

The Kinase Activity of Fibroblast Growth Factor Receptor 3 with Activation Loop Mutations Affects Receptor Trafficking and Signaling*

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Amino acid substitutions at the Lys-650 codon within the activation loop kinase domain of fibroblast growth factor receptor 3 (FGFR3) result in graded constitutive phosphorylation of the receptor. Accordingly, the Lys-650 mutants are associated with dwarfisms with graded clinical severity. To assess the importance of the phosphorylation level on FGFR3 maturation along the secretory pathway, hemagglutinin A-tagged derivatives were studied. The highly activated SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans) mutant accumulates in its immature and phosphorylated form in the endoplasmic reticulum (ER), which fails to be degraded. Furthermore, the Janus kinase (Jak)/STAT pathway is activated from the ER by direct recruitment of Jak1. Abolishing the autocatalytic property of the mutated FGFR3 by replacing the critical Tyr-718 reestablishes the receptor full maturation and inhibits signaling. Differently, the low activated hypochondroplasia mutant is present as a mature phosphorylated form on the plasma membrane, although with a delayed transition in the ER, and is completely processed. Signaling does not occur in the presence of brefeldin A; instead, STAT1 is activated when protein secretion is blocked with monensin, suggesting that the hypochondroplasia receptor signals at the exit from the ER. Our results suggest that kinase activity affects FGFR3 trafficking and determines the spatial segregation of signaling pathways. Consequently, the defect in down-regulation of the highly activated receptors results in the increased signaling capacity from the intracellular compartments, and this may determine the severity of the diseases.

Protein tyrosine kinase receptors play a fundamental role in the control of a variety of cellular processes during embryonic development; furthermore, they regulate many metabolic and physiological processes in a variety of tissues and organs (1–3). Indeed, aberrant receptor kinase activation results in severe diseases such as cancer, diabetes, cardiovascular diseases, and many others (4). Fibroblast growth factor receptors (FGFRs)¹

belong to the tyrosine kinase receptor (RTK) family. FGFRs are glycosylated transmembrane proteins that, upon binding with fibroblast growth factor ligands and heparin, dimerize and undergo interchain autophosphorylation of key tyrosine residues, thus transmitting signals into the cells (5–8). Tyrosine autophosphorylation sites in the cytoplasmic domain of the receptor serve as docking sites for the Src homology 2 domain of signaling proteins that are recruited and activated upon growth factor stimulation (9). FGFR3 is a member of the FGFR family and plays a pivotal role in skeletal development as a negative regulator of bone growth, because targeted disruption of the mouse FGFR3 gene causes a skeletal overgrowth (10, 11). In addition, mutations in FGFR3 are frequently involved in a variety of human skeletal disorders and cancer (12, 13).

Particular attention has been paid to the Lys-650 codon of FGFR3 located within a critical region of the tyrosine kinase domain activation loop. Amino acid substitutions at this codon result in the autophosphorylation of multiple tyrosine residues within the intracellular domain (14). Moreover, the identification of specific tyrosine residues involved in the activation of signal transducers and activators of transcription (STAT) proteins and mitogenesis has revealed the critical role exerted by tyrosine 724, whose substitution with phenylalanine abolishes receptor tyrosine phosphorylation (8). Interestingly, mutations at the Lys-650 codon are responsible for developmental disorders that display progressively increasing clinical severity (15). Two missense mutations, K650E and K650M, result in the strong constitutive activation of the FGFR3 tyrosine kinase and are associated with thanatophoric dysplasia type II (TDII), and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), two severe dwarfisms (16, 17). A third substitution K650N, although causing to a lesser degree the constitutive kinase activity of FGFR3, gives rise to the relatively mild hypochondroplasia (HCH) (18) (see Fig. 1A for a schematic representation). Although the clinical phenotypes of the diseases associated with the Lys-650 codon substitutions have been clearly characterized, the molecular bases that explain such diverse consequences remain to be elucidated. Based on our recent observation showing that the TDII mutation (K650E) hampers FGFR3 maturation (19), we have considered the hypothesis that different levels of receptor tyrosine phosphorylation could affect FGFR3 maturation differently along the secretory pathway, thus causing receptor-mediated signaling to be delivered from internal membranes.

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¹ The abbreviations used are: FGFR, fibroblast growth factor receptor; ACH, achondroplasia; BFA, brefeldin A; Endo H, endoglycosidase

H; ER, endoplasmic reticulum; GFP, green fluorescent protein; HA, hemagglutinin A; HCH, hypochondroplasia; HEK293, human embryonic kidney 293 (cells); Jak, Janus kinase; SADDAN, severe achondroplasia with developmental delay and acanthosis nigricans; STAT, signal transducers and activators of transcription; TDII, thanatophoric dysplasia type II; wt, wild type.

