Nuclear Translocation of Fibroblast Growth Factor (FGF) Receptors in Response to FGF-2

Pamela A. Maher

The Scripps Research Institute, La Jolla, California 92037

Abstract. Members of the FGF family of growth factors localize to the nuclei in a variety of different cell types. To determine whether FGF receptors are also present within nuclei and if this localization is regulated by FGFs, nuclei were prepared from quiescent and FGF-2–treated Swiss 3T3 fibroblasts and examined for the presence of FGF receptors by immunoblotting with an antibody produced against the extracellular domain of FGF receptor-1 (FGFR-1). Little or no FGFR-1 is detected in nuclei prepared from quiescent cells. When cells are treated with FGF-2, however, there is a time- and dose-dependent increase in the association of FGFR-1 immunoreactivity with the nucleus. In contrast, treatment with either EGF or 10% serum does not increase the association of FGFR-1 with the nucleus. When cell surface proteins are labeled with biotin, a biotinylated FGFR-1 is detected in the nuclear fraction prepared from FGF-2–treated, but not untreated, cells indicating that the nuclear-associated FGFR-1 immunoreactivity derives from the cell surface. The presence of FGFR-1 in the nuclei of FGF-2–treated cells was confirmed by immunostaining with a panel of different FGFR-1 antibodies, including one directed against the COOH-terminal domain of the protein. Fractionation of nuclei from FGF-2–treated cells indicates that nuclear FGFR-1 is localized to the nuclear matrix, suggesting that the receptor may play a role in regulating gene activity.

THE FGFs are a family of heparin-binding polypeptides that play a role in a wide array of biological processes, including cell growth, differentiation, angiogenesis, tissue repair, and transformation (for reviews see Baird and Bohlen, 1990; Burgess and Maciag, 1989; Wagner, 1991; Fernig and Gallagher, 1994). The FGFs interact with two classes of FGF receptors: high affinity receptors that bind FGFs with picomolar affinity and are thought to mediate the cellular responses to FGF, and low affinity receptors that bind FGFs with nanomolar affinity and are characterized by the presence of heparan sulfate moieties. At the present time, the high affinity FGF receptor family contains four members (for reviews see Johnson and Williams, 1993; Givol and Yayon, 1992; Partanen et al., 1993). In addition, alternative splicing can generate receptor isoforms that vary in their extra- or intracellular domains. Each of these receptors is capable of binding and responding to more than one type of FGF. Similar to other growth factor receptors, the FGF receptors possess intrinsic tyrosine kinase activity. Thus, FGF binding to the extracellular domain of a receptor rapidly leads to receptor autophosphorylation and substrate tyrosine phosphorylation. However, long-term treatment of cells with FGFs is necessary for cells to show a mitogenic response (Zhan et al., 1993; Presta et al., 1991) suggesting that signals other than those provided by direct receptor activation are required for cell proliferation.

Some of the mitogenic signals may be provided by FGFs themselves. Unlike other growth factors, internalized FGFs are surprisingly stable, persisting in treated cells for many hours (Walicke and Baird, 1991; Friesel and Maciag, 1988; Moenner et al., 1989). A number of studies have demonstrated the presence of FGF-1 (aFGF) or FGF-2 (bFGF) in the nuclei of cells (Florkiewicz et al., 1991; Tessler and Neufeld, 1990; Renko et al., 1990). In cells that produce FGF-2, the higher molecular mass isoforms (24, 23, and 22 kD) are found exclusively in the nucleus (Florkiewicz et al., 1991; Renko et al., 1990). The addition of 18 kD FGF-2 to target cell cultures results in uptake of a portion of the growth factor into the nucleus (Baldin et al., 1990; Walicke and Baird, 1991). In some cell types, the translocation of FGF-2 to the nucleus is cell cycle dependent (Baldin et al., 1990). Similar results are obtained after treating cells with FGF-1 (Zhan et al., 1992). Furthermore, there is some evidence that this translocation of FGF-2 to the nucleus is required for the induction of cell proliferation (Wiedlocha et al., 1994).

Since the pathways whereby FGFs travel from the cell surface to the nucleus have not been described, the studies

reported here were undertaken to determine if high affinity FGF receptors could play a role in this translocation. For these experiments, we used mouse Swiss 3T3 fibroblasts, which express relatively high levels (Maher, 1993) of FGF receptors and respond to treatment with FGF-2 by an increase in DNA synthesis (Pasquale et al., 1988). The nuclei of these cells were isolated and examined for the presence of FGF receptors. Quiescent fibroblasts have very low levels of nuclear receptors. After stimulation with FGF-2, however, we found a dose and time dependent increase in nuclear FGF receptors. At least some of these receptors appear to come from the cell surface. Within the nuclei, the receptors are found in the nuclear matrix, a region of the nucleus implicated in tissue- and cell type–specific gene expression (Dworetsky et al., 1990).

Materials and Methods

Cell Culture

Swiss 3T3 fibroblasts (No. CCL-92; American Type Culture Collection, Rockville, MD) were grown in DME containing 10% FCS and antibiotics. To prepare quiescent cells, Swiss 3T3 cells were grown to confluence and then incubated in 0.5% FCS for 48 h.

Antibodies

A rabbit polyclonal antibody to the carboxyl terminus of FGF receptor-1 (FGFR-1) was produced using a peptide containing the COOH-terminal 17 amino acids of human FGFR-1 coupled to keyhole limpet hemocyanin. The antibody was affinity purified by passage over a column of the peptide coupled to Affigel 102. A second rabbit polyclonal antibody to the COOH-terminal 15 amino acids of human FGFR-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and was used for the immunoprecipitation studies. A mouse mAb against the baculovirus-expressed extracellular domain of FGFR-1 produced in insect cells (Kiefer et al., 1991) was generated using standard techniques and has been described elsewhere (Hanneken et al., 1995).

