

# Different Tyrosine Autophosphorylation Requirements in Fibroblast Growth Factor Receptor-1 Mediate Urokinase-Type Plasminogen Activator Induction and Mitogenesis

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Among the seven tyrosine autophosphorylation sites identified in the intracellular domain of tyrosine kinase fibroblast growth factor receptor-1 (FGFR1), five of them are dispensable for FGFR1-mediated mitogenic signaling. The possibility of dissociating the mitogenic activity of basic FGF (FGF2) from its urokinase-type plasminogen activator (uPA)-inducing capacity both at pharmacological and structural levels prompted us to evaluate the role of these autophosphorylation sites in transducing FGF2-mediated uPA upregulation. To this purpose, L6 myoblasts transfected with either wild-type (wt) or various FGFR1 mutants were evaluated for the capacity to upregulate uPA production by FGF2. uPA was induced in cells transfected with wt-FGFR1, FGFR1-Y463F, -Y585F, -Y730F, -Y766F, or -Y583/585F mutants. In contrast, uPA upregulation was prevented in L6 cells transfected with FGFR1-Y463/583/585/730F mutant (FGFR1-4F) or with FGFR1-Y463/583/585/730/766F mutant (FGFR1-5F) that retained instead a full mitogenic response to FGF2; however, preservation of residue Y730 in FGFR1-Y463/583/585F mutant (FGFR1-3F) and FGFR1-Y463/583/585/766F mutant (FGFR1-4F<sub>bis</sub>) allows the receptor to transduce uPA upregulation. Wild-type FGFR1, FGFR1-3F, and FGFR1-4F similarly bind to a 90-kDa tyrosine-phosphorylated protein and activate Shc, extracellular signal-regulated kinase (ERK)<sub>2</sub>, and JunD after stimulation with FGF2. These data, together with the capacity of the ERK kinase inhibitor PD 098059 to prevent ERK<sub>2</sub> activation and uPA upregulation in wt-FGFR1 cells, suggest that signaling through the Ras/Raf-1/ERK kinase/ERK/JunD pathway is necessary but not sufficient for uPA induction in L6 transfectants. Accordingly, FGF2 was able to stimulate ERK<sub>1/2</sub> phosphorylation and cell proliferation, but not uPA upregulation, in L6 cells transfected with the FGFR1-Y463/730F mutant, whereas the FGFR1-Y583/585/730F mutant was fully active. We conclude that different tyrosine autophosphorylation requirements in FGFR1 mediate cell proliferation and uPA upregulation induced by FGF2 in L6 cells. In particular, phosphorylation of either Y463 or Y730, dispensable for mitogenic signaling, represents an absolute requirement for FGF2-mediated uPA induction.

## INTRODUCTION

Fibroblast growth factors (FGFs) comprise a family of at least 18 related heparin-binding polypeptides that play an essential role in the regulation of embryogenesis, angiogenesis, differentiation, and cell proliferation (Basilico and Moscatelli, 1992). In particular, basic

FGF (FGF2),<sup>1</sup> one of the prototypes of the family, induces, among other responses, cell proliferation and upregulation of urokinase-type plasminogen activator (uPA) production in different cell types, including endothelial cells and tumor cell lines (Presta *et al.*, 1988,

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<sup>1</sup> Abbreviations used: ERK, extracellular signal-regulated kinase; FGF2, basic fibroblast growth factor; FGFR, FGF receptor; JM, juxtamembrane; MEK, ERK kinase (also referred to as MAPK kinase); TK, tyrosine kinase; uPA, urokinase-type plasminogen activator.

1989). uPA upregulation is involved in different processes in which extracellular proteolysis is required, including cell migration, developmental tissue reorganization, angiogenesis, and invasive growth in normal and pathologic conditions (Dano *et al.*, 1985; Blasi and Verde, 1990; Tienari *et al.*, 1991; Kwaan, 1992).

FGFs interact on the cell surface with membrane-spanning tyrosine kinase FGF receptors (TK-FGFRs) classified as subclass IV (Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993). The first FGFR to be characterized (FGFR1/*flg* [Lee *et al.*, 1989]) is a single membrane-spanning molecule with three extracellular immunoglobulin (Ig)-like domains, an acidic box located between the first and the second Ig-like loop, a transmembrane domain, a juxtamembrane (JM) region, and an intracellular catalytic TK domain split by a 14 amino acid insertion. Since then, three other genes encoding TK-FGFRs have been discovered: FGFR2/*bek* (Dionne *et al.*, 1990), FGFR3 (Keegan *et al.*, 1991), and FGFR4 (Partanen *et al.*, 1991). Several RNA alternative spliced variants that structurally differ in the number of Ig-like loops and/or in the absence of the intracellular domain (soluble forms) were also described for FGFR1 and FGFR2 (Johnson and Williams, 1993). The growth factor binding site of FGFR appears to be located in the second half of the third Ig-like loop; three variants of this region, encoded by different exons, have been described: IIIa, IIIb, and IIIc. The IIIa sequence seems to be unique for the FGFR1-soluble receptor form (Johnson *et al.*, 1991), whereas IIIb and IIIc are found in FGFR1, FGFR2 (Johnson and Williams, 1993), and FGFR3 (Chellaiah *et al.*, 1994) membrane-spanning molecules. Binding studies from several laboratories indicate that IIIc variants show a broad spectrum of ligands (Johnson and Williams, 1993; Ornitz *et al.*, 1996). Indeed, we have shown that the IIIc variants of FGFR1, -2, -3, and -4 are all able to transduce uPA upregulation by FGF-1, FGF2, and FGF-4 in Chinese hamster ovary transfectants (Rusnati *et al.*, 1996).

As a general mechanism, binding of growth factors to cognate TK receptors and subsequent conformational alteration of the extracellular domain leads to receptor oligomerization (Ullrich and Schlessinger, 1990). The interactions between adjacent cytoplasmic domains lead to receptor autophosphorylation and activation of kinase function by allosteric mechanisms (Ullrich and Schlessinger, 1990). Autophosphorylation of TK receptors normally occurs at a conserved tyrosine residue located in the kinase domain that allosterically regulates the  $V_{\max}$  of the receptor and at various tyrosine residues distributed along the intracellular portion (Ullrich and Schlessinger, 1990). Phosphorylated tyrosines serve as docking sites for downstream signal transduction molecules containing either Src-homology 2 or phosphotyrosine-binding domains (Cantley *et al.*, 1991; Koch *et al.*, 1991; Mar-

golis, 1992; Pawson and Schlessinger, 1993; Pawson, 1995; Blaikie *et al.*, 1994; Kavanaugh and Williams, 1994; Kavanaugh *et al.*, 1995; Bork and Margolis, 1995). Thus, the capacity of growth factors to exert a complex array of biological responses on the same cell type is thought to reflect the capacity of different docking transducer proteins to associate with the activated TK receptor, leading to the switch of multiple intracellular signals (Pawson and Schlessinger, 1993).