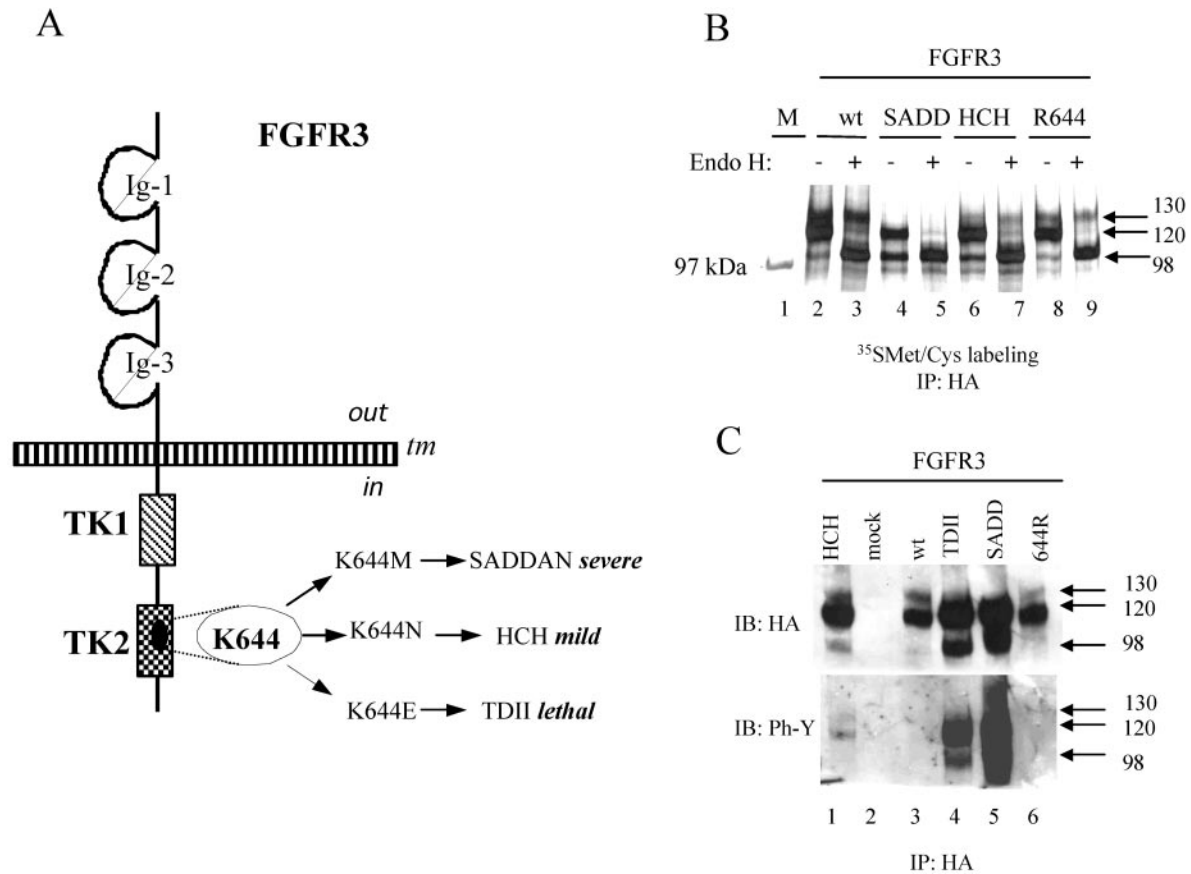


FIG. 1. Biosynthesis of FGFR3 mutants. *A*, schematic representation of the FGFR3 with the two tyrosine kinase domains (*TK1* and *TK2*) and the Lys-644 codon (corresponding to the human Lys-650). Relative substitutions are indicated with the severity of the disease. *K644*, Lys-644. *B*, [³⁵S]methionine metabolic labeling of HEK293 cells transfected with wt, SADDAN, HCH or Arg-644 (*R644*) FGFR3 mutants. *M*, molecular mass marker. Cell lysates were immunoprecipitated (*IP*) with anti-HA antibodies followed by autoradiography. The mature 130-kDa, intermediate 120-kDa, and unglycosylated 98-kDa forms are indicated. Immunocomplexes are treated (+) or not (-) with Endo H. *C*, tyrosine phosphorylation levels of the Lys-644 FGFR3 mutants. *R644*, K664R; *IB*, immunoblotting; *Ph-Y*, anti-phosphotyrosine antibodies.

Here we show that the two highly phosphorylated FGFR3 mutants, associated with severe pathologies, reside as immature glycosylated proteins in the ER and fail to be degraded. Furthermore, these mutants activate the Janus kinase (Jak)/STAT signaling pathway from the ER. Differently, the less activated HCH mutant, although presenting a delayed transition in the ER, cannot signal from this compartment and continues maturation to the cellular membrane. The results provide a model of the role played by different tyrosine kinase activation levels in controlling FGFR3 biosynthesis and maturation.

MATERIALS AND METHODS

FGFR3 Constructs—Murine FGFR3-HA cDNA was used in this study, and single point mutations were generated by polymerase chain reaction (PCR) as described previously for the FGFR3-TDII-HA (19). The K650M (SADDAN), K650N (HCH), and the Y724F substitutions corresponding to the K644M, K644N, and the Y718F transitions in mouse, respectively, were generated using the following oligonucleotides: Met-644, 5'-TACTACAAGATGACCACAAATG-3' (forward) and 5'-CATTTGTGGTCATCTGTAGTA-3' (reverse); Asn-644, 5'-TACTACAAGAATACCACAAATG-3' (forward) and 5'-CATTTGTGGTATTCTGTAGTA-3' (reverse); and Phe-718, 5'-ACATGACCTGTCATGATCATGC-3' (forward) and 5'-GCATGATCATGAACAGGTCATGT-3' (reverse). Each construct was transferred into the expression vector pRcCMV (Invitrogen) and confirmed by sequencing. The HCH- and SADDAN-green fluorescent protein (GFP) constructs were obtained by sub-cloning the cDNAs into the pEGFPN1 vector (Clontech) as described previously for the wt-GFP and the TDII-GFP (19).

Cell Culture and Transfections—HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Euroclone), 2 mM L-glutamine, and penicillin/streptomycin.

The RCS rat chondrosarcoma cells (21) were a gift by C. Basilico. They were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum with the addition of penicillin/streptomycin. Transient transfections in HEK293 and RCS cells were performed with LipofectAMINE-2000 (Invitrogen) according to manufacturer's instructions. Cells were collected after 48 h and analyzed.

Immunoprecipitation and Western Blot—Transfected cells were lysed in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) in the presence of a mixture of protease and phosphatase inhibitors, clarified by centrifugation, and subjected to immunoblotting; FGFR3 proteins were resolved on 7% tricine gels as described previously (19). The following antibodies were used: anti-HA (Roche Applied Science); anti-STAT1 E-23, anti-STAT3 K-15, and anti-STAT5 C-17 (Santa Cruz Biotechnology); antibodies recognizing the phosphorylated forms of STAT1/3/5 (Cell Signaling Inc.); and the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Polyclonal antibodies against Jak1 were a gift from C. Bovolenta.