Isolation of Nuclei

Nuclei were isolated from Swiss 3T3 fibroblasts by several different techniques. In all cases, the integrity and quality of the nuclei were evaluated by light microscopy. In most cases, the purity of the nuclear fractions was also assessed by immunoblotting with an anti-EGF receptor antibody and assaying 5' nucleotidase activity (Sigma Diagnostics, St. Louis, MO) as markers for plasma membrane contamination and by assaying acid phosphatase activity (Sigma Diagnostics) as a marker for lysosomal contamination. Neither EGF receptors (see Fig. 1) nor 5' nucleotidase activity were detected in the nuclear fraction, and <5% of the total acid phosphatase activity was associated with the nuclear fraction. For most experiments, we used a rapid method that allows isolation of nuclei from small numbers of cells (Schreiber et al., 1989). Briefly, cells in 35-mm culture dishes were rinsed twice with TBS and suspended in 200 μl cold homogenization buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) by gentle scraping with a rubber policeman. The cells were allowed to swell on ice for 15 min, at which time, 12.5 μl of a 10% solution of NP-40 was added and the tube was vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation for 30 s in a microfuge, washed twice with homogenization buffer containing NP-40, and resuspended in 200 μl homogenization buffer containing NP-40. Equal volumes of the nuclear and nonnuclear fractions were solubilized in 2.5× Laemmli sample buffer and were analyzed by SDS-PAGE and immunoblotting with the monoclonal anti-FGFR antibody (see below). For nuclear fractionation, membrane-depleted nuclei were isolated as described (Fey et al., 1986). Briefly, cells in 60-mm culture dishes were washed twice with PBS, suspended in CSK 100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1% Triton X-100, 0.5 mM CaCl₂, 10 mM Pipes, 1.2 mM PMSF, pH 6.8), incubated 7 min on ice, and nuclei were collected by centrifugation at 650 g for 5 min at 4°C. Nuclear matrices were isolated from membrane-depleted nuclei as described (Dworetsky et al., 1990).

The nuclei were treated first with a double detergent buffer (0.5% deoxycholate/1% Tween 40 in 10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, pH 7.4) to remove cytoskeletal elements and polyribosomes, then resuspended in CSK 50 buffer (50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1% Triton X-100, 0.5 mM CaCl₂, 10 mM Pipes, pH 6.8) containing 100 μg/ml DNase I and 50 μg/ml RNase A, incubated for 20 min at 37°C, and then ammonium sulfate was added to a final concentration of 0.25 M. After 15 min on ice, nuclear matrices were collected by centrifugation. In some experiments, the peripheral nuclear matrix was prepared by extracting intact matrices with 0.25 M ammonium sulfate and 40 mM DTT in CSK 50 buffer for 20 min at 37°C and collecting by centrifugation (Payrastre et al., 1992). Equal amounts of protein from all the fractions were solubilized in 2.5× Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with the monoclonal anti-FGFR-1 antibody. For immunoblotting, the transfers were stained with amido black to confirm the presence of equal amounts of protein in each lane and then blocked with 5% nonfat milk in TBS overnight before incubation with antibodies. The transfers were incubated overnight with the antibody (3 μg/ml), washed, and then incubated with rabbit anti–mouse IgG (1 μg/ml) for 2 h before the addition of ¹²⁵I-protein A (0.2 μCi/ml) for 2 h. The transfers were autoradiographed overnight at −70°C.

Biotinylation of Cell-surface Proteins

Quiescent Swiss 3T3 cells in 60-mm dishes were labeled for 30 min at 4°C with 0.5 mg/ml NHS–sulfo biotin (Vector Laboratories, Burlingame, CA) (Cole et al., 1987; Hurley et al., 1985), rinsed with ice-cold PBS, and then incubated for 60 min in DME with 0.5% FCS at either 4°C or 37°C in the absence or presence of FGF-2. The cells were rinsed in ice-cold TBS, and the nuclei were prepared as described above. The nuclei were solubilized by sonication in RIPA buffer (150 mM NaCl, 20 mM Na₂HPO₄, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM Na₃VO₄), and insoluble material was removed by centrifugation. Biotin-tagged proteins were precipitated from the nuclear and nonnuclear fractions overnight with avidin-agarose (Vector Laboratories) and then eluted from the beads by boiling for 5 min in 2.5× Laemmli sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting with the monoclonal anti-FGFR antibody, as described above.

Immunofluorescence Microscopy

Cells were treated as described in Results, rinsed in PBS, fixed for 5 min with 3% formaldehyde in PBS, and permeabilized for 5 min with 0.5% Triton X-100. Permeabilized cells were treated with either a rabbit anti–FGFR-1 antibody (10 μg/ml) in PBS/3% BSA or the monoclonal anti–FGFR-1 antibody (50 μg/ml) in PBS/3% BSA for 30 min, rinsed in PBS, labeled with either a mixture of rhodamine-conjugated, affinity-purified F(ab)₂ fragment of goat anti–rabbit IgG (8 μg/ml) and nitrobenzoxadiazole (NBD)-phallacidin (20 U/ml) in PBS/3% BSA (for the polyclonal antibody), or fluorescein-conjugated donkey anti–mouse IgG (for the mAb) for 10 min and mounted in 90% glycerol. In some cases, the polyclonal anti–FGFR-1 antibody was preincubated with the peptide antigen (100 μg/ml) before labeling of the cells. Labeled cells were examined with either a microscope (Carl Zeiss, Inc., Thornwood, NY) using a ×63, numerical aperture 1.4 planapochromat oil objective for standard immunofluorescence microscopy, or with a laser scanning confocal microscope (model MRC600; Bio Rad Laboratories, Hercules, CA) equipped with an argon/krypton mixed gas laser. Optical sections were taken in 400-nm steps in the z axis. Dual images were collected at 480 nm (NBD-phallacidin) and 560 nm (rhodamine) and photographed.