The multiple signal transduction pathways activated by FGFR1 are not fully elucidated. Recent observations have demonstrated that activation of FGFR1 induces tyrosine phosphorylation of the lipid-anchored docking protein FRS2 that forms a direct complex, as well as an Shp-2 tyrosine phosphatase-mediated complex, with Grb2/Sos, thus linking FGFR1 activation to the Ras/MAPK kinase (also referred to as MEK)/MAPK (also referred to as ERK) pathway (Wang *et al.*, 1996; Kouhara *et al.*, 1997). Moreover, FGFR1 activation leads to tyrosine phosphorylation of 80K-H protein, distinct from FRS2, that also binds to Grb2 (Goh *et al.*, 1996). Furthermore, a novel membrane-associated adapter protein, named SNT-2, has been shown to interact with FGFR1 *in vitro* and to be tyrosine-phosphorylated after FGFR1 stimulation *in vivo* (Xu *et al.*, 1998).

Seven autophosphorylation sites in the intracellular domain of FGFR1 have been identified: Y463, Y583, Y585, Y653, Y654, Y730, and Y766 (Mohammadi *et al.*, 1991, 1996a). Among them, phosphorylated tyrosine 766 has been shown to bind phospholipase C (PLC) $\gamma$  (Mohammadi *et al.*, 1991), although PLC $\gamma$  activation appears to be dispensable for cell proliferation, differentiation, and uPA upregulation induced by FGFs (Mohammadi *et al.*, 1991, 1992; Peters *et al.*, 1992; Spivak-Kroizman *et al.*, 1994; Huang *et al.*, 1995; Roghani *et al.*, 1996). Tyrosines 653 and 654 are crucial instead for receptor kinase activity, and their neutralization hampers receptor autophosphorylation (Mohammadi *et al.*, 1996a). Simultaneous multiple mutations of the autophosphorylation sites Y463, Y583, Y585, and Y730 do not perturb the ability of FGFR1 to mediate cell proliferation and differentiation (Mohammadi *et al.*, 1996a). The biological function(s) of these sites and their relationship with FGFR1-activated signal transduction pathways therefore remain undefined.

Previous results in our laboratory had shown that the mitogenic activity and the uPA-inducing capacity of FGF2 are mediated by different signal transduction pathways in cultured endothelial cells (Presta *et al.*, 1989). We observed also that various FGF2 mutants devoid of the capacity to upregulate uPA production still retain receptor-binding activity and full mitogenic capacity (Isacchi *et al.*, 1991; Presta *et al.*, 1992, 1993). Accordingly, we demonstrated that the interaction of FGF2 with FGFR1 is quantitatively and qualitatively different in mediating mitogenicity and uPA upregu-

lation in endothelial cells. Nevertheless, TK activity of FGFR1 is essential for both cellular responses (Rusnati *et al.*, 1996).

uPA gene expression induced by FGF2 in 3T3 fibroblasts requires the activation of the Ras/Raf-1/MEK/ERK<sub>2</sub>/JunD pathway (Besser *et al.*, 1995). Also, uPA upregulation is impaired in TK-deficient FGFR1-Y653/654F L6 transfectants (Roghani *et al.*, 1996). In the present work, we investigated the role played by different FGFR1 autophosphorylation sites in mediating FGF2-induced uPA upregulation. To this purpose, L6 myoblasts transfected with various FGFR1 mutants were evaluated for the capacity to upregulate uPA production after stimulation by FGF2. The results demonstrate that the uPA-inducing activity of FGF2 depends on tyrosine phosphorylation events in FGFR1 that include residues Y463 and Y730. Simultaneous mutagenesis of these residues abolishes FGF2-mediated uPA upregulation without affecting the mitogenic activity of the growth factor. Our findings shed a new light on the mechanism(s) responsible for the dissociation of the mitogenic activity of FGF2 from its uPA-inducing capacity.

## MATERIALS AND METHODS

### Site-directed Mutagenesis and Generation of Stable Cell Lines

Site-directed mutagenesis of FGFR1 cDNA was performed according to the protocol of the manufacturer (Amersham, Arlington Heights, IL). Oligonucleotide sequences and generation of single and multiple mutations on FGFR1 have already been described (Mohammadi *et al.*, 1996a). FGFR1-Y463/730F and FGFR1-Y583/585/730F mutants were generated by replacing the wild-type *Bst*EII fragment in FGFR1-Y730F with the corresponding mutagenized fragments from FGFR1-Y463F and FGFR1-Y583/585F mutants, respectively. All of the cDNAs were subcloned in pMJ30 and used together with pSV2neo to transfect L6 myoblasts by the calcium phosphate precipitation method (Chen and Okayama, 1987). G418-resistant colonies were screened for expression of FGFR1 by binding assay with <sup>125</sup>I-labeled FGF2 as a specific probe. Cell lines expressing similar levels of FGF receptors were used for further analysis. L6 parental and transfected cells were grown in DMEM supplemented with 10% FCS.

### [<sup>3</sup>H]thymidine Incorporation and Cell Proliferation Assays

Cells were seeded in 48-well plates at 20,000 cells/cm<sup>2</sup> in DMEM plus 10% FCS. After 48 h of serum starvation in DMEM plus 0.1% FCS, either FGF2 (30–100 ng/ml) or 10% FCS as a control was added to the wells. Twenty hours later, cells were incubated with [<sup>3</sup>H]thymidine (1 μCi/ml), and after an additional period of 6 h samples were directly precipitated in 5% trichloroacetic acid and incubated at 4°C for 1 h. Then cells were lysed in 0.5 M sodium hydroxide, and after neutralization with 1:10 (vol/vol) of 5 M hydrochloric acid and addition of liquid scintillator, they were analyzed for the amount of [<sup>3</sup>H]thymidine incorporated in a counting device (Beckman, Fullerton, CA). For the cell proliferation assay, cells were seeded in 96-well plates at 20,000 cells/cm<sup>2</sup> in DMEM plus 10% FCS. After 24 h of serum starvation in DMEM plus 0.1% FCS, cells were stimulated for 2 d with increasing concentrations of FGF2. Then cell number was estimated by a colorimetric assay after staining with

crystal violet. Briefly, cells were fixed for 20 min at room temperature with 2.5% glutaraldehyde, stained with 0.1% crystal violet in 20% methanol, and solubilized with 10% acetic acid. Then wells were read at 595 nm in a microplate reader against a calibration curve set up with a known number of cells.

