

The Thanatophoric Dysplasia Type II Mutation Hampers Complete Maturation of Fibroblast Growth Factor Receptor 3 (FGFR3), Which Activates Signal Transducer and Activator of Transcription 1 (STAT1) from the Endoplasmic Reticulum*

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The K650E substitution in the fibroblast growth factor receptor 3 (FGFR3) causes constitutive tyrosine kinase activity of the receptor and is associated to the lethal skeletal disorder, thanatophoric dysplasia type II (TDII). The underlying mechanisms of how the activated FGFR3 causes TDII remains to be elucidated. FGFR3 is a transmembrane glycoprotein, which is synthesized through three isoforms, with various degrees of N-glycosylation. We have studied whether immature FGFR3 isoforms mediate the abnormal signaling in TDII. We show that synthesis of TDII-FGFR3 presents two phosphorylated forms: the immature non-glycosylated 98-kDa peptides and the intermediate 120-kDa glycomers. The mature, fully glycosylated 130-kDa forms, detected in wild type FGFR3, are not present in TDII. Endoglycosidase H cleaves the sugars on TDII intermediates thus indicating their intracellular localization in the endoplasmic reticulum. Accordingly, TDII-FGFR3-GFP co-localizes with calreticulin in the endoplasmic reticulum. Furthermore, following TDII transfection, signal transducer and activator of transcription 1 (STAT1) is phosphorylated in the absence of FGFR3 ligand and brefeldin A does not inhibit its activation. On the contrary, the cell membrane-anchored FRS2 α protein is not activated in TDII cells. The opposite situation is observed in stable TDII cell clones where, despite the presence of phosphorylated mature receptor, STAT1 is not activated whereas FRS2 α is phosphorylated. We speculate that the selection process favors cells defective in STAT1 activation through the 120-kDa TDII-FGFR3, thus allowing growth of the TDII cell clones. Accordingly, apoptosis is observed following TDII-FGFR3 transfection. These observations highlight the importance of the immature TDII-FGFR3 proteins as mediators of an abnormal signaling in TDII.

The fibroblast growth factor receptor (FGFR)¹ family of receptor tyrosine kinases mediates growth, differentiation, and

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¹ The abbreviations used are: FGFR, fibroblast growth factor receptor; Endo-H, endoglycosidase H; BFA, brefeldin A; TDII, thanatophoric dysplasia type II; STAT1, signal transducer and activator of transcription 1; ER, endoplasmic reticulum; HA, hemagglutinin; wt, wild type;

cell migration in a wide range of cell types (1, 2). FGFR3 is one of four distinct members of the FGFR family that serve as high affinity receptors for more than 20 different fibroblast growth factors (FGFs) (3). Binding with a specific ligand together with heparan sulfate induces receptor dimerization, trans phosphorylation, and activation followed by receptor internalization and down-regulation (4–6). This leads to the controlled activation of specific signal transduction pathways (7). In particular, the membrane-linked docking proteins FRS2 α and FRS2 β function as major mediators of signaling by FGF (8). Recently, it has been shown that several human congenital skeletal disorders result from point mutations in the FGFR3. Indeed, mutations in different domains of FGFR3 are implicated in several clinically related forms of short-stature with graded severity including achondroplasia, hypochondroplasia, and the neonatal lethal syndrome thanatophoric dysplasia (TD) (9). TDII is one form of neonatal lethal dwarfism and results from a lysine (K) to glutamate (E) substitution at position 650 (644 in mouse) of FGFR3 (10). The K650E mutation is located in the activation loop of the kinase domain and is associated to strong, ligand-independent constitutive receptor autophosphorylation (11). In *in vitro* studies and in mice carrying the TDII mutation it was shown that the signal transduction and activator of transcription STAT1 is activated and translocated into the nucleus (12). This, together with activation of the cell cycle inhibitor p21^{waf1} was proposed as the molecular mechanism responsible for the TDII pathology (13). Furthermore, ligand-independent activation of the STAT signaling pathway was demonstrated in cultured TD cells and confirmed by immunodetection of activated STAT1 associated to apoptosis of chondrocytes in TD fetus (14, 15). However, the underlying mechanism of how the activated TDII-FGFR3 causes disrupted proliferation and organization of the cartilaginous growth plate of long bones in the TDII remains unclear.