FGF Receptor Immunoprecipitation and In Vitro Kinase Assays

Nuclear proteins were solubilized by sonication of isolated nuclei in Triton X-100 buffer (1% Triton X-100 in 50 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄). FGFR-1 was immunoprecipitated at 4°C overnight with the commercial anti–FGFR-1 antibody (0.5 μg/500 μl nuclear extract) or an equal amount of normal rabbit IgG, and the immunoprecipitates were collected on protein A–Sepharose and then washed 3× with 0.1% Triton X-100 in 20 mM Hepes, pH 7.5, 150 mM NaCl. In some experiments, 5 μg of the cognate peptide was included during the immunoprecipitation step. The immune complexes were resuspended in 50 μl in vitro phosphorylation buffer (150 mM NaCl, 20 mM Hepes, pH 7.5, 0.1% Triton X-100) containing 10 mM MnCl₂, 50 mM MgCl₂, 50 nM ATP, and 1

μCi [γ^{32}P]ATP. The reaction was carried out for 20 min at room temperature with continuous rotation and then terminated by the addition of an equal volume of 5× SDS sample buffer. The samples were separated on SDS-7.5% polyacrylamide gels, and the gels were fixed overnight in 25% methanol, 10% acetic acid, dried, and autoradiographed for 12–18 h.

Protein Tyrosine Kinase Assays

Protein tyrosine kinase activity in nuclei and nuclear matrices was assayed as described (Maher, 1991) using poly Glu/Tyr (4:1) as a substrate. The reactions were analyzed by SDS-PAGE and autoradiography, after which the substrate bands were cut from the gel and the incorporation of ^{32}P was quantified by Cerenkov counting.

Results

To determine if the treatment of cells with FGF-2 causes a redistribution of high affinity FGF receptors to the nucleus, quiescent Swiss 3T3 fibroblasts were treated for 60 min with FGF-2, and the cells separated into nuclear and nonnuclear fractions using a protocol for the rapid preparation of membrane-depleted nuclei (Schreiber et al., 1989). Inspection of isolated nuclei by light microscopy showed they were intact and free of membrane contamination (not shown). In addition, no 5′ nucleotidase activity or EGF receptors were associated with the nuclei (Fig. 1 B), although similar numbers of EGF and FGF receptors are found on Swiss 3T3 cells (unpublished data). Equal volumes from each fraction were analyzed for the presence of FGF receptors by immunoblotting with an mAb prepared against a baculovirus-expressed extracellular domain of FGFR-1 that is produced in insect cells. As shown in Fig. 1 A, little or no receptor is detectable in nuclei isolated from quiescent cells, whereas receptor is readily visualized in the nuclear fraction prepared from FGF-2–treated cells. In both cases, the majority of receptor is
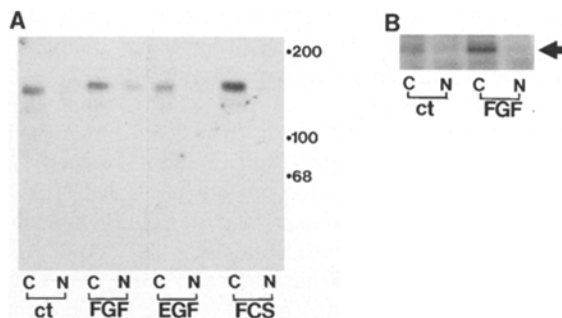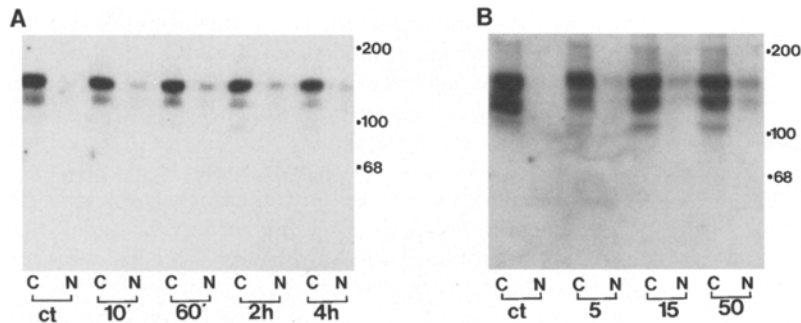

Figure 1. Presence of FGF receptors in nuclei of FGF-treated Swiss 3T3 fibroblasts. (A) Quiescent cells were untreated (ct) or treated with 15 ng/ml FGF-2, 15 ng/ml EGF, or 10% FCS for 60 min at 37°C. The cells were separated into nuclear (N) and nonnuclear (C) fractions as described (Schreiber et al., 1989), and equal volumes from each fraction were analyzed for the presence of FGF receptors by SDS-PAGE and immunoblotting with a mAb against the extracellular domain of FGFR-1. (B) Quiescent cells were untreated (ct) or treated with 15 ng/ml FGF-2 for 60 min at 37°C. The cells were separated into nuclear (N) and nonnuclear (C) fractions as described above, and equal volumes from each fraction were analyzed for the presence of EGF receptors by SDS-PAGE and immunoblotting with a mAb against the extracellular domain of EGFR. Arrowhead indicates position of EGFR. Similar results were obtained in three independent experiments of identical design. Molecular mass markers (in kilodaltons) are on the right.