### Plasminogen Activator and Northern Blot Assays

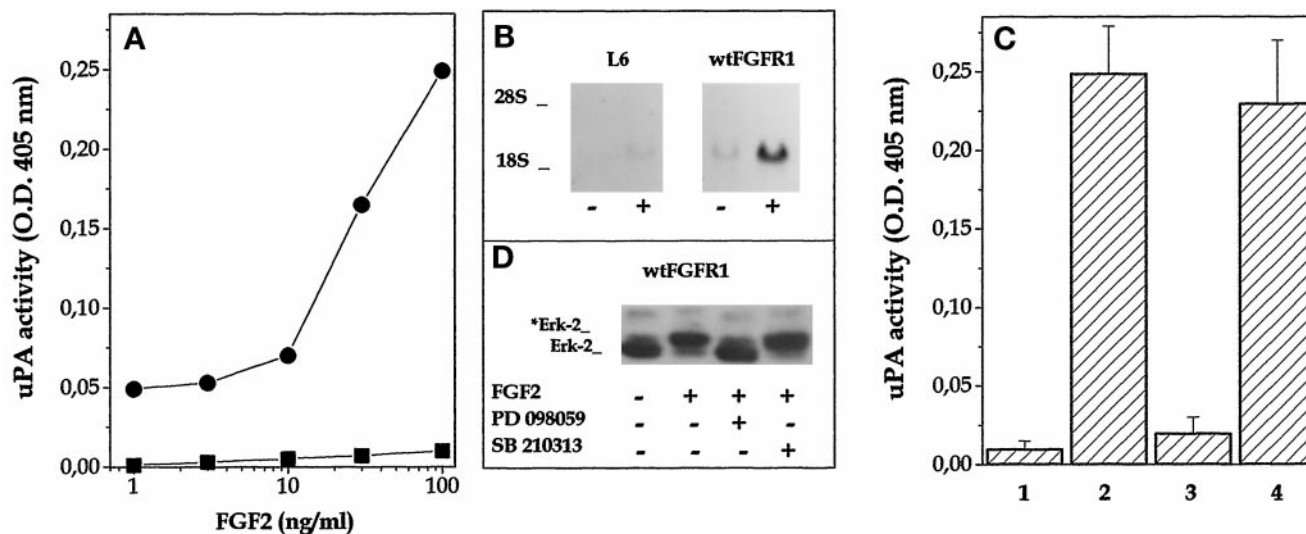
To evaluate the uPA-inducing activity of FGF2, L6 transfectants were seeded at 50,000 cells/cm<sup>2</sup> in DMEM containing 10% FCS. Twenty-four hours later, medium was replaced with DMEM plus 0.5% FCS in the absence or presence of FGF2. The day after, cell-associated uPA activity was measured using the plasmin chromogenic substrate H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide-acetate (American Diagnostica, Greenwich, CT) as described (Presta *et al.*, 1989). Steady-state levels of uPA mRNA were evaluated by Northern blot analysis of total RNA (20 μg/sample) according to standard procedures (Chomczynski and Sacchi, 1987; Sambrook *et al.*, 1989) using a murine uPA probe (kindly provided by P. Mignatti, New York University).

### Immunoprecipitation and Immunoblot Analysis

Cells expressing wild-type or mutant receptors were grown to 80–90% confluency in 10-cm-diameter dishes, treated with FGF2 without changing the medium, and maintained at 37°C for the indicated periods of time. At the end of the incubation, cells were washed briefly with ice-cold PBS and lysed in 1 ml of lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 1 mM sodium pyrophosphate). Cell lysates were centrifuged for 10 min at 15,000 × g, and protein concentration was determined. For immunoprecipitation, 1-mg aliquots of each sample were incubated overnight with the appropriate antibody and protein A-Sepharose. Immunocomplexes were washed three times with HNTG (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100) and then resuspended in 4× SDS-PAGE sample buffer and boiled for 5 min. For ERK<sub>2</sub> gel-shift analysis, 50-μg aliquots of each sample were directly boiled for 5 min in 4× SDS-PAGE sample buffer. All of the samples were then subjected to SDS-PAGE under reducing conditions. The gel was prepared according to Sambrook *et al.* (1989) except for ERK<sub>2</sub> shift analysis in which the pH of the separating gel was changed from 8.8 to 8.3 (Besser *et al.*, 1995). Proteins were transferred electrophoretically onto a PolyScreen polyvinylidene difluoride transfer membrane (New England Nuclear Life Science, Boston, MA) and immunoblotted with different antibodies. Membranes were incubated sequentially with horseradish peroxidase-conjugated secondary antibodies and with Renaissance chemiluminescence reagents (New England Nuclear Life Science) according to manufacturer's instructions and then exposed to Reflection films (New England Nuclear Life Science). The following antibodies were used in this study: anti-FGFR1 (C-15), anti-Jun-D, and antiphosphorylated ERK<sub>1/2</sub> antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Shc antibody was a gift from P.G. Pelicci (European Institute of Oncology, Milan, Italy); anti-ERK<sub>2</sub> antibody was a gift of Y. Nagamine (Friedrick Miescher Institute, Basel, Switzerland); and antiphosphotyrosine antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY).