Deglycosylation—Where indicated, immunoprecipitated complexes were resuspended in 50 mM sodium citrate, pH 5.5, containing 1% SDS and 1% β -mercaptoethanol and boiled for 10 min. Endoglycosidase H (Endo H) (New England Biolabs) was added to the recovered samples, and the reaction was carried out at 37 °C for 2 h.

Metabolic Labeling—Confluent cultures of HEK293 cells were starved in methionine/cysteine-free Dulbecco's modified Eagle's medium (Invitrogen) for 1 h prior to labeling with 200 μ Ci/ml [³⁵S]methionine/cysteine (Met/Cys) (Pro-Mix, Amersham Biosciences) for the indicated times. Where specified, the cells were treated with 5 μ g/ml brefeldin A (BFA) (ICN Biomedicals) or 3 μ g/ml monensin (Sigma) for 5 h. In pulse-chase experiments cells were labeled for 15 min, washed to remove the radioactive mix, and chased with complete medium for the indicated times.

Immunocytochemistry—HEK293 cells were grown on glass cover-

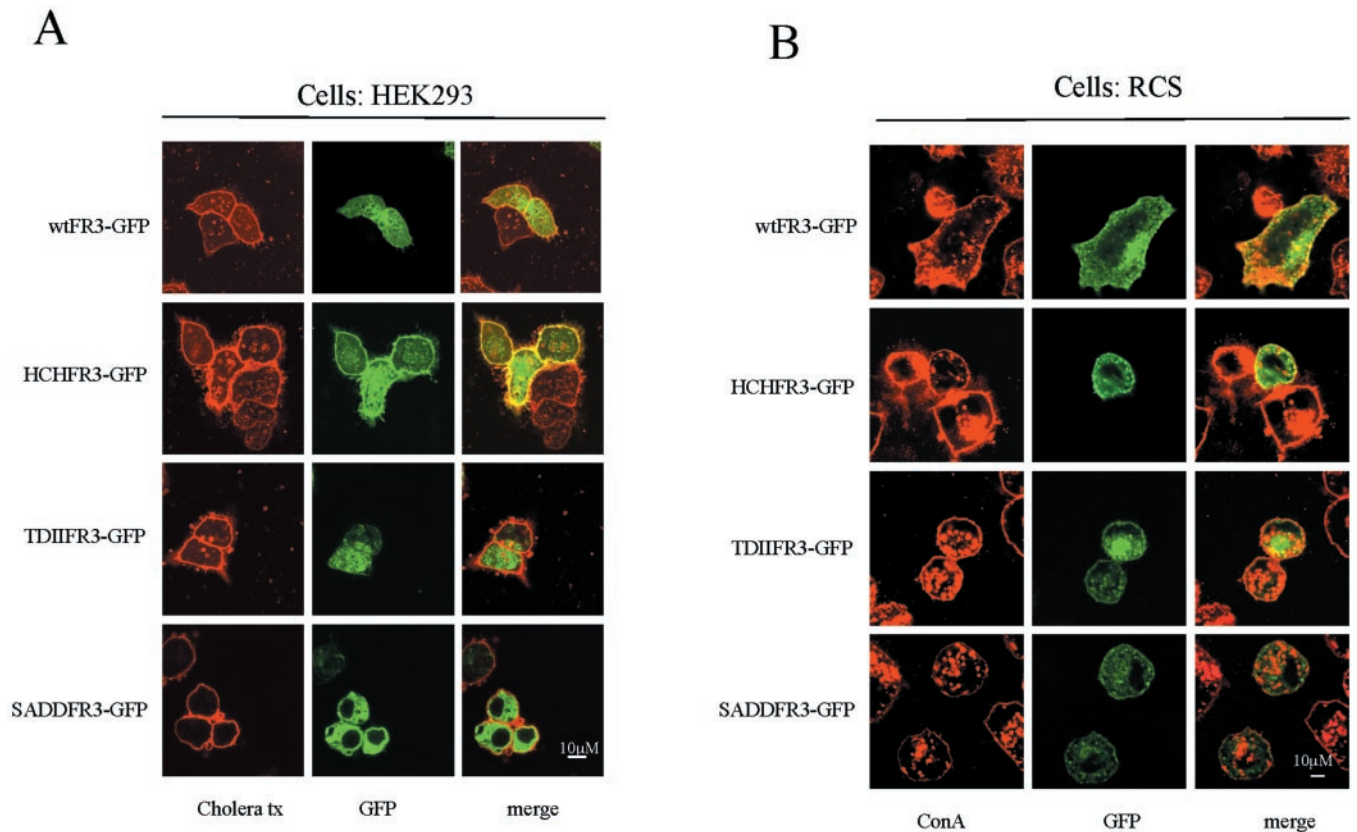


FIG. 2. **Membrane localization of wt and FGFR3 (FR3) mutants.** Wt-FGFR3-GFP or mutant FGFR3-GFP molecules (green) were transfected into HEK293 (A) or RCS (B) cells. At 48 h following transfection, cells were incubated with fluorescence-conjugated cholera toxin (Cholera tx) B peptides (red) or concanavalin A (ConA, red) and fixed. Merge analysis shows the presence of the wt and HCH-FGFR3 on the cell surface. Differently, the highly activated SADDAN and TDII mutants are not exposed on the cell surface. 10 μM magnification bars are indicated.

slips surfaced with 5 $\mu\text{g}/\text{ml}$ polylysine (Sigma) and transfected with mutant and wt-GFP constructs for 48 h. To visualize the plasma membrane, cells were rinsed once with phosphate-buffered saline and incubated for 30 min at 4 $^{\circ}\text{C}$ with 5 $\mu\text{g}/\text{ml}$ rhodamine-conjugated cholera toxin subunit B (Alexa 594, Molecular Probes), washed again in phosphate-buffered saline, and fixed with 4% paraformaldehyde. RCS cells were plated on glass coverslips in the absence of polylysine, transfected for 48 h, fixed with 4% paraformaldehyde and surface-labeled for 1 h. at 4 $^{\circ}\text{C}$ with 200 $\mu\text{g}/\text{ml}$ rhodamine-conjugated concanavalin A (Alexa 594, Molecular Probes). Analyses were performed at the confocal microscopy (Zeiss).