found in the soluble fraction that contains both solubilized plasma membranes and cytoplasm. The two bands detected with the antibody correspond to the two and three Ig loop forms of FGFR-1 (unpublished data). It is not clear why the amounts of the two forms of FGFR-1 differ slightly between experiments, but it may result from the use of different batches of Swiss 3T3 cells. Since these cells fairly rapidly lose their strong mitogenic response to FGFs because of a greatly increased basal growth rate, new batches of cells must be thawed quite frequently. The translocation of FGF receptor to the nucleus was specific: treatment with either EGF or 10% serum, two agents that also induce proliferation of the Swiss 3T3 cells, does not increase the levels of FGF receptor in the nucleus. Treatment of cells with FGF-1 in the presence of heparin also causes a similar increase in nuclear FGF receptors (not shown). The presence of FGF receptors in the isolated nuclei is unlikely to be an artifact of the technique used for nuclear isolation, since similar results were obtained using several different methods to prepare membrane-depleted nuclei (e.g., Fig. 6), and the biochemical results were confirmed by immunofluorescence microscopy (see Figs. 4 and 5).

The appearance of FGF receptors in the nucleus after treatment with FGF-2 was time and dose dependent (Fig. 2). Receptor is detected in the nucleus within 10 min of FGF-2 treatment, and maximal levels are reached within 1 h (Fig. 2 A). Extended treatment with FGF-2 (up to 10 h) does not result in a further increase in the level of nuclear FGFR-1 (not shown). Only a slight increase in nuclear FGF receptors is detected after a treatment with 1–5 ng/ml FGF-2, whereas a strong nuclear signal is observed after stimulation with 15 or 50 ng/ml FGF-2 (Fig. 2 B). Under the conditions used for these experiments, 15 ng/ml of FGF-2 is the optimal mitogenic dose for Swiss 3T3 fibroblasts (Pasquale et al., 1988).

To determine if the nuclear FGF receptor immunoreactivity derives from the cell surface, FGF receptors on the surface of quiescent 3T3 cells were labeled with an impermeable biotin analogue (Cole et al., 1987; Hurley et al., 1985) before treatment with FGF-2. Biotin-labeled, untreated, and FGF-2–treated cells were separated into nuclear and nonnuclear fractions, and the biotin-tagged proteins in each fraction were collected on avidin-agarose. The fractions were analyzed for the presence of FGF receptors by immunoblotting. As shown in Fig. 3, little or no biotin-tagged receptor is detected in the nuclei of quiescent cells. However, a biotin-tagged receptor is clearly detectable in nuclei isolated from FGF-2–treated cells. These experiments also demonstrate that a significant percentage (~30 %) of the cell-surface FGFR-1 migrates to the nucleus. No receptor is detectable in avidin-agarose precipitates that were prepared from cells not labeled with biotin. In addition, little or no biotin-tagged receptor is detectable in the nuclei of cells treated with FGF-2 at a low temperature (4°C).

Standard and confocal immunofluorescence microscopy were used to provide further evidence that FGF-2 stimulates the accumulation of FGF receptors in target cell nuclei. Quiescent Swiss 3T3 cells show a diffuse labeling for FGFR-1 (Fig. 4 A). The low level of plasma membrane staining is a result of the technique used to perme-

Figure 2. (*A*) Time-dependent accumulation of FGF receptors in the nuclei of Swiss 3T3 fibroblasts. Quiescent cells were untreated (*ct*) or treated with 15 ng/ml FGF-2 for 10 min, 60 min, 2 h, and 4 h before fractionation and analysis of equal volumes from each fraction for the presence of FGF receptors. (*B*) Dose-dependent accumulation of FGF receptor in the nuclei of Swiss 3T3 fibroblasts. Quiescent cells were untreated (*ct*) or treated for 60 min with 5, 15, or 50 ng/ml FGF-2 before fractionation and analysis of equal volumes from each fraction for the presence of FGF receptors. Similar results were obtained in three independent experiments of identical design. Molecular mass markers (in kilodaltons) are on the right.

abilize the cells. After treatment with FGF-2 for 10 min, some cells show an increase in perinuclear labeling (Fig. 4 *B*). After 60 min, most cells show strong nuclear labeling for FGFR-1 (Fig. 4 *C*). Little or no labeling is seen in cells labeled with blocked antibody (Fig. 4 *D*), providing further evidence for the specificity of the nuclear labeling. To provide additional confirmation for the localization of FGFR-1 within the nuclei of FGF-2–treated cells, the cells were optically sectioned using a confocal microscope. As shown in Fig. 5, the majority of the labeling for FGFR-1 is found within the nucleus. Some perinuclear staining is also observed. Qualitatively similar results were obtained with the monoclonal FGFR-1 antibody, although the intensity of labeling was weaker (not shown).

