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Enhanced Signaling and Morphological Transformation by a Membrane-Localized Derivative of the Fibroblast Growth Factor Receptor 3 Kinase Domain†

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Fibroblast growth factor (FGF) receptors (FGFRs) are membrane-spanning tyrosine kinase receptors that mediate regulatory signals for cell proliferation and differentiation in response to FGFs. We have previously determined that the Lys650→Glu mutation in the activation loop of the kinase domain of FGFR3, which is responsible for the lethal skeletal dysplasia thanatophoric dyplasia type II (TDII), greatly enhances the ligand-independent kinase activity of the receptor. Here, we demonstrate that expression of this construct induces a c-*fos* promoter construct approximately 10-fold but does not lead to proliferation or morphological transformation of NIH 3T3 cells. In contrast, the isolated kinase domain of activated FGFR3, targeted to the plasma membrane by a myristylation signal, is able to stimulate c-*fos* expression by 40-fold, induce proliferation of quiescent cells, and morphologically transform fibroblasts. This result suggests that the extracellular and transmembrane domains of FGFRs exert a negative regulatory influence on the activity of the kinase domain. Targeting of the activated kinase domain to either the cytoplasm or the nucleus does not significantly affect biological signaling, suggesting that signals from FGFR3 resulting in mitogenesis originate exclusively from the plasma membrane. Furthermore, our novel observation that expression of a highly activated FGFR3 kinase domain is able to morphologically transform fibroblasts suggests that dysregulation of FGFR3 has the potential to play a role in human neoplasia.

Fibroblast growth factor (FGF) receptors (FGFRs) are high-affinity membrane-spanning receptors for FGFs. FGFRs are normally catalytically inactive in the absence of FGF ligands. The binding of FGF to the extracellular domain of FGFRs, in the presence of heparan sulfate proteoglycans, induces the dimerization of two receptor molecules, allowing transphosphorylation of tyrosines within the activation loop of the intracellular tyrosine kinase domains. Activation loop phosphorylation greatly enhances the ability of FGFRs to autophosphorylate as well as to phosphorylate substrates which transmit biological signals into the cell leading to cell proliferation, differentiation, angiogenesis, or embryogenesis (4, 11, 21, 23). Although growth factor receptor-mediated signaling has traditionally been assumed to initiate from the plasma membrane, FGF-induced cell proliferation requires prolonged exposure to ligand (63), during which activated FGFRs relocalize to the perinuclear and/or nuclear compartments of the cell (35, 43, 44).

Point mutations in different domains of three of the four highly related FGFRs have been identified as causing human developmental abnormalities, including skeletal and cranial malformation syndromes (38, 40, 58). Recent work suggests that the biochemical mechanism underlying these syndromes is ligand-independent activation of the FGFR tyrosine kinase activity (58), and this constitutive signal transduction is postulated to cause premature and abnormal maturation of the affected long bones or cranial sutures. Although patients with skeletal dysplasias caused by activating FGFR germ line mutations do not have an apparent increase in tumor frequency, enhanced signaling through FGFRs has been implicated in tumor progression. For instance, amplification or ectopic expression of the genes encoding several FGFs has been found in neoplastic cells (39, 52, 55, 61), and overexpression of many of the FGFs results in morphological transformation of cell lines coexpressing FGFRs (10, 12, 33, 36, 64). Changes in the profile of FGFR1 and FGFR2 expression due to gene amplification, overexpression, or differential expression of splice variants have been noted in many tumor types (1, 14, 28, 60, 62). Furthermore, constitutively activated derivatives of FGFR1 and FGFR2 which morphologically transform cells in vitro have been described (29, 32). These results are consistent with an important role for dysregulated signaling through FGFR1 and FGFR2 in human cancer. Recently, a role for signaling through FGFR3 in human cancers has been suggested by the observation that a translocation leading to overexpression of FGFR3, often in combination with activating point mutations, occurs at high frequency in human multiple myeloma (8).

A point mutation in the activation loop of the kinase domain of FGFR3 is responsible for a heterozygous-lethal form of dwarfism, thanatophoric dysplasia type II (TDII) (53). This mutation, resulting in substitution of a Glu residue for a Lys at position 650, occurs very near the two highly conserved sites of activating autophosphorylation, Tyr647 and Tyr648. This mutation appears to play a role similar to activation loop autophosphorylation in relieving the inhibitory conformation of the kinase active site and strongly activates the tyrosine kinase activity of the receptor (56). In our previous work, we found that full-length FGFR3 bearing the Lys650→Glu TDII mutation was unable to morphologically transform NIH 3T3 fibroblasts (56), despite its greatly enhanced autophosphorylation activity. This suggested at least two possibilities, one being that activated FGFR3 signals much differently from FGFR1 and FGFR2 in that it is unable to connect to pathways leading to transformation, the other being that the full-length receptor

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†This paper is dedicated to Michael Li-Shan Lin, born 7 August 1997.

provides some sort of constraint that prevents sufficient signaling through mitogenic pathways to cause oncogenic transformation. In the case of the epidermal growth factor receptor and its counterpart, the viral oncogene v-*erbB*, deletion of the extracellular domain of the receptor, due to fusion with the viral *gag* protein, in combination with mutations within the cytoplasmic domain is necessary for full transforming potential (15, 49). We postulated that an analogous situation may exist with FGFR3, and thus we expressed the cytoplasmic domain of either wild-type or constitutively activated FGFR3 in the absence of the transmembrane and extracellular domains. We report here that the activated kinase domain of FGFR3 is able to morphologically transform NIH 3T3 fibroblasts when expressed at the plasma membrane but not when expressed in the cytoplasm or nucleus, suggesting that FGFR3 signaling may play an important role in the development of human cancers.