### ERK<sub>2</sub> In Vitro Kinase Assay

Cells expressing wild-type or mutant receptors were plated in a 60-mm dish (1.4 × 10<sup>6</sup> cells/dish), and 6 h later medium was changed to DMEM containing 1 mg/ml bovine serum albumin and 1 mg/ml transferrin. The day after, cells were treated for 20 min with 10 ng/ml FGF2 at 37°C and then lysed in ERK lysis buffer (50 mM β-Na glycerophosphate, 1.5 mM EGTA, pH 8.5, 2 mM sodium orthovanadate, 1 μM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml



**Figure 1.** FGF2-mediated uPA upregulation in FGFR1-transfected L6 cells requires ERK<sub>2</sub> activation. (A) Parental (■) and wt-FGFR1-transfected cells (●) were stimulated with different amounts of FGF2. Cell-associated uPA activity was evaluated after 24 h. For both cell lines, uPA activity measured in nonstimulated cells (ranging from 0.1 to 0.2 OD at 405 nm) was subtracted from all the values. (B) Northern blot analysis was performed using a murine uPA probe on total RNA extracted from parallel cultures incubated overnight with 10 ng/ml FGF2. Uniform loading of samples was judged by methylene blue staining of the filter. (C) wt-FGFR1-transfected cells were preincubated for 30 min at 37°C with no addition (1, 2) or with 100 μM PD 098059 (3) or 10 μM SB 210313 (4) before addition of 100 ng/ml FGF2 (2–4). After 24 h, cell-associated uPA activity was measured. uPA activity in control nontransfected L6 cells incubated under the same experimental conditions was subtracted from all the values. (D) Parallel cultures were lysed 20 min after FGF2 addition, and cell lysates were probed with anti-ERK<sub>2</sub> antibody in a Western blot. \*Erk-2 indicates the phosphorylated protein.

aprotinin, 1 μM benzamidine, 1% Nonidet P-40). Lysates were centrifuged for 10 min at 15,000 × *g*, and protein concentration was determined. Cell lysates (200 μg) were incubated with anti-ERK<sub>2</sub> antiserum and protein A-Sepharose for 2 h at 4°C. The immunoprecipitates were subjected to an *in vitro* kinase assay. The reaction was performed for 30 min at 37°C in 30 mM Tris, pH 8.0, 20 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 10 μM ATP, 15 μg per assay of myelin basic protein, and 0.1 μCi/sample of [<sup>32</sup>P]-labeled γATP. Samples were then boiled for 5 min in 4× SDS-PAGE sample buffer and subjected to SDS-PAGE (15%). The gel was dried and exposed to Reflection film.

## RESULTS

### *ERK<sub>1/2</sub> Activation and FGF2-mediated uPA Upregulation in FGFR1-transfected L6 Cells*

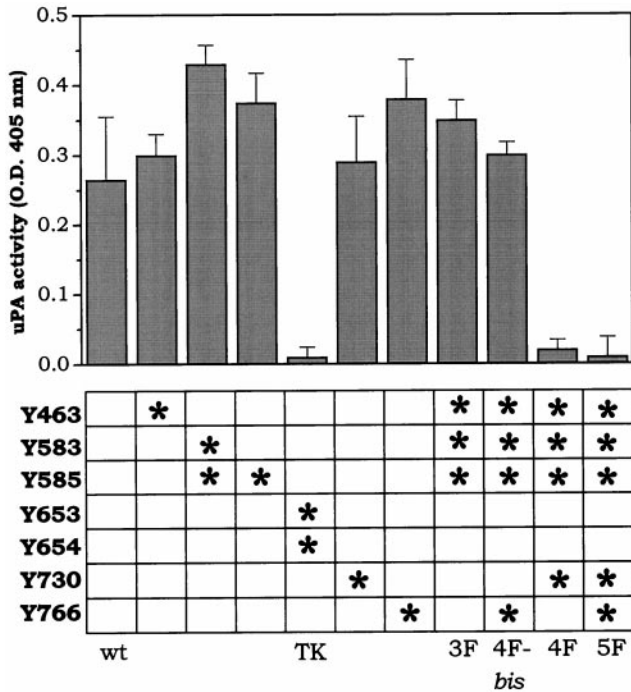
In agreement with previous observations (Roghani *et al.*, 1996), FGF2 induces a dose-dependent increase of cell-associated uPA activity in L6 cells transfected with wild-type FGFR1 cDNA (wt-FGFR1 cells) with an ED<sub>50</sub> equal to 20 ng/ml (Figure 1A). Accordingly, an increase in steady-state levels of uPA mRNA was observed in wt-FGFR1 transfectants after 24 h of treatment with 10 ng/ml FGF2 (Figure 1B). uPA upregulation by FGF2 was prevented in L6 transfectants incubated with 100 μM of the TK inhibitor 23 tyrphostin. On the contrary, the uPA-inducing activity exerted by FGF2 in wt-FGFR1 cells was not affected by down-regulation of PKC induced by a 16-h pretreatment with 500 ng/ml 12-*O*-tetradecanoyl phorbol 13-acetate

or by incubation with 50 μM of the protein kinase inhibitor *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide H-8 (Ido *et al.*, 1991) (our unpublished results).

The requirement for the activation of the Ras/Raf/MEK/ERK<sub>2</sub> pathway had been demonstrated for FGF2-mediated uPA upregulation in 3T3 fibroblasts (Besser *et al.*, 1995). To assess whether this holds true also for wt-FGFR1 transfectants, cells were incubated with FGF2 in the absence or presence of the MEK1 inhibitor PD 098059 (Alessi *et al.*, 1995). PD 098059 treatment prevents both ERK<sub>2</sub> phosphorylation and uPA upregulation in L6 cells (Figure 1, C and D). Specificity of the inhibition was demonstrated by the lack of effect of the p38 MAPK inhibitor SB 210313 (Cuenda *et al.*, 1995). In conclusion, FGF2-mediated uPA induction in L6 transfectants depends on TK-FGFR activity, requires ERK<sub>2</sub> phosphorylation, and is not mediated by PKC activation.

### *uPA Upregulation Is Abolished by Mutagenesis of Distinct FGFR1 Autophosphorylation Sites*

To assess the role of the different tyrosine residues of FGFR1 in uPA upregulation, L6 cells were independently transfected with different receptor mutants in which one or more of the seven identified autophosphorylation sites were mutagenized to phenylalanine



**Figure 2.** uPA-inducing capacity of different FGFR1 mutants. L6 myoblasts were independently transfected with wt-FGFR1 or with the FGFR1 mutants described in the bottom panel. In each column, an asterisk or asterisks mark the tyrosine autophosphorylation site(s) mutagenized to phenylalanine in the different mutants. Each cell line was stimulated with 100 ng/ml FGF2, and 24 h later cell-associated uPA activity was evaluated (top panel). For each cell line, uPA activity measured in the corresponding nonstimulated cells (ranging from 0.1 to 0.2 OD at 405 nm) was subtracted. Results represent the mean  $\pm$  SD of two independent experiments in triplicate. For each mutant, similar results were obtained for two independent clones.