Since theses studies clearly establish the presence of FGF receptors within the nuclei of fibroblasts after treatment with FGF-2, it was of interest to determine the subnuclear localization of the receptor. Membrane-depleted nuclei were isolated from untreated and FGF-treated cells and then fractionated further, as described in Materials and Methods. Using this approach, the majority of FGF receptor immunoreactivity is detected in the nuclear matrix (Fig. 6). Further fractionation of the nuclear matrix into peripheral matrix and internal matrix (Payrastre et al., 1992) demonstrates the presence of FGFR-1 immunoreactivity exclusively in the peripheral matrix (Fig. 6), a fraction that represents <5% of the total cellular protein. The membrane-depleted nuclei (Fig. 6, lanes *1* and *5*) isolated by this technique (Fey et al., 1986) are not as clean as those prepared by hypotonic lysis (Schreiber et al., 1989)

and shown in Figs. 1–3. Thus, a low level of FGF receptor is detected in the unfractionated nuclei isolated from untreated cells.

To determine if nuclear FGFR-1 retains tyrosine kinase activity, nuclei were solubilized by sonication in a detergent-containing buffer, FGFR-1 was immunoprecipitated with an antibody directed against the COOH terminus of FGFR-1, and the immunoprecipitate was assayed for autophosphorylation activity. As shown in Fig. 7, two high molecular weight phosphorylated bands, similar in size to the two FGFR-1 bands detected by immunoblotting (Figs. 1–3), are seen in receptor immunoprecipitates prepared with nuclei isolated from FGF-2–treated cells. These bands are greatly reduced or absent in receptor immunoprecipitates prepared with nuclei isolated from quiescent Swiss 3T3 cells. The additional protein bands that immunoprecipitate with FGFR-1 may represent nuclear substrates of the receptor. Only lower molecular weight phosphorylated bands are seen in control immunoprecipitates using normal rabbit IgG, and few or no phosphorylated bands are seen in control immunoprecipitates formed in the presence of the cognate peptide.

These results suggested that the translocation of FGF receptor to the nucleus might result in a general increase in tyrosine kinase activity in the nuclear fractions. To test this idea, nuclei, nuclear matrix, and peripheral nuclear matrix isolated from untreated and FGF-2–treated cells were assayed for tyrosine kinase activity using poly Glu/Tyr (4:1) as an exogenous substrate. Tyrosine kinase activity is increased in both the nuclei and nuclear matrix iso-
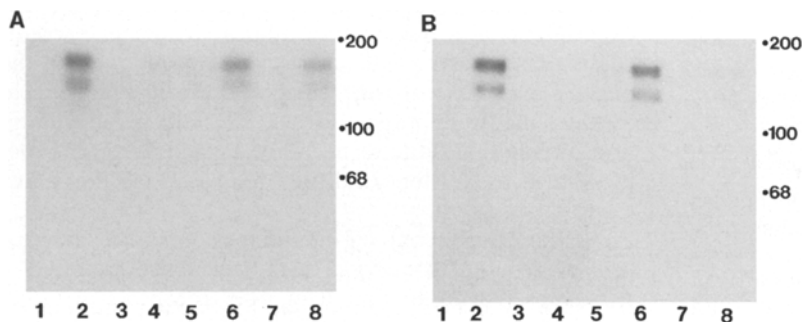
Figure 3. Translocation of FGF receptors from the cell surface to the nucleus. Quiescent Swiss 3T3 cells were unlabeled (lanes *1, 3, 5,* and *7*) or labeled (lanes *2, 4, 6,* and *8*) with NHS-sulfo biotin at 4°C, and then incubated for 60 min at either 37°C (*A*) or 4°C (*B*) in the absence (lanes *1–4*) or presence (lanes *5–8*) of 50 ng/ml FGF-2. Cells were fractionated, and biotin-labeled proteins in both the nuclear (lanes *3, 4, 7,* and *8*) and nonnuclear (lanes *1, 2, 5,* and *6*) fractions were precipitated with avidin-agarose. The precipitates were analyzed for the presence of FGF receptors by SDS-PAGE and immuno-