MATERIALS AND METHODS

Full-length and truncated FGFR3 expression constructs. The full-length wild-type and Lys650→Glu activation loop mutant human FGFR3 expression constructs [FL-Kin(Wt) and FL-Kin(Act), respectively] have been described previously (56). The FGFR3 cytoplasmic domain derivatives were constructed by fusion of the *Stu*I-*Xba*I fragment of the full-length receptors (amino acids 405 to 806) with complementary oligonucleotide pairs with *Hin*dIII-*Stu*I overhangs, which encode appropriate targeting signals and which recreate the intracellular juxtamembrane amino acids 399 to 404. The oligonucleotide pairs and kinase domain fragments were inserted into the pcDNA3 vector at the *Hin*dIII-*Xba*I sites in a three-part ligation. The targeting signals encoded by the oligonucleotides are as follows: for plasma membrane localization, the myristylation signal from c-Src, MGSSKSKPKDPSQR (2, 6) (PM constructs); for cytoplasmic localization, the c-Src myristylation signal with an inactivating mutation, MASSKSK PKDPSQR (2, 25) (Cyto constructs); and for nuclear localization, the bipartite nuclear localization signal from *Xenopus* nucleoplasmin, KRPAATKKAGQAK KKK (45), together with a translation initiation site (Nuc constructs). The targeting sequences were confirmed to be correct by sequencing. The kinase-inactive (KD) mutants were constructed by using a QuikChange site-directed mutagenesis kit from Stratagene, with the mutagenic primer GTCACCGTAGCC GTGCGCATGCTGAAAGACGATGCC, encoding a Lys508→Arg substitution at the catalytic lysine residue. The restriction fragment (*Xho*I-*Bst*EII) containing the mutation was completely sequenced and reinserted into the parental Wt or Act constructs to ensure that only the desired alteration was present. The Neu (Act) construct used as a control in certain experiments is the oncogenic rat $p185^{neu}$ expressed from the pSV2 vector (3).

Immunoprecipitation and in vitro kinase assays. Twenty-four hours prior to transfection, NIH 3T3 cells were plated at a density of 2×10^5 per 60-mm-diameter plate. The cells were transfected with 10 μg of plasmid DNA encoding each expression construct by use of a modified calcium phosphate transfection method (7). Eighteen hours after transfection, the cells were refed with Dulbecco's modified Eagle medium containing 10% calf serum. Four hours later, the medium was changed to Dulbecco's modified Eagle medium with 0.5% calf serum to reduce the presence of FGF in the medium, and the cells were incubated for a further 20 h. The cells were then lysed in 500 μl of Nonidet P-40 lysis buffer and immunoprecipitated with the C-terminal FGFR3 antibody as previously described (58). Immune complexes were collected on protein A-Sepharose beads and subjected to in vitro kinase reactions in the presence of [γ-^{32}P]ATP as previously described (57).

Immunoblotting. Lysates from cells expressing each FGFR3 construct, prepared as described above, were electrophoresed through sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels and transferred to nitrocellulose. The filters were incubated with anti-FGFR3 antiserum (Santa Cruz Biotechnology) and then with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham) and developed by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Mitogenesis assays. A total of 2×10^4 NIH 3T3 cells were plated in each well of a 24-well plate, and for each condition, transient transfections were performed in quadruplicate as described above with 1 μg of expression plasmid per well. The cells were grown in medium containing 10% calf serum for 24 h after being refed to allow them to reach confluence. Similar levels of expression of each construct were confirmed by indirect immunofluorescence (as described below) of cells growing on a coverslip in one well. The cells in the remaining three wells were then starved for 48 h in 1 ml of medium containing 0.2% calf serum. A 2-μCi amount of [^{3}H]thymidine (specific activity, 20 Ci/mmol; NEN) was added per well, and after 6 h the cells were rinsed extensively with phosphate-buffered saline, fixed in 10% trichloroacetic acid, and lysed in 0.3 ml of 0.3 N NaOH. The [^{3}H]thymidine incorporated into trichloroacetic acid-insoluble material was determined by scintillation counting. Results of one of three representative experiments are shown, with the average value from three wells indicated for each construct. On the basis of observations from indirect immunofluorescence, approximately 5 to 10% of the cells expressed each construct.

Focus assays. NIH 3T3 cells were plated onto 60-mm-diameter plates and transfected as described for in vitro kinase assays, except that the cells were not starved, and were split 1:12 onto 100-mm-diameter plates 30 h after being refed. The presence of foci of transformed cells was scored 14 days later, at which time the cells were fixed in methanol, stained with Giemsa stain, and photographed. Similar levels of initial expression of each construct were confirmed 24 h after transfection by indirect immunofluorescence.

Indirect immunofluorescence. To determine the subcellular localization of FGFR3 constructs, transiently transfected NIH 3T3 cells expressing each construct were fixed with 3% paraformaldehyde, permeabilized in 1% Triton X-100, and incubated with polyclonal antibodies directed against the C-terminal peptide of FGFR3 (Santa Cruz Biotechnology) and then with a fluorescein-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim). Both antibodies were used at a 1:1,000 dilution in 3% bovine serum albumin. Cell surface expression of full-length FGFR3 constructs was examined similarly, using extracellular antibody SB141 (a kind gift of Michael Hayman) in the absence of cell permeabilization.

Transcription assays. NIH 3T3 cells were transfected with 2 μg of the pFL700 reporter, containing the upstream 700 nucleotides from the c-*fos* promoter fused to the luciferase gene (18), together with 8 μg of each FGFR3 expression construct. After being refed, the cells were starved for 48 h in medium containing 0.5% calf serum. Luciferase assays were performed with the Luciferase Assay System from Promega, according to the manufacturer's instructions. The data shown in each figure are the averages of duplicate transfections from a single representative experiment, one of three performed with each construct.