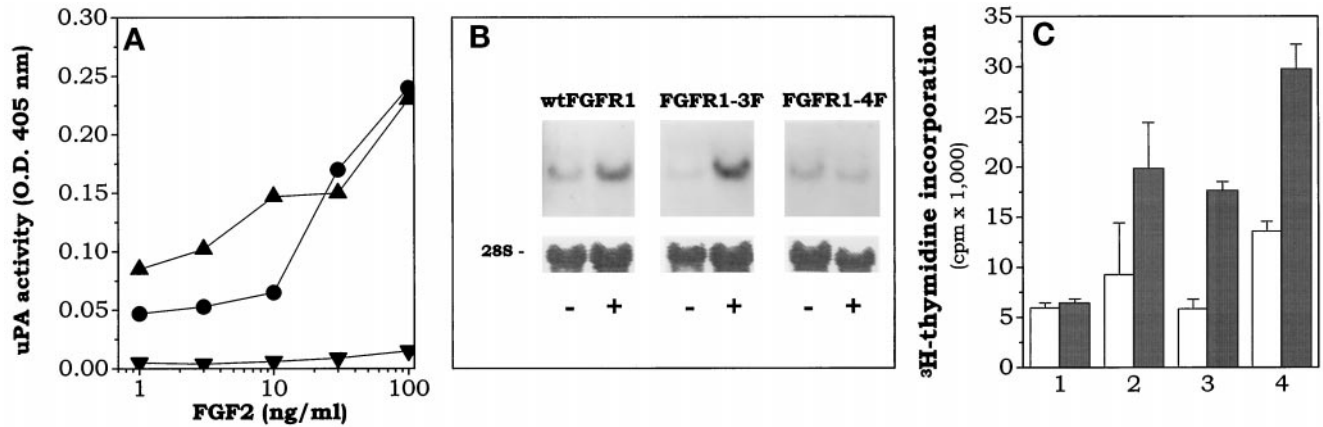
(Mohammadi *et al.*, 1996a). After G418 selection and <sup>125</sup>I-FGF2 binding analysis, two independent clones for each transfection, bearing 40,000–100,000 receptors/cell, were incubated for 24 h with 100 ng/ml FGF2. At the end of the incubation, cell monolayers were lysed, and cell-associated uPA activity was measured. The results are summarized in Figure 2. No uPA upregulation was observed in cells transfected with the TK-defective mutant FGFR1-Y653/654F, whereas single point mutations in autophosphorylation sites Y463, Y585, Y730, or Y766 did not affect the uPA-inducing capacity of FGF2. FGF2 was able to induce a significant increase of cell-associated uPA activity also in cells transfected with FGFR1-Y583/585F mutant, FGFR1-Y463/583/585F mutant (FGFR1-3F cells), or FGFR1-Y463/583/585/766F mutant (FGFR1-4Fbis cells); however, uPA upregulation by FGF2 was hampered in cells transfected with FGFR1-Y463/583/585/730F mutant (FGFR1-4F cells) or with FGFR1-Y463/583/585/730/766F mutant (FGFR1-5F cells).

On this basis, FGFR1-3F and FGFR1-4F clones, which differ for the absence or presence of the Y730F mutation, were analyzed further. FGF-1 induces [<sup>3</sup>H]thymidine incorporation in L6 myoblasts transfected with wt-FGFR1 or FGFR1-4F and FGFR1-5F mutants (Mohammadi *et al.*, 1996a). When [<sup>3</sup>H]thymidine incorporation and cell-associated uPA activity were measured in parallel cultures of wt-FGFR1, FGFR1-3F, and FGFR1-4F cells exposed to FGF2, the results shown in Figure 3 were obtained. As anticipated, FGF2 stimulates DNA synthesis in all cell lines, but only wt-FGFR1 and FGFR1-3F cells respond to the growth factor with an increase in uPA activity. This latter observation was confirmed by Northern blot analysis of total RNA isolated from L6 transfectants that demonstrated a significant increase in the steady-state levels of uPA mRNA in wt-FGFR1 and FGFR1-3F cells incubated with 10 ng/ml FGF2 for 24 h, but not in FGFR1-4F transfectants (Figure 3B). Interestingly, we did not observe any significant differences in the rate of receptor downregulation and FGF2 internalization among wt-FGFR1, FGFR1-3F, and FGFR1-4F transfectants (Dell’Era, unpublished observations). Finally, control experiments demonstrated that FGFR1-4Fbis and FGFR1-5F cells behave similarly to FGFR1-3F and FGFR1-4F transfectants, respectively (our unpublished results).

**Signal Transduction Pathway Analysis of FGFR1-3F and FGFR1-4F Mutants**

When wt-FGFR1, FGFR1-3F, and FGFR1-4F cells were exposed to FGF2, lysed, immunoprecipitated with anti-FGFR1 antibody, and immunoblotted with antiphosphotyrosine antibodies, mutated receptors underwent tyrosine autophosphorylation, albeit to a lesser extent than the wild-type receptor, because of the neutralization of three or four of the autophosphorylation sites (Figure 4B). Also, tyrosine phosphorylation of a receptor-associated 90-kDa protein was observed in all of the cell lines (Figure 4B). This protein does not cross-react with antibodies directed against Stat3 (Faris *et al.*, 1996), 80K-H (Goh *et al.*, 1996), or FRS2 (Kouhara *et al.*, 1997) (our unpublished results). Reprobing the same membrane with anti-FGFR1 antibody confirmed the presence of similar amounts of the receptor in all the samples (Figure 4A). In agreement with previous observations (Mohammadi *et al.*, 1996a), we found that both FGFR1-3F and FGFR1-4F mutants retain the capacity to tyrosine-phosphorylate Shc (Figure 4C).

