGFR-1β Are Precursors of the High Mr Forms of These Receptors—Because the low Mr, form of FGFR-1α (p125) and FGFR-1β (p100) are unable to respond to exogenous FGF-1 and autophosphorylate on tyrosine residues, we examined the possibility that there existed a product-precursor relationship between the high and low Mr, forms of FGFR-1α and FGFR-1β. In order to address this issue, pulse-chase analysis of [35S]methionine-labeled FGFR-1α and FGFR-1β L6 myoblast transfectants was performed. Using FGFR-1 immunoprecipitation of the FGFR-1α L6 myoblast transfectants, we observed that after a 15-min incubation with [35S]methionine, the radiolabel was present only in the p125 of FGFR-1α (Fig. 3). After 1 h, the p145 form of FGFR-1α was detected, and after 165 min, the label was present exclusively in the p145 form of FGFR-1α. The use of this strategy with FGFR-1β-transfected L6 myoblasts revealed a similar relationship between the early appearance of the p100 form of FGFR-1β and a significant increase in the presence of the p120 form of FGFR-1β very late in the chase (Fig. 3). These data suggest that the p125 form is the precursor of the p145 product of FGFR-1α, and similarly,
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The p100 form is the precursor of the p120 product of FGFR-1β.

The High and Low Mr Forms of FGFR-1α and FGFR-1β May Undergo Differential N-Glycosylation in Vitro—Because the high M\(_r\) forms of FGFR-1α and FGFR-1β are derived from their corresponding low M\(_r\) forms and FGFR-1 does contain N-glycosylation sites (41), we questioned whether the high M\(_r\) forms of FGFR-1α (p145) and FGFR-1β (p120) are post-translational modifications of the low M\(_r\) forms of FGFR-1α (p125) and FGFR-1β (p100). Pretreatment of \(\text{[}^{35}\text{S}\text{]}\)methionine-labeled FGFR-1α and FGFR-1β L6 myoblast transfectants with tunicamycin followed by immunoprecipitation with FGFR-1 antibody revealed that FGFR-1α and FGFR-1β are synthesized as proteins with apparent M\(_r\) of 110 and 95 kDa, respectively (Fig. 4). In addition, we did not observe high M\(_r\) forms of FGFR-1α and FGFR-1β under these conditions. Similarly, using an in vitro kinase assay, it was possible to demonstrate that treatment of the FGFR-1α and FGFR-1β immunoprecipitates with N-glycosidase yielded a significant decrease in M\(_r\), in which the p145 and p125 form of FGFR-1α were converted to p110 and the p120 and p100 forms of FGFR-1β were converted to p95 (data not shown). These data suggest that the p145 and p125 forms of FGFR-1α and the p120 and p100 forms of FGFR-1β are synthesized as a result of post-translational N-glycosylation of the p110 and p95 forms of FGFR-1α and FGFR-1β, respectively.

FGFR-1β but Not FGFR-1α-transfected Cells Exhibit Morphological Changes upon the Stimulation with FGF-1—We examined the FGFR-1α and FGFR-1β L6 myoblast transfectants for FGFR-1-dependent morphological changes. As shown in Fig. 5 (A and B), the treatment of the FGFR-1α L6 myoblast transfectants with FGF-1 did not result in a change in monolayer phenotype. In contrast, similar treatment of the FGFR-1β L6 myoblast transfectants exhibited a pronounced morphologic change as manifested by the less flattened monolayer appearance and the presence of well rounded yet anchored cells (Fig. 5, C and D). This phenotype was exhibited in at least 50% of the FGFR-1β L6 myoblast transfectant population. Immunofluorescence analysis using anti-vinculin antibodies revealed a significant decrease in the level of focal adhesion sites in the FGFR-1β (Fig. 5, E and F) but not in the FGFR-1α (data not shown) L6 myoblast transfectants.