RESULTS

Localization of the FGFR3 kinase domain to different subcellular compartments. In order to study the role of signaling through the FGFR3 kinase domain in the absence of the extracellular and transmembrane domains, and in the absence of a requirement for addition of ligand, several constructs were made. The cytoplasmic domain of either wild-type human FGFR3 or the activation loop mutant containing the Lys650→Glu substitution, extending from amino acids 399 to 806, was cloned in frame with one of the following targeting signals: the myristylation signal from c-Src (2, 6) to direct localization to the inner surface of the plasma membrane; a mutant myristylation signal, containing a Gly2→Ala substitution (2, 25) to direct cytoplasmic localization; or the bipartite nuclear localization signal from *Xenopus* nucleoplasmin (45) to direct nuclear localization. Kinase-inactive derivatives (Lys508→Arg) of membrane-targeted Wt and Act constructs were also generated [PM-KD(Wt) and PM-KD(Act)]. These constructs, as well as constructs expressing the full-length wild-type and Lys650→Glu mutant receptors described previously (56), are shown schematically in Fig. 1.

Each construct was transiently transfected into NIH 3T3 fibroblasts, and protein expression and localization were examined by indirect immunofluorescence using an antibody directed against the C terminus of FGFR3. As shown in Fig. 2, each protein was expressed predominantly at the expected location in the cell. The full-length constructs were observed at the cell surface, as well as in the secretory pathway (Fig. 2B and H). For these constructs, surface expression was also examined in nonpermeabilized cells with an antibody directed against the extracellular domain of FGFR3 (Fig. 2A and G). The myristylated derivatives (PM) exhibited a staining pattern consistent with expression at the inner surface of the plasma membrane (Fig. 2C, D, I, and J), whereas the Cyto derivatives exhibited more-diffuse cytoplasmic staining (Fig. 2E and K). Both the Nuc-Kin(Wt) and the Nuc-Kin(Act) constructs were localized predominantly to the nucleus (Fig. 2F and L).

The FGFR3 (Act) kinase domain is active in the absence of the extracellular and transmembrane domains. We wished to examine whether FGFR3 kinase domain derivatives were able to autophosphorylate in the absence of the normal extracellular and transmembrane domains and from different subcellular

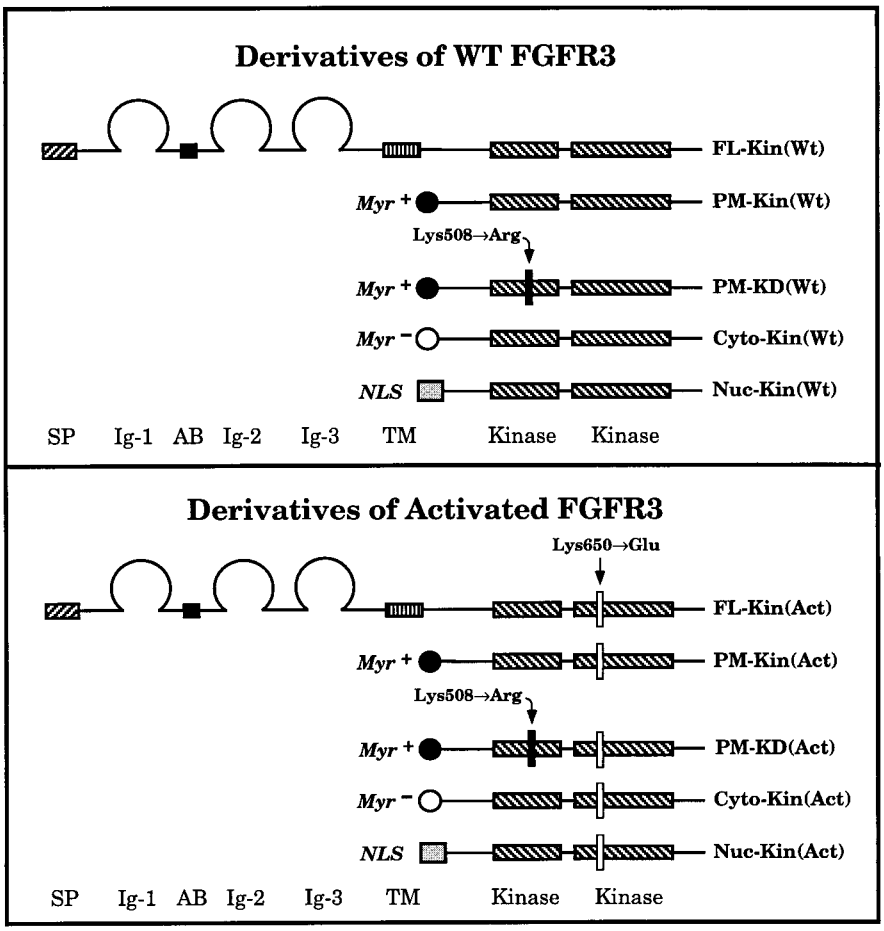

FIG. 1. Schematic representation of FGFR3 derivatives bearing the wild-type kinase domain [Kin(Wt)] sequence or the Lys650→Glu activating mutation [Kin (Act)]. Kinase-inactive derivatives (KD) (Lys508→Arg) of membrane-targeted Wt and Act constructs are also shown. The full-length (FL) receptor has a signal peptide (SP), three immunoglobulin-like loops (Ig-1 to Ig-3), an acid box (AB) between the first and second Ig loops, a membrane-spanning domain (TM), and an intracellular split tyrosine kinase domain. The intracellularly targeted derivatives include the entire coding sequence for the cytoplasmic domain of wild-type or activated FGFR3 fused to either the myristylation signal from Src (Myr^+) for localization to the inner surface of the plasma membrane (PM), a mutant myristylation signal (Myr^-) for localization to the cytoplasm (Cyto), or the nucleoplasmin nuclear localization signal (NLS) for localization to the nucleus (Nuc).