blotting with the mAb against the extracellular domain of FGFR-1. Similar results were obtained in three independent experiments of identical design. Molecular mass markers (in kilodaltons) are on the right.
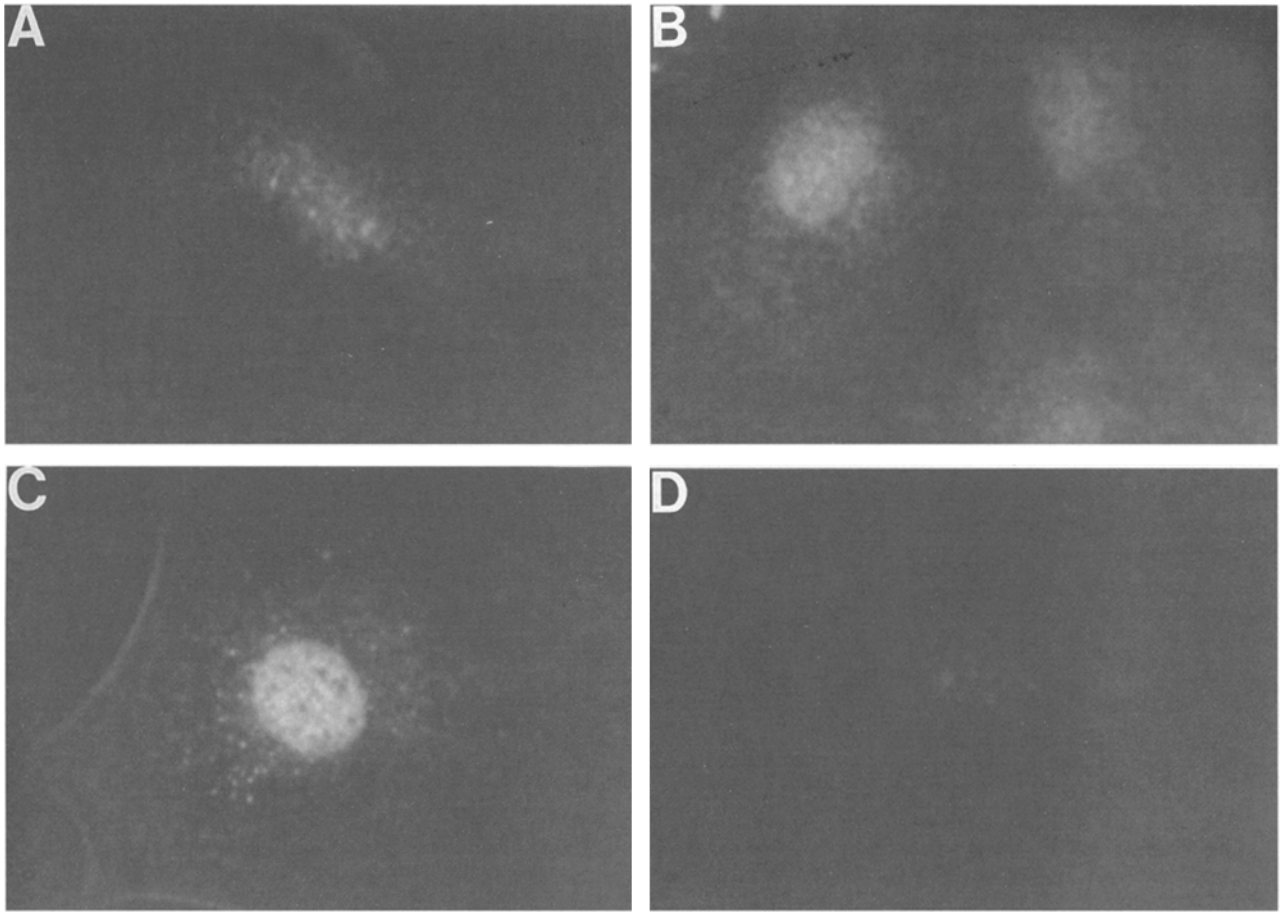
Figure 4. Indirect immunofluorescence labeling of Swiss 3T3 fibroblasts for FGF receptors. Quiescent cells were untreated (*A*) or treated for 10 min (*B*) or 60 min (*C* and *D*) with 50 ng/ml FGF-2, and then labeled with the rabbit polyclonal FGFR-1 antibody in the absence (*A–C*) or presence (*D*) of the cognate peptide. Similar results were obtained in three independent experiments of identical design.

lated from FGF-treated cells, and is highest relative to untreated cells in the peripheral nuclear matrix fraction (Table I). The increase in tyrosine kinase activity is consistent with the presence of FGFR-1 in these fractions (Fig. 6). The marginal increase in tyrosine kinase activity seen in whole nuclei isolated from FGF-2–treated cells probably reflects the presence of multiple tyrosine kinases in whole nuclei, as well as the presence of low levels of FGF receptor in nuclei isolated from untreated cells by this technique.

Discussion

The results presented here provide strong evidence for an FGF-2–dependent translocation of high affinity FGF receptors to the nuclei of fibroblasts. Their subnuclear association with the nuclear matrix suggests that FGF receptors may play a direct role in regulating gene transcription. In addition, these findings may help explain a number of paradoxes regarding the effects of FGF on cells (e.g., Zhan et al., 1993; Wiedlocha et al., 1994).

Several lines of evidence indicate that the FGF-2–dependent association of FGF receptors with nuclei is unlikely to be an artifact. First, several different methods for preparation of nuclei give similar results. In addition, fractionation of the nuclei indicates that the vast majority of

nuclear FGF receptor is located in the nuclear matrix. Since preparation of this nuclear fraction removes >95% of the cellular proteins, any protein that is nonspecifically associated with the nucleus would be unlikely to persist throughout this fractionation. Furthermore, nuclear FGF receptors can be detected both by biochemical analysis of the nuclei by either immunoblotting or immunoprecipitation and in vitro phosphorylation, and by immunocytochemistry with two distinct FGF receptor antibodies. Finally, the presence of FGF receptors in the nuclei is ligand dependent, with little or no receptor detected in the nuclei of quiescent cells.