FGFR-1β but Not FGFR-1α Exhibits a Reduced Efficiency of Nuclear Translocation of Exogenous FGF-1—It is well described that the exogenous FGF prototypes traffic to a nuclear locale in a receptor-dependent manner during the entire G\(_1\) transition period (5, 6, 12, 42). In an attempt to determine the contribution of the FGFR-1α and FGFR-1β isoforms as potential mediators of this trafficking event, FGFR-1α and FGFR-1β L6 myoblast transfectants were examined for their ability to partition exogenous \(\text{[}^{125}\text{I}\text{]}\)FGF-1 between cytosol and nuclear fractions during the immediate-early (2 h) and mid-to-late (6 h) G\(_1\) phase of the cell cycle. In these experiments, the transfected L6 myoblasts were pretreated at 37 °C with cold FGF-1 for the periods of time described in Fig. 6, washed with heparin, and treated with \(\text{[}^{125}\text{I}\text{]}\)FGF-1 for 2 h, after which the cells were lyzed and cytosol and nuclear fractions prepared. As shown in Fig. 6, FGFR-1α L6 myoblast transfectants exhibited a significant level of nuclear-associated \(\text{[}^{125}\text{I}\text{]}\)FGF-1 after 2 and 6 h of exposure to cold FGF-1. In contrast, FGFR-1β L6 myoblast transfectants exhibited a reduction in the level of \(\text{[}^{125}\text{I}\text{]}\)FGF-1 associated with the nuclear fraction (Fig. 6). These data suggest that the isoforms of FGFR-1β expressed in the FGFR-1β L6 myoblast transfectants are unable to efficiently translocate exogenous FGF-1 from the cell surface to the nucleus during the immediate-early and mid-to-late G\(_1\) phase of the cell cycle. These data also imply that the p145 FGFR-1α isoform may be responsible for the receptor-dependent trafficking of exogenous FGF-1 from the cell surface to the nucleus.

FGFR-1α but Not FGFR-1β Isoforms Are Associated with the...
We have previously reported the nuclear association of FGFR-1α in FGFR-1α-transfected and control NIH3T3 cell and L6 myoblasts (8) and confirmed the presence of the p125 and p145 FGFR-1α isoforms in the nuclear fraction of control L6 myoblasts using both the nonspecific FR-1 antibody and the highly specific monoclonal antibody M2F12, which recognized the first Ig-like loop of FGFR-1α (data not shown). Because the FGFR-1β L6 myoblast transfectants exhibited a reduced efficiency of exogenous FGF-1 nuclear trafficking when compared with FGFR-1α transfectants, we sought to determine whether FGFR-1β exhibited a reduction in the perinuclear trafficking. Thus, the perinuclear association of FGFR-1β was studied in FGFR-1β L6 myoblast transfectants using the nondiscriminatory anti-FGFR-1 antibody FR-1 for immunoprecipitation followed by the amplification in an in vitro kinase reaction and resolution of the phosphorylated proteins by radioautography. As shown in Fig. 7, the p120 and p100 translation products of the FGFR-1β gene were present in the cytosolic fraction and absent in the nuclear fraction. However, the presence of endogenous p125 and p145 isoforms of FGFR-1α was detected both in cytosolic and nuclear fractions. These data suggest that the absence of the FGFR-1β isoforms in the nuclear fraction may be the result of a reduction in the efficiency of the FGFR-1β to associate with the nuclear fraction and argue that the first Ig-like loop may contain the structural information requisite for perinuclear trafficking. We also examined the importance of glycosylation for the nuclear association of FGFR-1α in...
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FIG. 6. Ligand-chase analysis of [125I]FGF-1 intracellular traffic in FGFR-1α and FGFR-1β L6 myoblast transfectants. Serum-starved untransfected L6 myoblast FGFR-1α and FGFR-1β transfectants were stimulated with nonradioabeled FGF-1 (40 ng/ml) and heparin (10 ng/ml) for 2 or 6 h at 37 °C, after which the transfectants were washed with heparin (10 μg/ml) and exposed to [125I]FGF-1 for an additional 2 h at 37 °C (8). The transfectants were harvested, lysed, and fractionated into cytosol (cyt) and nuclear (nuc) fractions, and the fate of [125I]FGF-1 was analyzed by 15% (w/v) SDS-PAGE. The ratio of cytosol to nuclear lysate in the sample load was 1 to 50. Serum-starved transfectants not exposed to nonradiolabeled FGF-1 but exposed to [125I]FGF-1 for 2 h (0 h), and cells exposed to [125I]FGF-1 at 4 °C for 2 h (4°) served as controls for the 2- and 6-h ligand chase.