compartments. To this end, NIH 3T3 cells were transiently transfected with the constructs described above, and after 16 h in 0.5% serum-containing medium, lysates were generated which were immunoprecipitated with FGFR3 antiserum. Immune complexes were tested for in vitro kinase activity in the presence of [γ-^{32}P]ATP. As previously reported (56), FL-Kin (Act) (Fig. 3A, lane 6) exhibits significant constitutive autophosphorylation, whereas FL-Kin(Wt) (Fig. 3A, lane 2) does not. Each of the cytoplasmic constructs bearing the Lys650→Glu mutation, whether localized to the plasma membrane (Fig. 3A, lane 7), the cytoplasm (lane 8), or the nucleus (lane 9), was also active in this in vitro kinase assay, whereas their wild-type counterparts were not. The Lys508→Arg mutation in the active site of the kinase domain abolished the kinase activity (Fig. 3C) of the PM-Kin(Act) derivative, as anticipated.

As a control for loading and expression, parallel lysates were immunoblotted with an antibody to FGFR3, revealing comparable levels of protein expression for all constructs. The full-length and kinase domain FGFR3 derivatives are indicated in Fig. 3B, and the myristylated derivatives are shown in Fig. 3D.

FGFR3 constructs localized to different subcellular compartments differentially activate expression from the c-*fos* promoter. In response to FGF stimulation, quiescent NIH 3T3 cells rapidly induce immediate-early genes and reenter the cell cycle (23). To assess the ability of the wild-type and constitutively activated FGFR3 derivatives to send signals leading to immediate-early gene activation, each construct was cotransfected with a luciferase reporter construct driven by the promoter for the immediate-early gene c-*fos* (Fos-Luc) (18), and induction of luciferase was measured. The membrane-localized wild-type kinase domain of FGFR3 [PM-Kin(Wt)] was able to induce approximately 12-fold-more expression from this promoter than was the full-length wild-type plasmid (Fig. 4A). This result suggests that the extracellular and transmembrane domains of FGFR3 may negatively regulate the activity of the kinase domain in some way in the absence of ligand. Removal of the extracellular and transmembrane domains from the full-length receptor was not sufficient for signaling through this pathway, however, unless the protein was also targeted to the inner surface of the plasma membrane, as evidenced by the fact that Cyto-Kin(Wt) and Nuc-Kin(Wt) constructs did not stimulate Fos-Luc expression.

Activation of signaling by the PM-Kin(Wt) derivative was unexpected, as no increase in kinase activity was detected either by an in vitro kinase assay (Fig. 3A) or by examination of the profile of tyrosine-phosphorylated proteins in lysates from

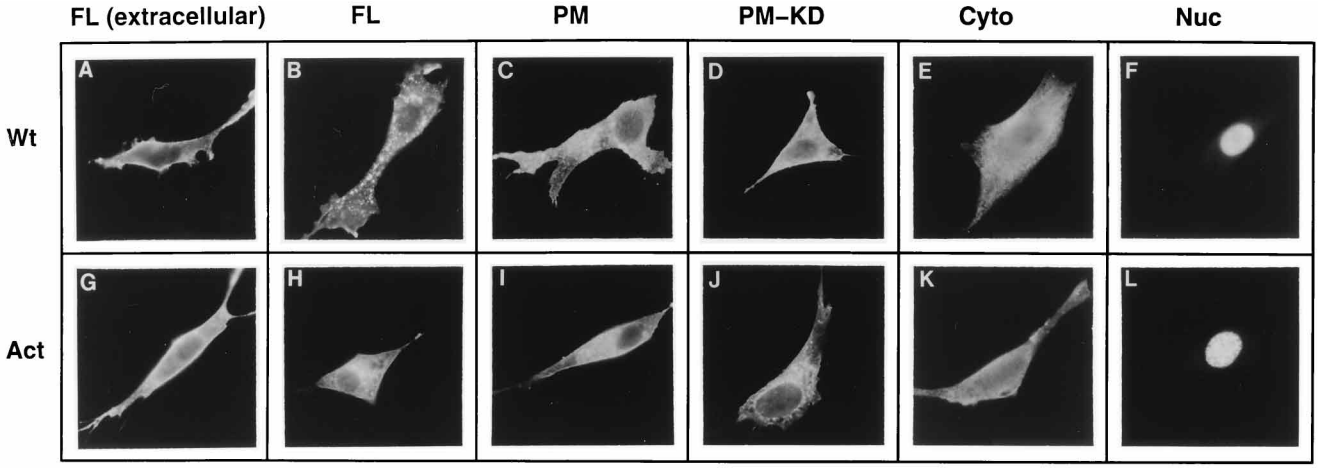

FIG. 2. Localization of FGFR3 derivatives by indirect immunofluorescence. Permeabilized NIH 3T3 cells transiently transfected with the indicated constructs were incubated with antiserum directed against the intracellular C-terminal peptide of FGFR3 and a fluorescein-conjugated goat anti-rabbit polyclonal antiserum (B to F and H to L). Predominant localization of the FL and PM derivatives to the plasma membrane, the Cyto derivatives to the cytoplasm, and the Nuc derivatives to the nucleus is observed. Nonpermeabilized NIH 3T3 cells expressing the FL derivatives were incubated with antibody SB141 directed against an extracellular epitope of FGFR3 and then with a fluorescein-conjugated goat anti-rabbit polyclonal antiserum to confirm cell surface localization of these constructs.

cells transfected with this construct, in contrast to each of the Kin(Act) derivatives (data not shown). In order to assess whether the observed c-*fos* promoter induction requires the kinase activity of this construct, a derivative, PM-KD(Wt), bearing a mutation of the catalytic lysine, Lys508→Arg, was tested in this assay. A dramatic reduction in Fos-Luc expression was observed, suggesting that signaling by PM-Kin(Wt) is indeed dependent on its kinase activity, at least transiently, towards its substrates and that in vitro tyrosine kinase activity and in vivo tyrosine phosphorylation may not fully reflect the biological activity of a particular construct.