The FGF-2–dependent translocation of FGF receptors observed in this study is distinct from the FGF-1–dependent association of FGF receptors with the perinuclear region of NIH 3T3 cells (Prudovsky et al., 1994). For example, the time course of FGF-2–dependent FGF receptor translocation to the nucleus is faster than that reported for FGF-1 stimulation of receptor translocation in NIH 3T3 cells (Prudovsky et al., 1994). Maximal translocation is observed by 1 h after the addition of FGF-2 with no further increase with longer treatment times, whereas at least a 9 h treatment with FGF-1 is required for maximal association of FGF receptors with the nuclei of NIH 3T3 cells. A more significant difference between the two studies is the ultimate location of translocated receptor. Using immunocy-
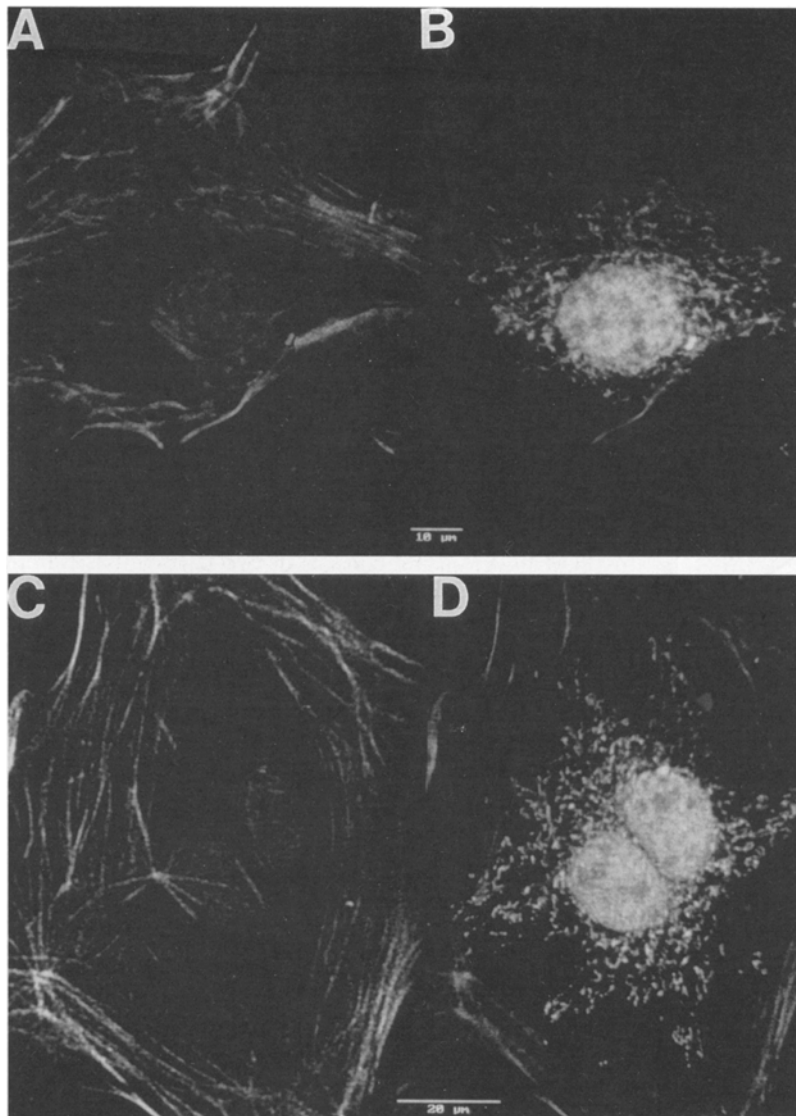
Figure 5. Confocal immunofluorescence microscopy of FGF receptors in FGF-2–treated Swiss 3T3 cells. Cells treated for 60 min with 50 ng/ml FGF-2 were labeled with the rabbit polyclonal FGFR-1 antibody (*B* and *D*) and NBD-phallacidin (*A* and *C*), and were examined with a Bio Rad MRC600 laser scanning confocal microscope. Median nuclear optical sections are shown.

tochemistry, enzymology, and biochemistry, I detect FGF receptors within the nucleus associated with the nuclear matrix. In response to FGF-1, receptors are translocated to a perinuclear region (Prudovsky et al., 1994). Although this difference could be caused by different receptor antibodies, I obtain the same immunocytochemical results with two different antibodies. It is unlikely the difference is caused by differences in the FGF receptors on the two cell types, since Swiss 3T3 fibroblasts express predominantly FGFR-1 (unpublished results), similar to the NIH 3T3 cells. The nuclear FGFR-1 seen in the Swiss 3T3 cells also appears to be distinct from the nuclear FGFR-3 observed in breast epithelial cells (Johnston et al., 1995). In the latter case, the receptor in the nucleus is a smaller, novel, intracellular form of FGFR-3 whereas, in the Swiss 3T3 cells, nuclear FGFR-1 is caused by ligand-dependent translocation of the receptor from the cell surface to the nucleus.

Although this is the first report of the FGF-2–dependent translocation of FGF receptors into the nuclei of cells, other growth factor receptors have been reported to be translocated to the nucleus in a ligand-dependent manner (Hopkins, 1994). Insulin receptors (Podlecki et al., 1987; Marti et al., 1991), EGF receptors (Jiang and Schindler, 1990; Rakowicz-Szulczynska et al., 1989; Holt et al., 1994), HER2/neu (Xie and Hung, 1994), IL-1 receptors (Curtis et al., 1990), and growth hormone receptors (Lobie et al., 1994) have been shown to associate with the nucleus using a variety of techniques. In general, the time course for translocation of these receptors is similar to that observed in this study. Maximal levels are reached within 1–2 h after the addition of the ligand to the cells. In the case of EGF receptors, a specific interaction with chromatin (Rakowicz-Szulczynska et al., 1989) has been proposed.

Our results indicate that the majority of FGF receptor in the nucleus of FGF-2–treated cells arises from the cell surface. Furthermore, several pieces of evidence indicate that this receptor is full length. First, the size of the receptor as detected by immunoblotting is consistent with full-length receptor. Second, nuclear receptor can be detected using either an antibody to the extracellular domain or an antibody to the COOH-terminal domain of the receptor. These data imply that a specific nuclear transport mechanism for the FGF receptor must exist. Specific nuclear
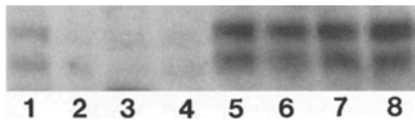
Figure 6. Fractionation of Swiss 3T3 fibroblast nuclei. Quiescent cells were untreated (lanes *1–4*) or treated for 60 min with 50 ng/ml FGF-2 (lanes *5–8*). Membrane-depleted nuclei (lanes *1* and *5*), double detergent–treated nuclei (lanes *2* and *6*), nuclear matrix (lanes *3* and *7*), and peripheral nuclear matrix (lanes *4* and *8*) were isolated as described (Fey et al., 1986; Dworetsky et al., 1990; Payrastre et al., 1992), and equal amounts of protein from each fraction were analyzed for the presence of FGF receptors by SDS-PAGE and immunoblotting with the mAb against the extracellular domain of FGFR-1. Similar results were obtained in five independent experiments of identical design. Molecular mass markers (in kilodaltons) are on the right.