FIG. 7. The appearance of FGFR-1 tyrosine-kinase activity in the cytosol and nuclear fraction of FGFR-1β L6 cell transfectants. Serum-starved FGFR-1β L6 cell transfectants were stimulated for 10 h with 40 ng/ml FGF-1 in the presence of 10 μg/ml heparin. The cells were lysed and fractionated into cytosolic and nuclear fractions as described under “Experimental Procedures.” In this experiment, the nuclear fraction was solubilized by DNase and SDS treatment as described previously (8), and the cytosol (cyt) and nuclear (nuc) fractions were immunoprecipitated with the FR-1 antibody (i) described in the legend to Fig. 1. The in vitro kinase reaction was initiated as described under “Experimental Procedures,” and the reaction products (5 μl of cytosol and 40 μl of nuclear lysate) were resolved by 7.5% (w/v) SDS-PAGE analysis. The positions of the relevant bands are indicated with arrows.

FIG. 8. FGFR-1 isoform tyrosine kinase activity in FGFR-1α and FGFR-1β L6 myoblast transfectants treated with tunicamycin. Serum-starved FGFR-1α and FGFR-1β L6 myoblast transfectants were treated with FGF-1 (40 ng/ml) and heparin (10 μg/ml) for 10 h in the absence or the presence of tunicamycin (10 μg/ml). Cells were harvested, lysed, and fractionated into cytosol and nuclear fractions as described under “Experimental Procedures.” The nuclear fraction was solubilized by DNase and SDS treatment as described previously (8), and the cytosol (cyt) and nuclear (nuc) fractions were immunoprecipitated with the FR-1 antibody described in the legend to Fig. 1. The in vitro kinase reaction was initiated as described under “Experimental Procedures,” and the reaction products (5 μl of cytosol and 40 μl of nuclear lysate) were resolved by 7.5% (w/v) SDS-PAGE analysis. The positions of the relevant bands are indicated with arrows.

The nuclear trafficking properties of the FGFs are well described and may involve the function of two pathways. The endogenous pathway of nuclear trafficking appears to be limited to the high M, forms of FGF-2 (18–22) and FGF-3 (16, 17, 23), whose expression is regulated by alternative translational control at upstream CUG start sites. FGF-1 is excluded from this pathway because the open-reading frame encoding FGF-1 is flanked by termination codons (42), and the identification and characterization of a cytosol-retention sequence in FGF-1 is consistent with its presence in the cytosol as an endogenous translation product. Although the function of the endogenous pathway of FGF nuclear trafficking appears to be associated with the repression of cell growth, the exogenous pathway of FGF nuclear trafficking is a receptor-dependent process (5, 12–14) and has been associated with the stimulation of cell growth. However, the potential function of FGF trafficking to the nucleus by the exogenous pathway as a repressor of cell proliferation has not been eliminated. Indeed, the requirement for the continual presence of extracellular FGF-1 (5) and FGF-2 (6) during the entire G1 transition period for the initiation of maximal DNA synthesis and the remarkable long-term (months) stability of nuclear-associated FGF-1 following cell proliferation in vivo (43) imply that the exogenous pathway of FGF nuclear trafficking may be both permissive for cell proliferation in the short term yet may ultimately be repressive for cell proliferation in the long term.