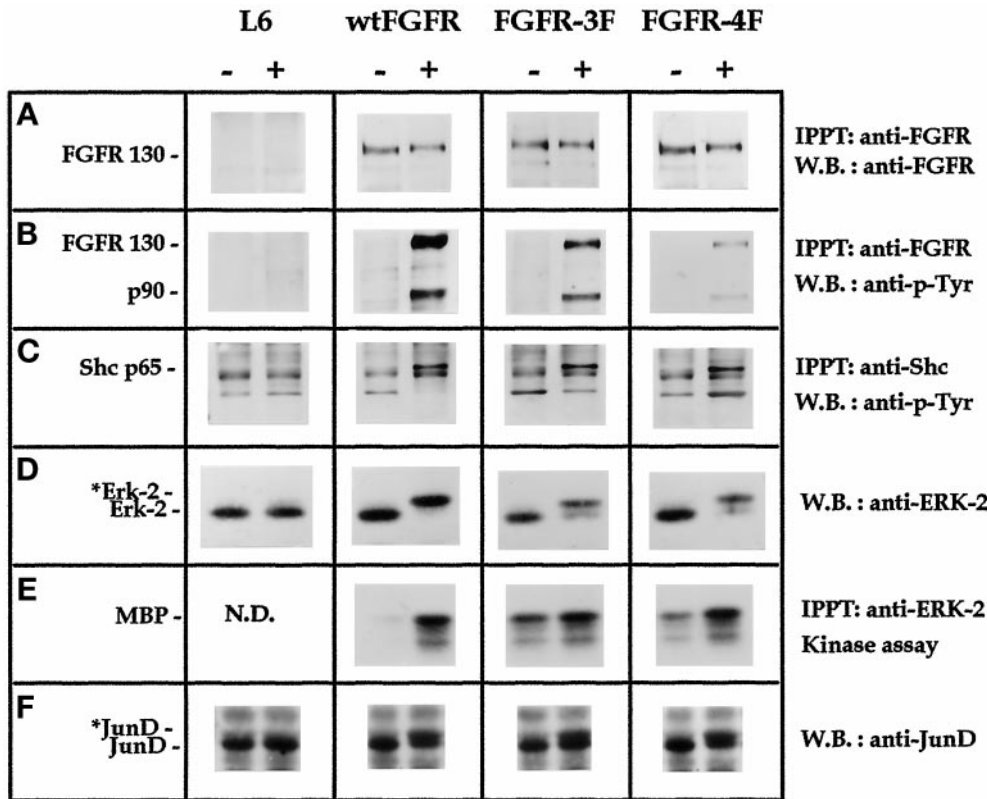
Activation of the Ras/Raf-1/MEK/ERK<sub>2</sub>/JunD pathway is required for uPA upregulation by FGF2 (see above and Besser *et al.*, 1995). On this basis, the capacity of receptor mutants to activate the last two steps of this pathway was investigated after 20 min of incubation of wt-FGFR1, FGFR1-3F, and FGFR1-4F



**Figure 3.** Dissociation of uPA upregulation from mitogenicity in L6 transfectants. (A) wt-FGFR1 (●), FGFR1-3F (▲), and FGFR1-4F (▼) cell lines were stimulated with FGF2. Cell-associated uPA activity was evaluated after 24 h. For each cell line, uPA activity measured in corresponding nonstimulated cells (ranging from 0.1 to 0.2 OD at 405 nm) was subtracted from all the values. (B) Parallel cultures were incubated overnight in the absence or presence of 10 ng/ml FGF2. Then, total RNA was extracted and analyzed by Northern blot using a murine uPA probe. Uniform loading of the gel was judged by methylene blue staining of the filter (28S). (C) Parental L6 myoblasts (1), wt-FGFR1 (2), FGFR1-3F (3), and FGFR1-4F (4) cell lines were seeded in 48-well plates, starved for 48 h in the presence of 0.1% FCS, and treated with 30 ng/ml FGF2 (gray bar) or vehicle (open bar) for 20 h. Then [<sup>3</sup>H]thymidine was added, and 6 h later samples were collected and [<sup>3</sup>H]thymidine incorporation was evaluated. Data are the mean ± SD of three determinations.

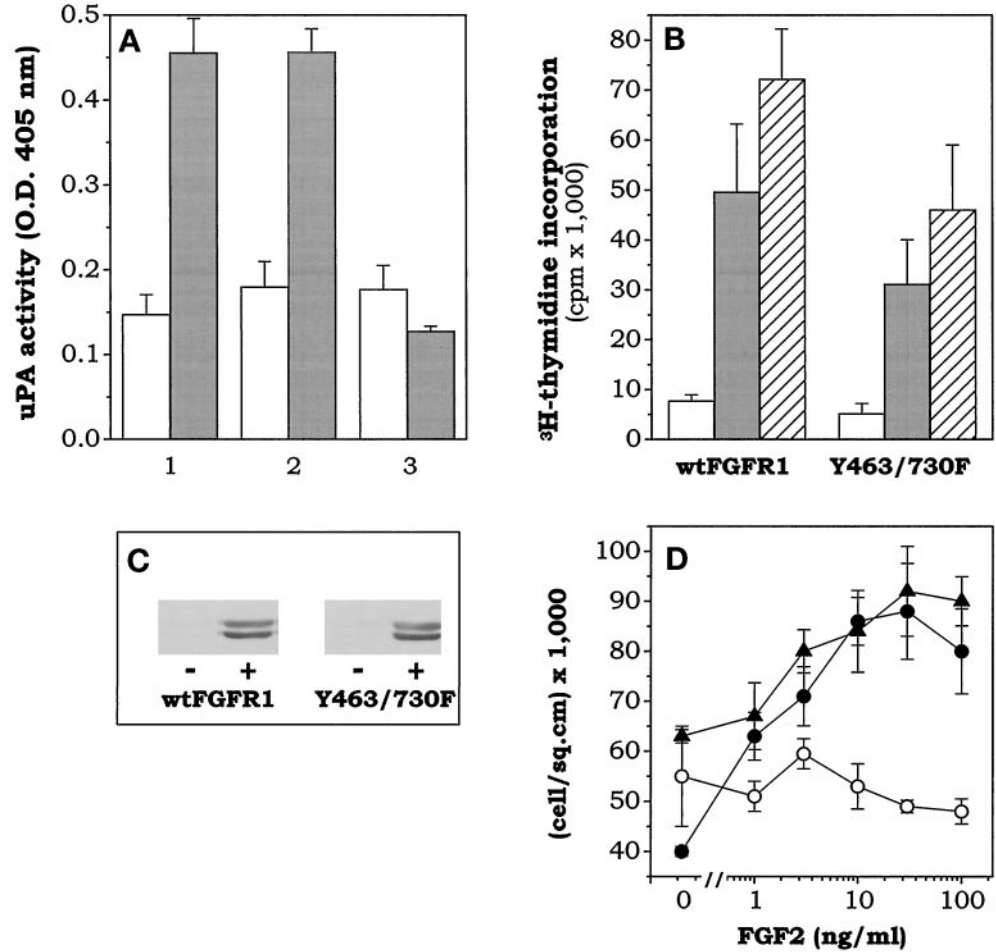
cells with 10 ng/ml FGF2. Activation of ERK<sub>2</sub> was evident in all of the cell lines as a mobility shift of the protein by immunoblotting with anti-ERK<sub>2</sub> antibody