Full-length, activated FGFR3 induced expression from the c-*fos* promoter 10-fold more strongly than did FL-Kin(Wt). The PM-Kin(Act) construct was the most active in this assay, leading to a 40-fold-higher induction of the c-*fos* promoter. Signaling activity was abolished by introduction of the Lys508→Arg mutation [PM-KD(Act)] and greatly reduced by targeting of the Kin(Act) constructs to either the nucleus or the cytoplasm. These results suggest that removal of the extracellular and transmembrane domains, targeting to the plasma membrane, and constitutive activation of the tyrosine kinase by the Lys650→Glu mutation combine for maximal signaling, leading to c-*fos* promoter induction.

As a control for transfection efficiency and expression of each construct in this assay, parallel lysates were examined by immunoblotting with FGFR3 antiserum (Fig. 4B).

The activated, membrane-localized kinase domain from FGFR3 stimulates DNA replication. To examine whether the FGFR3 derivatives which activated signaling from the c-*fos* promoter were also able to cause progression through the cell cycle in growth-arrested fibroblasts, each construct was transiently expressed in NIH 3T3 cells, which were then starved in low (0.2%) serum for 48 h before the addition of [^{3}H]thymidine for 6 h. The incorporation of radioactivity into cells, indicative of DNA replication, was measured. Of the FGFR3 derivatives examined, only the PM-Kin(Act) construct resulted in significant DNA synthesis (Fig. 5). Interestingly, neither the FL-Kin(Act) construct nor the PM-Kin(Wt) derivative, which both upregulated expression from the c-*fos* promoter (Fig. 4A), was detectably mitogenic. It is not yet clear whether the differing abilities of these constructs to signal immediate-early gene induction and mitogenesis reflect a qualitative difference in signaling by these constructs or simply a quantitative difference.

Expression of the wild-type or activated FGFR3 kinase domains, targeted to either the nucleus or the cytoplasm, had little effect on DNA replication. As a control, transient expression of oncogenic Neu resulted in high levels of DNA synthesis, comparable to expression of PM-Kin(Act).

The activated, membrane-localized kinase domain of FGFR3 is able to morphologically transform NIH 3T3 fibroblasts. As described in the introduction, enhanced signaling through FGFR1 and FGFR2 has been shown to result in morphological transformation of fibroblasts in vitro (29, 32) and has been correlated with human tumor progression (1, 14, 28, 60, 62). Recently, dysregulation of FGFR3 has been associated with human multiple myeloma (8), although the oncogenic potential of FGFR3 has not been directly demonstrated. In order to examine the transforming ability of our FGFR3 derivatives, each construct was transfected into NIH 3T3 cells, and after 2 weeks the plates were scored for the presence of foci of transformed cells. Strikingly, expression of the PM-Kin(Act) construct resulted in the formation of numerous, large foci (Fig. 6H), indicating that FGFR3, under appropriate conditions, has the potential to transform cells. As a positive control, expression of oncogenic Neu resulted in a similar number of smaller foci (Fig. 6F). Expression of FGFR3 derivatives localized to the nucleus or cytoplasm or of full-length constructs failed to elicit focus formation (Fig. 6). The PM-KD(Act) construct also was not transforming (data not shown), consistent with a role for the kinase activity of FGFR3 in mitogenesis and morphological transformation.

DISCUSSION

FGFR3 has the potential to act as an oncogene. In this report, we have demonstrated that morphological transformation of NIH 3T3 cells can occur in response to expression of a highly activated derivative of FGFR3, providing the first direct evidence that human FGFR3 has the potential to act as an oncogene. Recently, it has been proposed that dysregulation of FGFR3 can be an oncogenic event in human multiple my-