RESULTS

FGFR3 Maturation Is Inhibited in the SADDAN but Not in the HCH Mutant—The murine FGFR3-HA with the SADDAN and HCH substitutions were transfected into HEK293 cells. Following [^{35}S]methionine/cysteine metabolic labeling, protein extracts were immunoprecipitated with anti-HA antibodies and analyzed on tricine gels. Similarly to the TDII mutant (19), the mature 130-kDa band was not detected in the SADDAN-FGFR3, whereas the mannose-rich 120-kDa and the native 98-kDa forms were well represented (Fig. 1B, lane 4). Differently, the HCH receptor, like the wt FGFR3, exhibited the complete biosynthetic pattern composed by the three receptor isoforms of 98, 120, and 130 kDa (Fig. 1B, lanes 2 and 6). We next examined whether the 120-kDa species from the different mutants were located in the ER. For this purpose, the immunoprecipitated receptors were subjected to Endo H digestion. Indeed, Endo H cleaves sugars on high mannose-rich intermediates resident in the ER. As shown previously for the TDII-FGFR3, the 120-kDa isoforms of both SADDAN and HCH were Endo H-sensitive, thus confirming their location within the ER compartments (Fig. 1B, lanes 5 and 7).

To investigate whether activating mutations located in dif-

ferent domains of the FGFR3 could affect receptor maturation, experiments were performed with FGFR3 carrying point mutations associated with achondroplasia. For this purpose, transfection experiments with the transmembrane G380R and G369C mutants were performed. The results showed that the achondroplasia mutants presented a low level of autophosphorylation and were able to process the mature 130-kDa forms (not shown).

We further questioned whether a positive charge at the 644 residue was a mandatory requirement for correct receptor maturation. For this reason, the K644R-FGFR3 mutant was generated. As shown in Fig. 1B, lanes 8–9, the K644R mutant was processed into the mature 130-kDa form, thus confirming the importance of a positive charge for the normal FGFR3 secretory pathway.

Experiments aimed at determining the constitutive phosphorylation of the SADDAN and HCH receptors were performed. For this purpose, lysates from transfected cells were immunoprecipitated with anti-HA antibodies and analyzed by Western blot with anti-phosphotyrosine antibodies. The results shown in Fig. 1C confirmed that both mutations cause receptor autophosphorylation, although to a different extent, with the HCH receptor being less reactive to anti-phosphotyrosine antibodies (lane 1) than the SADDAN and TDII mutants (lanes 4 and 5). As expected, the K644R mutant was not phosphorylated (Fig. 1C, lane 6). This latter observation indicates the requirement of a positive charge in position 644 to maintain the receptor in its unphosphorylated state, a condition ensuring a correct biosynthesis.

Cell Membrane Localization of the HCH Mutant Receptor—To visualize the localization of the HCH and SADDAN proteins within the cell, these mutants were fused with a GFP, transfected into HEK293 and chondrocytic RCS cells,

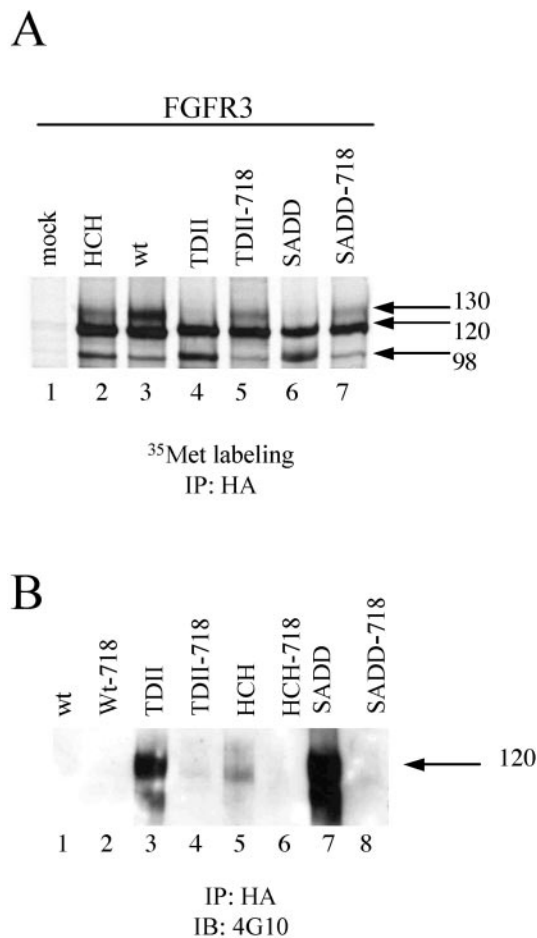


FIG. 3. Effects of the Y718F substitution on FGFR3 maturation and phosphorylation. *A*, HEK293 cells transfected with TDII and SADDAN double mutants are [³⁵S]Met/Cys metabolically labeled, immunoprecipitated (*IP*) with anti-HA antibodies, and exposed for autoradiography. The 130-kDa mature forms are present in the TDII and SADDAN Tyr-718 double mutants. *B*, abrogation of the kinase activity is detected in the TDII and SADDAN FGFR3 double mutants as indicated. *IB*, immunoblotting.

and compared with the wt-GFP and the TDII-GFP molecules. The plasma membrane of HEK293 and RCS cells was labeled with rhodamine-cholera toxin peptides and concanavalin A, respectively. The pictures presented in Fig. 2 show that the SADDAN mutant is only located intracellularly, whereas the HCH mutant appears clearly exposed on the cell surface, as proven by the strong co-localization with cholera toxin.

Role Played by the Critical Tyr-718 in FGFR3 Maturation—To explore whether the autophosphorylation of TDII and SADDAN receptors plays a role in the control of the maturation process, the tyrosine in position 718 was replaced with phenylalanine. The mature 130-kDa band appeared in both the TDII-Y718F and the SADDAN-Y718F mutants (Fig. 3*A*, lanes 5 and 7), indicating that full maturation of the receptor was restored. The state of autophosphorylation by the double mutants was determined by Western blot analysis. In Fig. 3*B*, lanes 4 and 8, it is shown that the Y718F substitution abrogates autophosphorylation of the intrinsic mutant receptor. Thus, the inhibition of the autocatalytic property of the mutated FGFR3 allows full maturation of the receptor. The introduction of the Y718F mutation in HCH-FGFR3 resulted as well in the abrogation of its constitutive phosphorylation (Fig. 3*B*, lane 6) but did not modify its biosynthetic profile (Fig. 3*A*, lane 2).