(Figure 4D) and as an increased enzymatic activity in a kinase assay (Figure 4E). Control experiments confirmed that FGF2-activated FGFR1-4F<sub>bis</sub> and



**Figure 4.** Signal transduction analysis of FGFR1 mutants. Parental L6 myoblasts, wt-FGFR1, FGFR1-3F, and FGFR1-4F cells were nonstimulated (-) or treated (+) either for 5 min (A, B, and C) or 20 min (D, E, and F) with 100 ng/ml FGF2. (A) Anti-FGFR1 immunoprecipitates were analyzed for FGFR1 expression using anti-FGFR1 antibodies. (B) Anti-FGFR1 immunoprecipitates were analyzed for tyrosine phosphorylation using antiphosphotyrosine (P-Tyr) antibodies. (C) Anti-Shc immunoprecipitates were analyzed for tyrosine phosphorylation. (D) Cell lysates (50 μg of total protein) were analyzed with anti-ERK<sub>2</sub> antibodies. \*Erk-2 indicates the phosphorylated protein. (E) Anti-ERK<sub>2</sub> immunoprecipitates were analyzed for kinase activity using myelin basic protein (MBP) as substrate. (F) Cell lysates (50 μg of total protein) were analyzed with anti-JunD antibodies. \*JunD indicates the phosphorylated protein. N.D., not determined.

**Figure 5.** Residues Y463 and Y730 are implicated in uPA upregulation but not mitogenesis by FGF2. (A) wt-FGFR1 (1), FGFR1-Y583/585/730F (2), and FGFR1-Y463/730F (3) transfectants were assayed for cell-associated uPA activity after a 24-h incubation in the absence (open bar) or presence (gray bar) of 100 ng/ml FGF2. (B) wt-FGFR1 and FGFR1-Y463/730F transfectants were seeded in 48-well plates, starved for 48 h in the presence of 0.1% FCS, and then treated with no addition (open bar), 100 ng/ml FGF2 (gray bar), or 10% FCS (dashed bar) for an additional 20 h. Then, [<sup>3</sup>H]thymidine was added, and 6 h later samples were collected and [<sup>3</sup>H]thymidine incorporation was evaluated. (C) Parallel cultures were lysed 20 min after FGF2 addition, and cell lysates (30 μg of protein) were probed with antiphosphorylated ERK<sub>1/2</sub> antibody in a Western blot. (D) Parental L6 cells (○), wt-FGFR1 (●), and FGFR1-Y463/730F (▲) transfectants were seeded in 96-well plates at 20,000 cells/cm<sup>2</sup> in DMEM plus 10% FCS. After 24 h of serum starvation in DMEM plus 0.1% FCS, cells were stimulated with increasing concentrations of FGF2. Cells were counted after 2 d. For each mutant, similar results were obtained for two independent clones in all the assays.



FGFR1-5F also undergo autophosphorylation, associate with and phosphorylate the 90-kDa protein, and activate ERK<sub>2</sub> (our unpublished results). Furthermore, FGF2 treatment modifies JunD protein in wt-FGFR1, FGFR1-3F, and FGFR1-4F cell lines (Figure 4F).

**Simultaneous Mutagenesis of Residues Y463 and Y730 in FGFR1 Is Sufficient to Dissociate uPA Induction from Mitogenesis**

The above data point to a role for Y730 phosphorylation in uPA upregulation, although in a complex context attributable to the simultaneous mutation of residues Y463, Y583, and Y585; however, mutation of Y730 alone is not sufficient to abolish FGF2-mediated uPA induction in L6 transfectants (see Figure 2). On this basis, L6 cells were transfected with FGFR1 mutants bearing the mutation Y730F together with the single mutation Y463F or with the double mutation Y583/585F. As shown in Figure 5, FGF2 was unable to cause a significant increase of uPA activity in FGFR1-Y463/730F cells, whereas FGFR1-Y583/585/730F cells were fully responsive. Neverthe-

less, in agreement with the results obtained with FGFR1-4F and FGFR1-5F mutants, both FGFR1-Y463/730F and FGFR1-Y583/585/730F mutants were able to stimulate ERK<sub>1/2</sub> phosphorylation and DNA synthesis in response to FGF2. Moreover, FGF2 exerted a dose-dependent mitogenic response in FGFR1-Y463/730F transfectants similar to that elicited in wt-FGFR1 cells (Figure 5D). In conclusion, phosphorylation of residues Y463 and Y730 is dispensable for mitogenic signaling but essential for uPA upregulation by FGF2 in FGFR1 transfectants.

**DISCUSSION**

FGF2 induces, among other responses, cell proliferation and uPA upregulation in different cell types, including endothelial cells (Presta *et al.*, 1989; Rusnati *et al.*, 1996), NIH 3T3 fibroblasts (Besser *et al.*, 1995), and tumor cell lines (Coltrini *et al.*, 1995). Previous results in our laboratory had shown that the mitogenic activity and the uPA-inducing capacity of FGF2 are medi-

ated by distinct signal transduction pathways in cultured endothelial cells (Presta *et al.*, 1989). We observed also that site-directed mutagenesis of FGF2 leads to growth factor mutants that retain full receptor binding and mitogenic activity but are devoid of the capacity to upregulate uPA production (Isacchi *et al.*, 1991; Presta *et al.*, 1992, 1993). Finally, we demonstrated that the interaction of FGF2 with endothelial cell FGFR1 that is able to elicit a mitogenic response is quantitatively and qualitatively different from uPA upregulation requirements (Rusnati *et al.*, 1996).

The results presented here suggest that the observed dissociation between mitogenic activity and uPA-inducing capacity of FGF2 reflects different tyrosine autophosphorylation requirements of FGFR1. Indeed, FGFR1-Y463/730F, FGFR1-4F, and FGFR1-5F mutants retain the capacity to mediate the FGF2 mitogenic signal, although they have lost the ability to stimulate uPA upregulation. It must be pointed out that the inhibition of the uPA-inducing activity of these FGFR1 mutants does not appear to be the mere consequence of a nonspecific decrease of the overall tyrosine autophosphorylation capacity of FGFR1. Indeed, FGFR1 mutants bearing three (FGFR1-Y583/585/730F) or four (FGFR1-4F*bis*) tyrosine substitutions are still able to transduce uPA upregulation in L6 cells, whereas the simultaneous mutation of residues Y463 and Y730 is sufficient to abolish this activity.