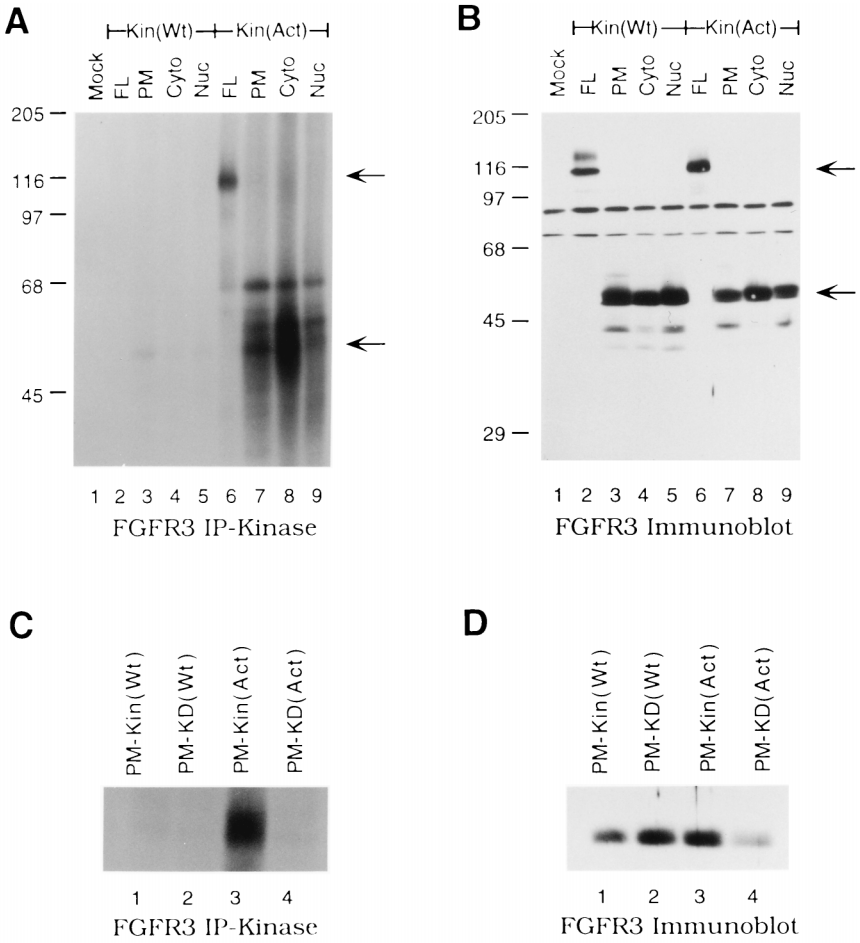

FIG. 3. In vitro tyrosine kinase activity of the FGFR3 derivatives. (A) Autophosphorylation assay. NIH 3T3 cells transiently expressing each indicated construct were lysed and immunoprecipitated with antiserum directed against the C-terminal peptide of FGFR3. Immunoprecipitates were subjected to in vitro kinase reactions in the presence of [γ-^{32}P]ATP and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The positions of the full-length FGFR3 and the intracellular derivatives are indicated (top and bottom arrows, respectively). Each of the Kin(Act) derivatives (lanes 6 to 9) is highly active in this assay. (B) FGFR3 immunoblot. Lysates from (A) were electrophoresed through an SDS-PAGE gel and transferred to nitrocellulose. The filters were incubated with FGFR3 antiserum and developed by enhanced chemiluminescence. The expression level of all constructs was similar. The positions of the full-length and intracellular FGFR3 derivative are indicated (top and bottom arrows, respectively). Sizes (in kilodaltons) are shown on the left. (C) Autophosphorylation assay. The kinase-inactive, membrane-targeted derivatives and their PM-Kin(Wt) and PM-Kin(Act) counterparts were subjected to in vitro kinase reactions as described for panel A. (D) FGFR3 immunoblot. Lysates from panel C were examined for expression of FGFR3 derivatives as described for panel B.

eloma (8). In these studies, a translocation that results in the juxtaposition of the FGFR3 locus near the immunoglobulin H switch region was observed in about 25% of multiple myeloma tumors and cell lines examined, resulting in high-level expression of FGFR3. Additionally, two cell lines and one primary tumor with this t(4;14) translocation were demonstrated to selectively overexpress an FGFR3 allele with activating mutations, either K650E, K650M, or Y373C, previously observed to occur in skeletal dysplasias (47, 53, 54). It will be of interest to determine whether abnormal activation of FGFR3 signaling, either by overexpression, point mutation, truncation, establishment of an autocrine loop with overexpressed ligand, or some combination of these events, plays a role in the etiology of other cancers, as we predict from our results.

The germ line mutation in FGFR3 which causes the skeletal dysplasia TDII results in defects in chondrocyte maturation and central nervous system development (53), rather than in cancer. This may be because in these affected tissues, where FGFR3 expression levels are highest (41), FGFR3 activation is coupled to signaling pathways leading to differentiation or growth arrest, rather than to proliferation. For instance, constitutive activation of STAT1 and increased expression of the cell cycle inhibitor p21WAF1/CIP1 have been observed in cartilage cells from a TDII fetus but not a normal fetus (51). Additionally, in other cells from TDII patients, such as fibroblasts and lymphoid cells in which FGFR3 signaling may normally be coupled to mitogenesis, the level of expression and constitutive activation of the K650E receptor may not be sufficient to stimulate unregulated proliferation, consistent with our observation that the FL-Kin(Act) FGFR3 derivative does not transform NIH 3T3 cells.

We observed that partial activation of FGFR3 signaling in vitro could be accomplished either by removal of the extracellular and transmembrane domains of the plasma membrane-localized wild-type kinase domain or by the Lys650→Glu mutation in the full-length receptor. Greater levels of promoter activation, as well as mitogenesis and transformation, however, required both the removal of the extracellular and transmembrane domains and an activating mutation in the kinase domain. These multiple regulatory constraints apparently prevent