HCH-FGFR3 Presents a Delayed Transition in the ER—We have investigated turnover of the mutant receptors. To address

this issue, *de novo* receptor synthesis was studied by pulse-chase experiments in cells transfected with the wt-HA, and mutant receptors. The immature 120-kDa species could be detected in the wt and in the three mutant receptors within 15 min (Fig. 4*A*, lane 1). After this short labeling time, chase points at 1, 2, 3, and 5 h were performed. The mature 130-kDa species clearly appeared at 1 h of chase in the wt-FGFR3 (Fig. 4*A*, lane 2). At longer chase points we observed the gradual processing of the wt 120-kDa forms into the mature 130-kDa proteins (Fig. 4*A*, lanes 3 and 4). By 5 h both the 120- and 130-kDa wt-FGFR3 forms were almost completely processed (Fig. 4*A*, lane 5). A totally different situation was observed with the TDII and SADDAN mutants in which the mature 130-kDa forms were completely absent. Differently, the 120-kDa species remained unchanged at each chase point (Fig. 4*A*, lanes 2–5) and comparable in amount to that of the pulse point (lane 1). These data indicate that both SADDAN and TDII mutations block the processing of receptor in its immature high mannose-rich forms. On the contrary, the HCH receptor was slowly processed into the 130-kDa forms, and only after a 10-h chase was the HCH receptor completely processed (lane 8). At 10 h of chase both TDII and SADDAN proteins were still present, whereas the wt proteins had completely disappeared by 7 h (lane 7). These data indicate that the cells can eliminate the HCH molecules but not the TDII or the SADDAN proteins.

To study the turnover of the double TDII, SADDAN, and HCH-Y718F mutants, pulse-chase experiments were performed. In Fig. 4*B* it is shown that the abrogation of Tyr-718 allows the FGFR3 mutants to be turned off after 5 h of chase.

Jak/STAT Pathway Is Activated from Different Cellular Compartments—STAT proteins have been found in their activated forms in TDII fetuses (20), and a role for those proteins in FGFR3 signaling has been reported (21). We have studied whether the different Lys-644 substitutions exhibited the same ability to activate STAT1, STAT3, and STAT5 proteins. Furthermore, we questioned whether the activation of STATs occurred from the same cellular compartment occupied by the FGFR3 mutants. STAT proteins were analyzed from HEK293 cells transfected with FGFR3 molecules prior to and after treatment with BFA in order to block the traffic in and out of the ER/Golgi apparatus (22). The results indicate that STAT1, STAT3, and STAT5 proteins were phosphorylated by the three mutated receptors and not, as expected, by the wt FGFR3 (Fig. 5, *A* and *B*). However, only the TDII and SADDAN mutants were able to activate STAT proteins in the presence of BFA (Fig. 5, *A* and *B*, lanes 4 and 8). Conversely, the HCH mutant was unable to activate the STAT proteins in cells treated with BFA (Fig. 5, *A* and *B*, lane 6), thus indicating that the immature 120-kDa HCH isoform cannot signal, despite the fact that it is phosphorylated and abundantly present in the ER. Jak proteins have been shown to be the major activators of the STATs. Therefore, we proceeded in our analysis by assaying whether the different mutants also activated Jak1. The results are shown in Fig. 5*C*. The three mutants were able to activate Jak1, although Jak1 appeared to be weakly phosphorylated by the HCH mutant (Fig. 5*C*, lane 6). However, BFA did not modify Jak1 activation in TDII and SADDAN (Fig. 5*C*, lanes 5 and 9), whereas the HCH receptor, when constrained in the ER, could not activate Jak1 (lane 7).

It has been shown previously that substitutions of all non-activation loop tyrosine residues with Phe rendered the TDII-FGFR3 inactive and that adding back the single Tyr-718 restored its ability to stimulate PI3K activation and phosphorylation of Shp2, mitogen-activated protein kinase, STAT1, and STAT3 (8). To confirm that our TDII, SADDAN, and HCH Y718F mutants were unable to activate STAT1,