transport is generally thought to occur via nuclear localization sequences (NLS) (Silver, 1991; Dingwall and Laskey, 1991). Although a close examination of the primary sequence of FGFR-1 does not reveal the presence of any obvious NLS, there are two sequences in the receptor that have weak homology to well-characterized NLS (Dingwall
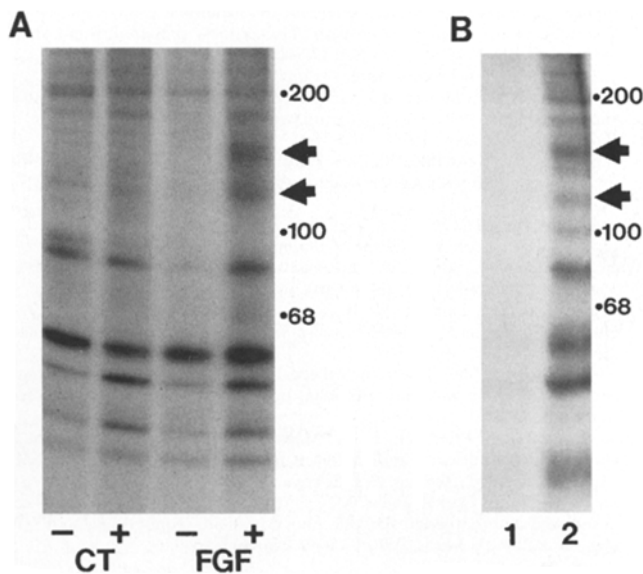
Figure 7. Tyrosine kinase activity of nuclear FGFR-1. (*A*) Quiescent cells were untreated (*CT*) or treated for 60 min with 50 ng/ml FGF-2 (*FGF*). Nuclei were isolated as described (Schreiber et al., 1989), and nuclear proteins were solubilized by sonication in Triton X-100 buffer. Immunoprecipitates were prepared using either an antibody specific for the COOH terminus of FGFR-1 (+) or normal rabbit IgG (−), and the immune complexes were phosphorylated with [γ^{32}P]ATP and analyzed by SDS-PAGE and autoradiography. (*B*) Quiescent cells were treated with 50 ng/ml FGF-2 for 60 min, and nuclear proteins were solubilized as described in (*A*). Immunoprecipitates were prepared using an antibody specific for the COOH terminus of FGFR-1 in the presence (*1*) or absence (*2*) of the cognate peptide. The immune complexes were phosphorylated and analyzed as described in *A*. Arrowheads indicate phosphorylated bands corresponding to FGFR-1. Similar results were obtained in three independent experiments of identical design. Molecular mass markers (in kilodaltons) are on the right.

Table I. Nuclear Tyrosine Kinase Activity

	Control	+FGF-2
Nuclei	1.0	1.14 ± 0.31
Nuclear matrix	1.0	2.14 ± 0.32
Peripheral nuclear matrix	1.0	2.22 ± 0.22

Protein tyrosine kinase activity in nuclei and subnuclear fractions was assayed in untreated cells (control), and cells that were treated with 50 ng/ml FGF-2 for 60 min (+FGF-2) as described in Materials and Methods. The results for FGF-2–treated cells are presented relative to the activity in the equivalent fraction from untreated cells. The data are the average from three independent experiments ±SD.

and Laskey, 1991). The first potential NLS is located between the first and second Ig loops of FGFR-1 and consists of a pair of basic amino acids separated from a basic cluster, in which three out of the next six amino acids are basic, by a stretch of seven amino acids. The second potential NLS is located in the cytoplasmic juxtamembrane domain of FGFR-1 and consists of two pairs of basic amino acids separated by a stretch of 18 amino acids. Alternatively, since proteins that have had their nuclear targeting sequences deleted can still be transported to the nucleus as part of a protein complex (Dingwall and Laskey, 1991), a similar process could apply to the FGF receptor. It is not known how the internalized receptor is shuttled through a vesicular pathway, eventually arriving at the nuclear membrane. There is no precedent for this type of trafficking pathway, except in the reports of other nuclear-associated growth factor receptors (see above). Consistent with internalization, the cell surface to nucleus pathway does not function at low temperatures (Fig. 3).

On a final note, the observation that translocated FGF receptors concentrate in the nuclear matrix is particularly intriguing. This matrix is thought to be instrumental in the regulation and coordination of gene expression (Fey and Penman, 1988; Dworetsky et al., 1990; Stuurman et al., 1990). Its composition is distinct in different tissues and in different cell types, and can change dramatically as a function of differentiation (Fey and Penman, 1988; Dworetsky et al., 1990; Stuurman et al., 1990). The presence of FGF receptors in this particular nuclear fraction suggests that they could be playing a role in regulating the expression of a set of FGF-2–dependent genes. If so, the identification of specific nuclear substrates for different FGF receptor isoforms may help explain the specificity of the mitogenic and differentiation response of cells to similar signaling pathways.

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