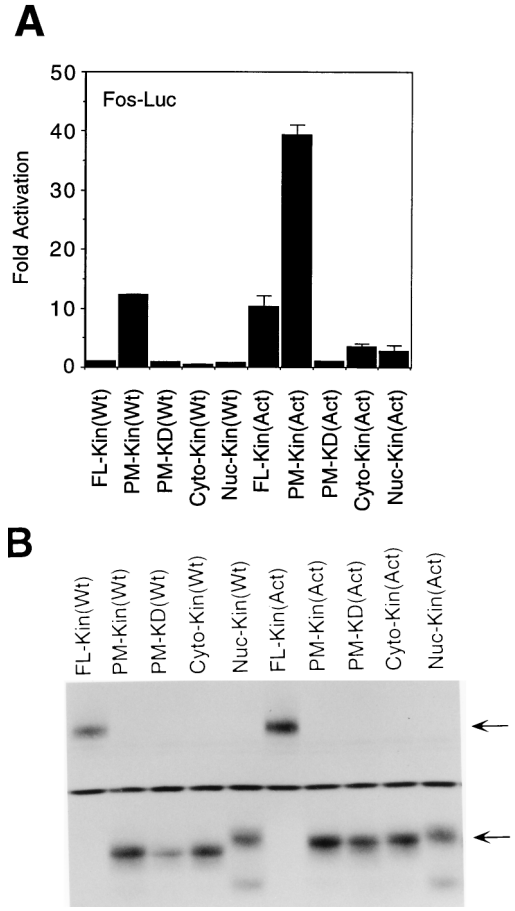

FIG. 4. Induction of the c-*fos* promoter by FGFR3 derivatives. (A) NIH 3T3 cells were cotransfected with the indicated expression plasmids together with a luciferase reporter construct driven by the 700 upstream nucleotides of the c-*fos* promoter, Fos-Luc. After 48 h in low (0.5%) serum, the cells were lysed and luciferase activities were measured. Fold activation represents the average value from each expression construct, compared to the average value from FL-Kin(Wt)-transfected cells. (B) Lysates from cells transfected in parallel were immunoblotted with FGFR3 antiserum to assess the level of expression of each construct, as described in the legend to Fig. 3B.

inappropriate mitogenic signaling through FGFR3 and perhaps explain why oncogenic forms of FGFRs have rarely been observed.

The extracellular and transmembrane domains of FGFR3 provide constraints on the biological activity of the kinase domain. Removal of the extracellular and transmembrane domains of FGFR3 could have several consequences that result in the enhanced signaling observed with the membrane-localized kinase derivatives. It has been proposed that divalent cations and heparan sulfate proteoglycans present in the extracellular matrix, which interact with the extracellular domain of FGFRs, cooperate to maintain the receptors in a conformation that restricts *trans* activation of the intracellular kinase domains (26). Normally, these restrictions would be transiently overcome by the binding of FGFs. Removal of the extracellular domain may mimic ligand effects by allowing juxtaposition and subsequent *trans* activation of the kinase domains (26, 50). Consistent with these observations, the X-ray crystal structure of the FGFR1 kinase domain suggests that dimers and perhaps tetramers may form between isolated FGFR cytoplasmic domains (37). It will be of interest to determine whether the PM-Kin(Wt) and PM-Kin(Act) derivatives are able to signal as monomers or as multimeric complexes.

In addition to increased activation of the kinase domains, removal of the extracellular and transmembrane domains and targeting via a myristylation signal may allow greater lateral movement along the plasma membrane, providing greater access to membrane-localized substrates or perhaps even access to alternative substrates.

In human tumors, chromosomal translocations resulting in fusions between novel proteins and growth factor receptors such as the platelet-derived growth factor receptor (13), Ret (5), and Met (46) have been frequently observed. In these chimeras, various portions of the ligand-binding domain of the normal receptor are replaced with novel sequences, leaving the kinase domain intact. Some of these fusion partners apparently provide motifs that lead to dimerization of the fusion protein and activation of the tyrosine kinase domain (46, 48). It is not clear for some of these oncogenic fusion proteins whether the subcellular localization of the kinase domain is also altered. In the case of the v-*erbB* oncogene, the EGFR kinase domain remains anchored at the plasma membrane through its transmembrane domain, although much of the extracellular domain is deleted, and additionally, there are activating mutations within the kinase domain (15). For both the v-*erbB* oncogene and, as shown here, FGFR3, the loss of the normal ligand-binding domain together with activating mutations within the kinase domain is required for maximal in vitro transforming activity.

The FGFR3 kinase domain mediates proliferative signals from the plasma membrane. Growth factor receptor signaling has traditionally been thought to initiate exclusively at the plasma membrane, with internalization of ligand and receptor serving primarily to downregulate the biological signal. Initiation of DNA synthesis in response to FGF, however, requires continuous exposure to ligand during the entire G_1 phase of the cell cycle (63), during which activated FGFRs have been demonstrated to stably relocalize to intracellular compartments. For instance, FGFR1 is translocated from the cell sur-

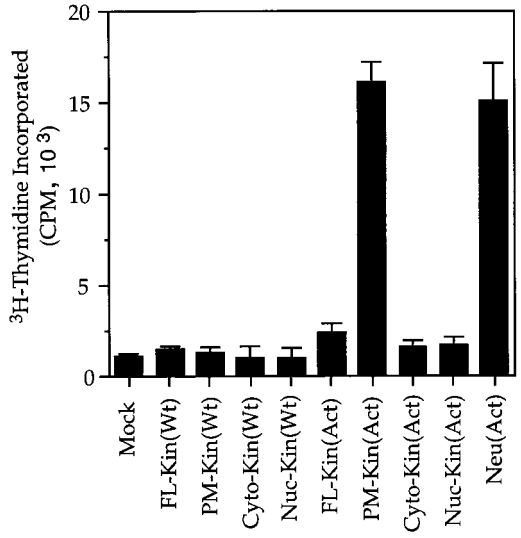

FIG. 5. DNA synthesis by cells transiently transfected with FGFR3 derivatives. NIH 3T3 cells expressing each FGFR3 construct or p185^{neu} [Neu(Act)] were starved for 48 h and incubated with [^{3}H]thymidine for 6 h, and the radioactivity incorporated into DNA was measured. Values shown are the averages for triplicate samples. The PM-Kin(Act) derivative of FGFR3 strongly induced DNA synthesis.