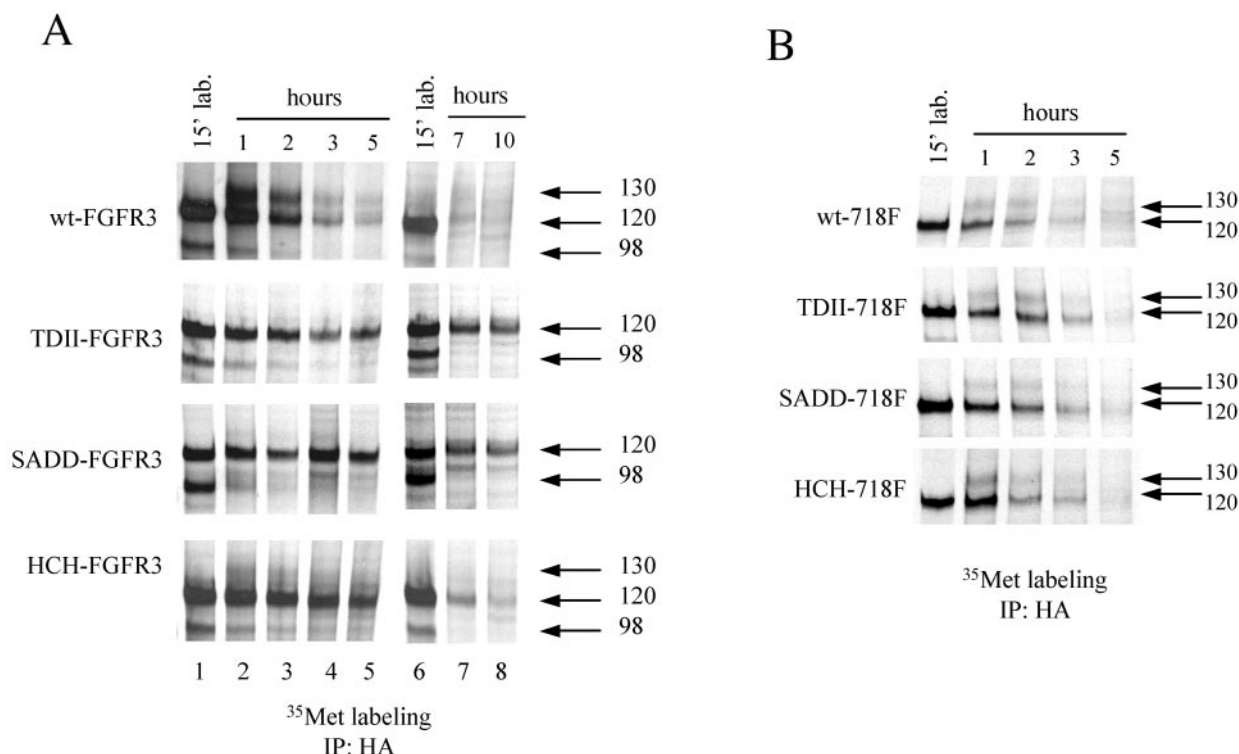


FIG. 4. **wt-FGFR3 and FGFR3 mutants turnover.** *A*, *de novo* receptor synthesis was studied by pulse-chase experiments performed with [³⁵S]Met/Cys metabolic labeling of HEK293 cells transfected with wt-HA, TDII-HA, HCH-HA, and SADDAN-HA mutants. The labeled proteins were immunoprecipitated (IP) with anti-HA antibodies, analyzed by gel electrophoresis, and exposed for autoradiography. The three receptor forms are indicated, as well as the 15-min labeling time (15' lab.) and the chase time intervals (hours). The data shown are representative of three different experiments. *B*, *de novo* receptor synthesis was studied by pulse-chase experiments performed with [³⁵S]Met/Cys metabolic labeling of HEK293 cells transfected with wt-Y718F (718F), TDII-Y718F, HCH-Y718F, and SADDAN-Y718F mutants and analyzed as for panel A.

transfection experiments were performed. As expected, STAT1 failed to be activated by the three FGFR3 variants (Fig. 5D).

Jak1 Is Directly Recruited from the ER by the SADDAN Mutant—We were intrigued by the possibility that Jak1 could be directly recruited by the immature and highly phosphorylated proteins located in the ER. To determine such a direct interaction, we assessed whether Jak1 co-immunoprecipitated with the mutant FGFR3. We found that the SADDAN mutant strongly interacts with Jak1, whereas under the same conditions no wt FGFR3 molecules co-immunoprecipitated with Jak1, as shown in Fig. 6

The HCH Mutant Signals from the trans-Golgi—We have shown that the HCH receptor does not activate the STAT proteins from the ER. Therefore, we hypothesized that either the receptor has to reach the cell membrane to signal or it becomes able to transduce the signal as soon as it exits the ER.

To study whether the HCH receptor could transduce the signal from the Golgi compartment, we have treated cells expressing either the HCH or the wt receptors with monensin, a drug acting at the cellular level as a secretion blocker (23). Monensin completely abolished the presence of both receptors on the cell surface, as visualized by the loss of merge with the fluorescent cholera toxin (compare Fig. 7A with Fig. 2). The cell lysates were subsequently analyzed with anti-STAT1 antibodies to assess whether the Golgi-trapped receptors were able to signal (Fig. 7B). Furthermore, to prove that monensin does not mimic the effects of BFA, we compared the effects on STAT1 activation between monensin and BFA. We show that the HCH mutant activated STAT1 in cells treated with monensin (Fig. 7B, lane 6) and not in the presence of BFA (lane 5). This observation implies that the change in sugar composition during the transition from the ER to the Golgi compartment may determine the acquisition of signaling abilities by the HCH receptor.

DISCUSSION

The study presented herein is consequent to our previous observations on the effects that a single, highly activating mutation in the FGFR3 associated with the severe TDII has on the biosynthesis of the mutated receptor (19). Based on this earlier work, we argued that the different levels of the intrinsic kinase activity of the receptor could affect the FGFR3 biosynthesis differently. Therefore, in an attempt to establish a relationship between phosphorylation and intracellular trafficking of FGFR3, our studies were addressed to the Lys-644 substitutions whose effects on the receptor phosphorylation levels have been described (15).

Our results show that SADDAN-FGFR3 biosynthesis exhibits a dramatic stop in maturation where the high mannose-rich intermediate accumulates in the ER (as in TDII-FGFR3). However, FGFR3, with activating mutations in the transmembrane domain (the achondroplasia G380R and G369C substitutions), did not show an unpaired biosynthesis. A possible explanation is that achondroplasia activation is related to a higher stability of the receptor on the cell membrane (27, 29) rather than to a high level of tyrosine phosphorylation. These results further support our observation that high levels of tyrosine kinase activity rather than other means of receptor activation affect FGFR3 trafficking. We cannot presently rule out that the unpaired trafficking is exclusively restricted to the disruption of the kinase activation loop caused by the Lys-644 mutations. We also show that the positive charge is required at position 644 of FGFR3 for correct processing into the mature, fully glycosylated 130-kDa form, as the K644R mutant behaves like the wt FGFR3. Indeed, the original basic residue (Lys-644) plays a critical role in stabilizing the FGFR3 activation loop in an inactive conformation because different mutations of this res-