Residues Y653 and Y654 are essential for the TK activity of FGFR1, whereas Y766 has been shown to bind PLC $\gamma$  (Mohammadi *et al.*, 1991, 1996a). Previous observations showed that the simultaneous mutation Y653/654F hampers FGF2-mediated uPA induction in L6 transfectants, whereas mutation Y766F is ineffective (Roghani *et al.*, 1996). Our results confirm and extend these observations by showing that also mutations Y585F and Y583/585F do not affect uPA upregulation by FGF2. Residues Y583 and Y585 belong to the kinase insert domain of FGFR1. A FGFR1 chimera in which the kinase insert of FGFR1 was replaced by the kinase insert of PDGF receptor (kindly provided by L. Claesson-Welsh, Uppsala, Sweden) retains the capacity to mediate uPA upregulation in stable Chinese hamster ovary transfectants (our unpublished observations), although no homology in amino acid sequences flanking autophosphorylated tyrosine residues exists between the kinase insert domains of the two receptors. These findings indicate that autophosphorylation sites Y583 and Y585, like Y766, are dispensable for uPA induction by FGF2.

Our data point instead to a role for residues Y463 and Y730 in uPA upregulation. This conclusion is inferred from the observation that among the mutants tested, only those characterized by the simultaneous mutation of both residues (FGFR1-Y463/730F, FGFR1-4F, and FGFR1-5F) were unable to upregulate uPA in response to FGF2. In contrast, the mutation of

only one of them, as it occurs in FGFR1-Y463F, FGFR1-Y730F, FGFR1-Y583/585/730F, FGFR1-3F, and FGFR1-4F*bis*, as well as FGFR1-Y463/766F (Dell'Era, unpublished observations), does not affect the uPA-inducing capacity of the receptor. Thus, our results suggest a redundancy in FGFR1 tyrosine residues implicated in uPA upregulation, autophosphorylation sites Y463 and Y730 being able to sustain uPA induction despite clear differences in their flanking amino acid sequences. Similar conclusions have been drawn for activation of STAT-5 nuclear import and DNA binding activity mediated by interleukin-2 receptor (Gaffen *et al.*, 1996).

Y463 is located in the JM domain of FGFR1. Autophosphorylation sites in the JM region of insulin, PDGF, and nerve growth factor receptors have been shown to be involved in signal transduction (White *et al.*, 1988; Mori *et al.*, 1993; Obermeier *et al.*, 1994). Recently, the JM domain of FGFR1 has been demonstrated to play an important role in FGF1-mediated neurite outgrowth in PC12 cells transfected with a FGFR1 JM domain-FGFR3 chimera (Lin *et al.*, 1998). This receptor chimera also causes a sustained phosphorylation of ERK<sub>2</sub> and FRS2 protein without affecting cell proliferation; however, this capacity appears to be independent of Y463 phosphorylation (Lin *et al.*, 1998). Also, the yeast two-hybrid protein-protein interaction assay has demonstrated the capacity of FRS2 to interact with a JM region of FGFR1 lacking Y463 (Xu *et al.*, 1998). Accordingly, no significant differences in FRS2 phosphorylation was observed in Grb2 immunoprecipitates from lysates of wt-FGFR1 and FGFR1-Y463F transfectants (Dell'Era, unpublished observations).

FGFR1, FGFR2, FGFR3, and FGFR4 are all able to mediate FGF2-induced uPA upregulation in stable Chinese hamster ovary transfectants (Rusnati *et al.*, 1996). Accordingly, Y730 is conserved in the four FGFRs in all of the species cloned so far. This residue has a YMXM motif that resembles the consensus binding site for the SH2 domain of the regulatory subunit of PI 3-kinase; however, neither direct association of PI 3-kinase with FGFR nor its activation after ligand binding has been demonstrated (Wennstrom *et al.*, 1992). It has been proposed that the Y730 counterpart in chicken FGFR (Y728) is a minor site for Shc binding (Ward *et al.*, 1996). Nevertheless, FGF2 causes Shc phosphorylation in FGFR1-4F cells. Finally, crystallography of the kinase domain of FGFR1 has shown that Y730 is buried (Mohammadi *et al.*, 1996b), suggesting that autophosphorylation of this site requires a conformational change of the intracellular portion of FGFR1 after ligand interaction. It is possible that the modality of ligand/receptor interaction may decide the exposure and consequent phosphorylation of Y730, possibly explaining the inability of various mi-



togenic FGF2 mutants to induce uPA upregulation (Isacchi *et al.*, 1991; Presta *et al.*, 1992, 1993).

The activation of the Ras/Raf/MEK/ERK<sub>2</sub>/JunD pathway is required for FGF2-mediated uPA upregulation in NIH 3T3 cells that constitutively express FGFR1 (Besser *et al.*, 1995). Accordingly, we have observed that the MEK1 inhibitor PD 098059 (Alessi *et al.*, 1995) prevents ERK<sub>2</sub> phosphorylation and uPA upregulation in wt-FGFR1 transfectants, thus indicating that ERK<sub>2</sub> activation is required for uPA induction also in L6 cells. The same pathway is activated by FGF2 in FGFR1-Y463/730F, FGFR1-4F, and FGFR1-5F cells; nevertheless, uPA is not induced. This observation suggests that ERK<sub>2</sub> activation is necessary but not sufficient for FGF2-induced uPA upregulation and that the activation of a second as yet unidentified signal transduction pathway might be required. This hypothesis is in keeping with the incapacity of FGF2 to induce uPA production, as well as fibrin gel invasion, in murine aortic endothelial cell cultures, although they respond to FGF2 with a rapid activation of ERK<sub>2</sub> and cell proliferation (Bastaki *et al.*, 1997). Also, we have found that various mitogenic FGF2 mutants, characterized by the incapacity to induce uPA upregulation in endothelial cells (Isacchi *et al.*, 1991; Presta *et al.*, 1992, 1993), are still able to stimulate ERK<sub>1/2</sub> phosphorylation in a manner similar to the wild-type growth factor (Presta and Bastaki, unpublished observations).

In conclusion, we have demonstrated the possibility of dissociating the mitogenic activity of FGF2 from its uPA-inducing capacity by mutational analysis of the seven characterized autophosphorylation sites in FGFR1. Residues Y463 and Y730, dispensable for cell proliferation, are implicated in uPA upregulation by FGF2. To our knowledge, our findings represent the first demonstration for a role of these tyrosine residues in FGFR1 activity.

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