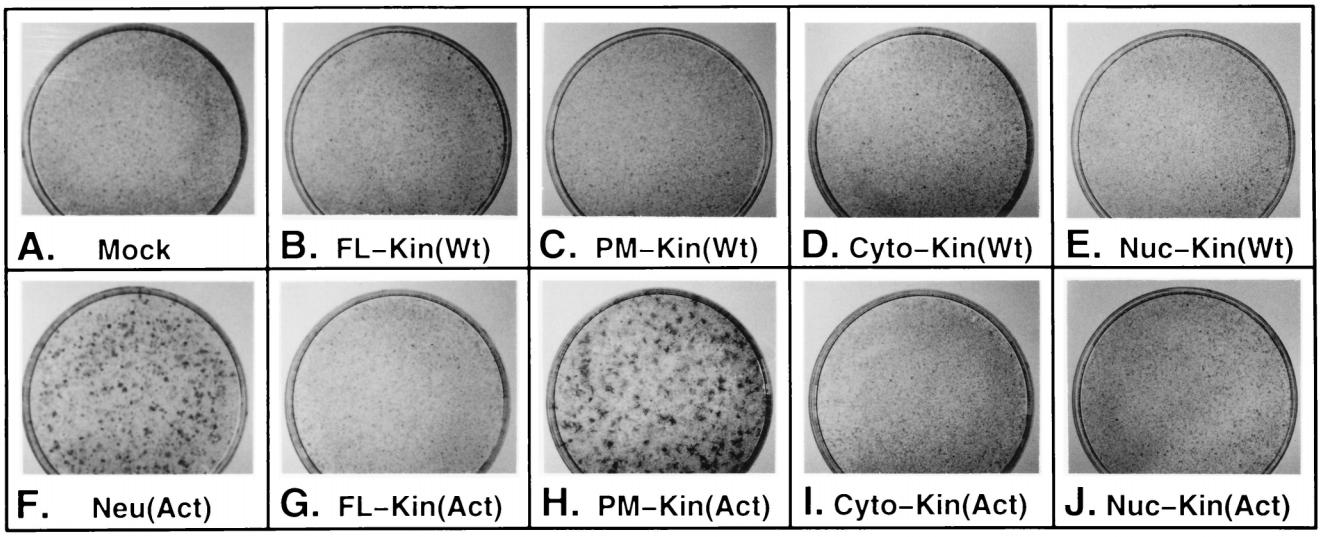

FIG. 6. Focus formation assay. NIH 3T3 cells were transfected with the indicated expression constructs, and after 14 days, the cells were fixed with methanol, stained with Giemsa stain, and photographed. Expression of PM-Kin(Act) (H) and Neu(Act) (F) resulted in the formation of numerous foci of transformed cells.

face to the nucleus in Swiss 3T3 cells in response to FGF-2 (35) and to the perinuclear region in NIH 3T3 cells in response to FGF-1 (43, 44). It has been suggested that these activated receptors may directly play a role in regulating gene expression from these intracellular locations (20, 35, 43). Alternatively, the translocation of FGFRs in response to ligand binding may primarily provide a means of internalizing FGF ligands, which themselves have been proposed to play a role in mitogenic signaling from the nucleus (19, 59).

Intracellular isoforms of FGFRs, which presumably possess intact, functional kinase domains, have also been observed. A splice variant of FGFR3 lacking both the signal peptide and the transmembrane domain has been detected in the nucleus in both normal and transformed breast epithelial cells (24). Furthermore, a hypoglycosylated, intracellular isoform of FGFR1 has been observed in embryonic tissues (34), and splice variants of FGFR1 and FGFR4 lacking the signal peptide and first immunoglobulin-like domain, with an intracellular localization, have been predicted (16, 17).

The role of the kinase activity of FGFRs in these intracellular compartments in mitogenesis has been difficult to establish, as ligand is normally required both for kinase activation and for intracellular trafficking of the receptor. The fact that FGFR3 can be constitutively activated by the Lys650→Glu point mutation suggested a means of directly determining whether FGFR tyrosine kinase domains can mediate mitogenic signals from alternative locations within the cell. Our results indicate, however, that plasma membrane localization of FGFR3 is required for transducing signals which lead to the induction of immediate-early gene expression and DNA synthesis. One pathway recently confirmed to be used by FGFR3 to regulate cell proliferation is the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway (27). Activation of FGFR3 was shown to result in the recruitment of two different Grb2/Sos adapter complexes, one involving phosphorylated Shc and the other involving phosphorylation of a 90-kDa protein, 80K-H, and a novel 66-kDa protein (27). Consistent with our observation that FGFR3 mitogenic signaling initiates at the plasma membrane, relocalization of either the Ras activator, Sos (2), or the Ras effector, Raf (30), to the plasma membrane is necessary and sufficient for activation of the MAPK cascade.

Localization of either the wild-type or the mutant kinase domain of FGFR3 to the nucleus or cytoplasm stimulated expression from the c-*fos* promoter only slightly and had no effect on mitogenesis. There may indeed be a role for intracellularly localized FGFR3 splice variants or for FGFR internalization in response to ligand binding, as suggested by others (20, 35, 43), but our results suggest that the kinase activity of these intracellular receptors is not sufficient for stimulation of cell cycle progression.

There is evidence to suggest that many different membrane receptors are expressed both at the cell surface and within the cell (20). For instance, in addition to FGFRs, tyrosine kinase receptors for epidermal growth factor (22) and insulin (42) have been observed to accumulate in the nucleus in response to ligand, as have receptors for growth hormone (31) and interleukin 1 (9). As with FGFRs, it has previously been difficult to dissociate effects of ligand activation and of ligand-dependent receptor trafficking in these systems. The experiments described here suggest a general means of examining the role of isolated receptor cytoplasmic domains in signaling from intracellular compartments.

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