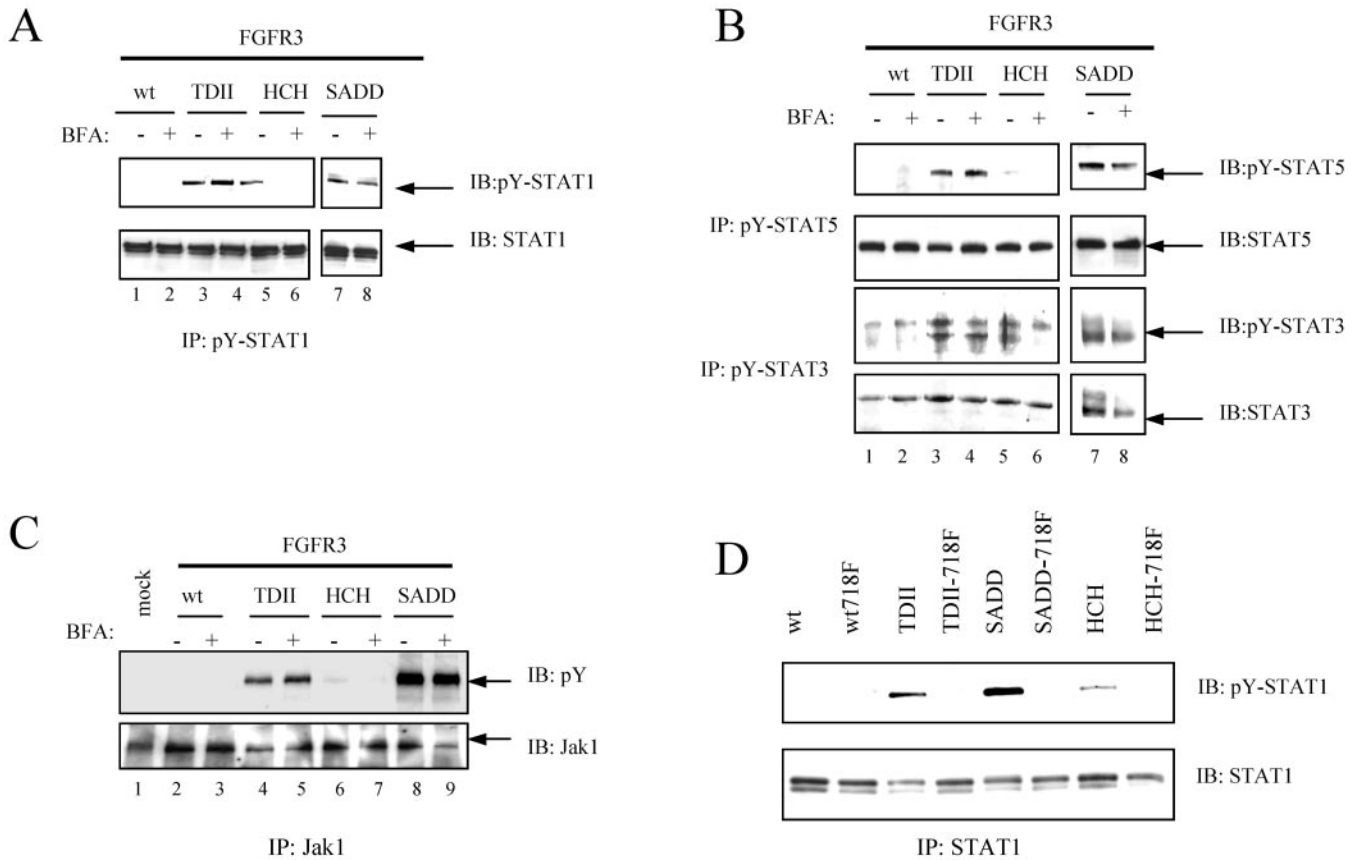


FIG. 5. Jak/STAT activation by FGFR3 mutants. A, activation of STAT1 in wt and the Lys-644 mutants with (+) or without (-) BFA treatment. Transfected HEK293 cells were immunoprecipitated (IP) with anti-STAT1 antibodies and immunoblotting (IB) performed with anti-STAT1 or anti-phosphotyrosine STAT1 (pY-STAT1) antibodies. The HCH mutant does not activate STAT1 in the presence of BFA (lane 6). B, same as in panel A, but anti-phosphotyrosine STAT5 (pY-STAT5) and STAT3 (pY-STAT3) antibodies were used. The HCH mutant failed to activate both STAT5 and STAT3 when cells were treated with BFA (+) (lane 6). C, Jak1 activation by the FGFR3 mutants. HCH shows faint Jak1 phosphorylation (lane 6) that it is not present upon treatment with BFA (+) (lane 7). D, STAT1 is not activated by the SADDAN (SADD) and TDII-Y718F (718F) mutants. Protein extracts were immunoprecipitated with anti-STAT1 antibodies. Immunoblotting was performed with anti-STAT1 or anti-phospho-tyrosine STAT1 antibodies as indicated.

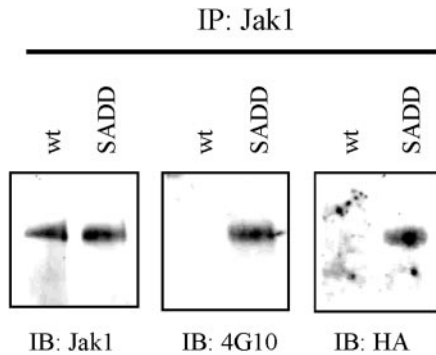


FIG. 6. Jak1 is directly recruited by the SADDAN-FGFR3 mutant. A, upon transfection with wt or SADDAN-FGFR3, protein extracts were immunoprecipitated (IP) with anti Jak1 antibodies. Immunoblotting (IB) with Jak1, 4G10 and HA antibodies were performed as indicated. The experiment shows that Jak1 is recruited by the SADDAN (SADD) mutant (under these conditions Jak1 co-migrates with the 120-kDa SADDAN isoform).

idue constitutively activate the tyrosine kinase to varying degrees (14). With our experiments we show that a positively charged residue is required to maintain the inactive conformation of the activation loop, therefore resembling a wt phenotype (28). It remains that the receptor is not autophosphorylated with the K644R substitution, thus suggesting that the kinase activity affects the receptor maturation process.