Because FGF isoform expression is a result of alternating gene splicing (27–31), it has been difficult to anticipate their individual contribution to the exogenous FGF nuclear trafficking pathway. Indeed, our data suggest that the FGFR-1α and FGFR-1β isoforms do exhibit differential biochemical properties that are both permissive and restrictive of their involvement in the endogenous FGF trafficking pathway. Analysis by pulse-chase, N-glycosylation treatment, and tunicamycin interference argues that the high and low M, FGFR-1α and FGFR-1β isoforms are the products of common precursor forms; the p145 and p125 FGFR-1α isoforms are ultimately derived from a common p110 precursor, whereas the p120 and p100 FGFR-1β isoforms are derived from a p95 precursor. The p125 form of FGFR-1α and the p145 FGFR-1α product are both the result of N-glycosylation, and it appears that the p125 FGFR-1α isoform is the precursor for the mature p145 FGFR-1α product. Likewise, the p120 FGFR-1β and p100 FGFR-1β isoforms are also
the result of N-glycosylation of the common p95 FGFR-1a precursor, and the p100 FGFR-1b isoform is the precursor for the mature p120 FGFR-1b product. Although the mature p145 FGFR-1a isoform appears to be involved in the regulation of FGF-1 nuclear trafficking, the mature p120 FGFR-1b isoform does not. However, both forms of the mature p145 FGFR-1a and p120 FGFR-1b are able to recognize exogenous FGF-1 and are functional as FGF-1-induced tyrosine kinases. Thus it appears that the amino-terminal Ig-like loop is involved in the sorting of FGFR-1a but not FGFR-1b to a perinuclear locale during the G1 phase of the cell cycle. This observation is consistent with the recent report that the p110 FGFR-1 isoform is present in breast epithelial cells in the cytosolic compartment (44). Likewise, the requirement for N-glycosylation within the amino-terminal FGFR-1a Ig loop for perinuclear trafficking is also consistent with this conclusion. However, the presence of a ligand-induced and functional FGFR-1 tyrosine kinase are not sufficient to ensure the function of the FGF-1 nuclear trafficking pathway.

The perinuclear traffic of the p125 FGFR-1a isoform in L6 myoblasts is also interesting because FGF-1 neither binds the p125 FGFR-1a isoform nor activates its intrinsic tyrosine kinase, even though the enzymatic activity of the p125 FGFR-1a isoform is functional in an in vitro kinase reaction. Although we do not understand the mechanism of the p125 FGFR-1a isoform perinuclear transport, it is possible that this isoform may be competent to enter the trafficking pathway by dimerization with the ligand-binding competent p145 FGFR-1a isoform.

We chose to study FGFR-1 isoform traffic in the L6 myoblast because they are not responsive to exogenous FGF and are considered to be FGFR-deficient (45). Their classification as a FGFR-deficient cell line is based upon their inability to bind radiolabeled FGF (45), and we have also been able to detect a [125I]FGF-1-FGFR complex using competitive covalent radioligand cross-linking method. However, Hawker and Granger, using covalent ligand-receptor cross-linking and immunoblot analysis (46), have recently reported low levels of FGFR in the L6 cells. We also have been able to demonstrate by FGFR-1 immunoprecipitation and enzymatic amplification in an in vitro kinase assay the presence of the p125 FGFR-1a and p145 FGFR-1a isoforms in untransfected L6 myoblasts (8). Interestingly, these FGFR-1a isoforms appear to traffic independent of the presence of exogenous FGF-1. Although these data are consistent with the results obtained from FGFR-1a and FGFR-1b L6 myoblast transfectants, they do suggest that the L6 myoblast may be able to express low levels of an extracellular member of the FGFR gene family. Because the rat DNA sequences for the majority of the FGFR gene family are not available, it has not been possible to utilize either reverse transcriptase-polymerase chain reaction or RNase protection analysis to assess this possibility at least at the mRNA level.