Analysis of the SADDAN-Y718F and TDII-Y718F double mutants shows that the maturation of the receptor is restored,

STAT1 is not activated, and the mutants are turned off. Indeed, tyrosine kinase activity is completely abolished in both derivatives. This result suggests that the tyrosine 718 plays a critical role in the final conformation of the receptor, bypassing the effects introduced by the point mutations (8). Therefore, we conclude that ligand-independent phosphorylation is the primary cause for the defects in maturation associated with the TDII and SADDAN substitutions. Furthermore, these data establish a correlation between the high autophosphorylation levels of immature FGFR3 and the prevention of its correct export from the ER to the Golgi compartment. Of note, though, is that such strong premature autophosphorylation of TDII and SADDAN has no effect on the transport of the primary amino acid structure into the ER. However, upon the addition of the first sugars, these mutant receptors become incapable of pursuit along the secretory pathway. One possible explanation is that immature SADDAN and TDII isoforms are trapped in the ER because they form complexes with other factors. Considering that tyrosine kinase receptors exert their function by recruiting Src homology 2 targets (3), we speculate that the highly activated mutants may recruit, at the wrong time and in the wrong place, the (ER) protein(s) that retain the receptor within the ER. In support of this hypothesis is the observation that Jak1 is directly recruited by the SADDAN receptor from the ER. A further dramatic consequence related to SADDAN (and TDII) mutants is that they fail to be turned over. Accordingly, a defect in receptor degradation has been recently described for the achondroplasia mutation (24). Because the du-

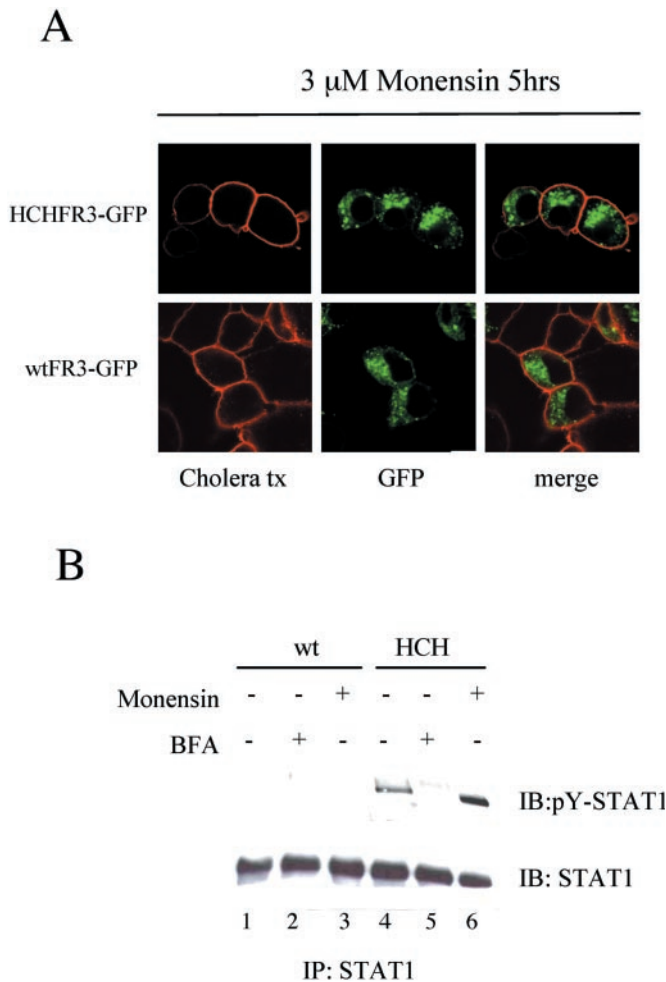


FIG. 7. The HCH mutant signals before reaching the cell membrane. *A*, monensin inhibits exposure of both wt-GFP and HCH-GFP FGFR3 (*FR3*) on the cell membrane. Confocal analysis is performed by labeling cells with cholera toxin (*Cholera tx*). The merged image clearly shows that the green proteins do not reach the membrane. *B*, under the same conditions STAT1 activation is monitored with (+) or without (-) monensin and BFA treatment. Immunoblotting (IB) with anti-phosphotyrosine STAT1 antibodies recognized activated STAT1 in HCH mutant in cells treated with monensin (+). BFA (+) does not allow STAT1 activation. IP, immunoprecipitation.

ration of signaling could be critical for a cellular response, failure in the mechanisms that attenuate receptor signaling or down-regulate receptor transcription (25) may have devastating effects, thus justifying the severity of the diseases. The situation observed for the HCH-FGFR3 mutant is different. Indeed, the HCH receptor matures, although it shows a delayed transition in the ER and is completely processed. A possible simple explanation is that the weak HCH kinase activity may not allow the interaction with potential targets from the ER. Accordingly, the HCH mutant does not activate the Jak/STAT pathway from the ER.

Our results point to a further important issue consequent to the unpaired trafficking, *i.e.* the cellular compartment from which FGFR3 activates the signaling pathway. It is well known that, upon binding with a specific ligand, the wt FGFR3 becomes phosphorylated and triggers the activation of many sig-

naling factors, among which are phospholipase C γ , SHP-2, FRS2 α , and the STAT proteins (26, 8). Because activation of the STATs has been undoubtedly associated with the severe TDII disease, we used STAT1/3/5 activation as markers for signaling activation. In this paper we show that all three mutant receptors are able to phosphorylate the STATs but not, however, from the same cellular compartments. Indeed, we show that whereas the severe SADDAN and TDII mutants signal from the ER, the low activated HCH receptor does not, as proven by the treatment with BFA. Therefore, we hypothesized that the modification in sugar composition that characterizes the passage from the ER to the Golgi compartment (*i.e.* the removal of mannoses in favor of the final sugar composition) could promote a change in conformation that will activate the signaling cascade. By using monensin, we proved that the HCH proteins acquire signaling properties as soon as they exit the ER. We conclude that the levels of phosphorylation highly influence the biosynthesis of the receptor and, consequently, the cellular location for signaling. Finally, our results focus attention on the importance of the spatial segregation of signaling pathway by a mutated TK receptor involved in many human diseases.

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