The observation that the p120 FGFR-1b and p100 FGFR-1b isoforms do not traffic to a perinuclear locale argues that differential biological properties associated with the FGFR-1a and FGFR-1b isoforms (32, 33) should be considered relative to their differential intracellular trafficking properties. Indeed, this may be particularly informative with regard to differences between established cell lines and diploid cell strains. Likewise, the intracellular trafficking patterns of other members of the FGFR gene family where equivalent alternatively spliced FGFR isoforms have been characterized (27, 33) may also likely provide structural correlates to biological patterns of differential isoform expression (44). Because the differential trafficking of the FGFR-1a isoforms occurs during the G1 transition period as a structurally intact and functional tyrosine kinase, it is possible that FGFR-1a isoforms traffic from the plasma membrane via a novel mechanism. The perinuclear trafficking of the FGFR-1a isoforms is also interesting because we have described a biphasic interaction between FGFR-1 and Src during the immediate-early and mid-to-late G1 phase of the cell cycle (47). We have also reported that FGF-1 is able to regulate the phosphorylation of the Src substrate, cortactin, during these time periods (48) as well as induce a biphasic association between Src and cortactin (47). Interestingly, (i) cortactin is a F-actin-binding protein (49), (ii) Src (50) and related tyrosine kinases (51, 52) are also associated with adhesion sites and traffic to a perinuclear locale during the G1 transition period, and (iii) the cytoskeleton is linked to adhesion sites (53). In addition, FGFR-1a has been reported to exhibit associative properties with the glycosaminoglycans, heparin (34). Because (i) members of the FGFR gene family are well documented as heparin/heparan sulfate proteoglycan-binding growth factors (1–3), (ii) the heparan sulfate proteoglycans are localized at adhesion sites (54), (iii) many of the adhesion macromolecules contain heparin-binding domains (55, 56), and (iv) the low affinity heparin/heparan sulfate proteoglycans appear to be essential to propagation of a FGF-induced mitogenic signal (57–60), it is possible that these sites may contain regulatory features for biologically astute FGF-FGFR interactions. The observations that (i) the heparin-binding domain in FGFR-1a has also been recognized for a homology with cell adhesion molecules and (ii) antibodies against this domain block biological signal responses specific for these cell adhesion molecules (61) are also consistent with this premise.

Unlike FGFR-1a transfectants, L6 myoblasts transfected with FGFR-1b acquired a FGF-1-dependent morphology resembling highly malignant tumor cells including a well rounded shape and a decrease in the level of focal adhesion sites. This observation is consistent with the report that the induction of FGFR-1a and not FGFR-1b expression may be involved in the regulation of the malignant potential of astrocytes (32). However, the mechanisms responsible for this activity of FGFR-1b (but not FGFR-1a) is unclear, and two alternative pathways are suggested. First, there may be a differential association of FGFR-1a and FGFR-1b with focal adhesion sites, and these differences may involve the ability of the FGFR-1 isoforms to associate with cell matrix and/or cell adhesion molecules. Alternatively, the nuclear trafficking of FGF-1 provided by FGFR-1a but not by FGFR-1b could potentially downregulate the morphological effects that are triggered by exogenous FGF-1. Indeed, it has been suggested that the intranuclear trafficking of endogenous FGF-1 (2), FGF-2 (18), and FGF-3 (17) not only decreases cell proliferation but also induces a more flattened monolayer phenotype. Thus, it is possible that the intranuclear trafficking of exogenous FGF-1 provided by FGFR-1a may play a feedback role that regulates the mitogenic activity of FGF-1 and thus limit the development of a malignant phenotype. The absence of this feedback by FGF-1-induced signaling of FGFR-1b may result in an attenuation of the metastatic potential.

We have also noted that the first Ig-like loop in the FGFR-1a isoforms does not contain a sequence with structural similarity to known nuclear localization signals (62), and this is consistent with our prior observation that the translocation of FGFR-1a is restricted to a perinuclear locale in NIH 3T3 cells (8). Thus, the role of the first Ig-like loop in the FGFR-1a isoforms may be limited to the trafficking of the appropriate exogenous FGF ligand to a perinuclear locale that would enable the exogenous FGF ligand to utilize its structural nuclear localization signal for nuclear (5, 42) and perhaps nuclear translocation (13, 14). Indeed, the reduced efficiency of exogenous FGF-1 nuclear trafficking exhibited by point mutants in
the FGF-1 nuclear localization sequence (12) are consistent with this suggestion.

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The Nuclear Trafficking of Extracellular Fibroblast Growth Factor (FGF)-1 Correlates with the Perinuclear Association of the FGF Receptor-1 α Isoforms but Not the FGF Receptor-1 β Isoforms

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