In this study we have investigated whether additional means by TDII mutation in FGFR3 could affect receptor signaling. Biosynthesis of FGFR3 is characterized by three isoforms with various degrees of N-glycosylation: a 98-kDa unglycosylated native protein, a 120-kDa intermediate membrane-associated glycoprotein, and a 130-kDa mature glycoprotein (16). In particular, we have questioned whether TDII-FGFR3 intermediates played a role in the signal transduction. We show herein that the K644E mutation causes the immature phosphorylated TDII-FGFR3 intermediate glycomers to activate STAT1 from the ER. As far as we know, this is the first report of a tyrosine

GFP, green fluorescent protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PARP, poly(ADP)ribose polymerase.

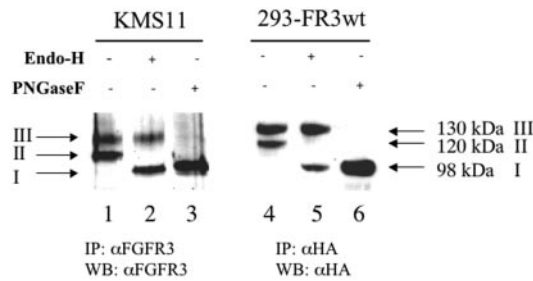


FIG. 1. Biosynthesis of FGFR3. Cell lysates from KMS11 and 293wtFR3-HA cell lines were immunoprecipitated as indicated and analyzed by Western blot with anti-FGFR3 (lanes 1–3) or anti-HA (lanes 4–6) antibodies. Bands I, II, and III correspond to the 98-, 120-, and 130-kDa forms, respectively. Untreated (lanes 1 and 4) or treated with Endo-H (lanes 2 and 5) or PNGase F (lanes 3 and 6) as indicated. HA-tagged FGFR3 and endogenous FGFR3 from KMS11 cells show the same protein profile.

kinase receptor that signals from within the cell in its immature form. We discuss that a novel intracellular signal transduction pathway may be relevant in the pathogenesis of FGFR3-mediated TDII.

MATERIALS AND METHODS

FGFR3 Constructs—Murine FGFR3 cDNA (17) was kindly provided by C. Basilio. The cDNA was subcloned into pBSKII (Stratagene) and the K644E (TDII) mutation was obtained by polymerase chain reaction (PCR) using the following primers: 5'-GAGGAACAGCTCACCTGCA-3' (mFR3F1), 5'-CATTTGTGGTCTCCTTGTA-3' (mFR3644R), 5'-TACTACAAGGAGACCACAAATG-3' (mFR3644F), and 5'-CAGGAACAGCTCACCTGCA-3' (mFR3R1). The 5'-half of the wild type mFR3 was amplified with primers mFR3F1 and mFR3644R, whereas the 3'-half of the same cDNA was amplified with primers mFR3644F and mFR3R1. 5' and 3' PCR products were then mixed and amplified again with primers mFR3F1 and mFR3R1. After cleavage with *Bst*EII and *Bgl*III enzymes the secondary PCR product was cloned into pBSKIIImFR3 that had been cleaved with the same two enzymes. The structure of the DNA construct was confirmed by sequencing, and both the wild type and the K644E mFGFR3 cDNAs were then subcloned into the expression vector pRcCMV (Invitrogen). A double HA sequence was introduced in frame at the 3' end of both the wt and the TDII mutant mFR3 cDNAs as now described. A first PCR product was obtained by combining 5'-GAGGAACAGCTCACCTGCA-3' (mFR3F1) and 5'-GGGAGCGTAATCTGGAA-CATCGTATGGGTACGTCGAGGTCCCCGGT-3' (mFR3HaR, containing the first HA sequence and the half-site of a *Sma*I restriction enzyme sequence), and the obtained amplified DNA was digested with *Bst*EII. The second HA sequence was obtained by excising it from the yeast vector pYX (R&D System) using *Sma*I and *Nhe*I. Both inserts were purified and ligated to the pRcCMVmFR3 digested with *Bst*EII and *Xba*I. Positive clones were verified by sequencing analysis. Finally, mFGFR3-GFP were obtained by subcloning the cDNAs into the vector pEGFPN1 (Clontech).

Cell Culture and Transfections—Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Euroclone), 2 mM L-glutamine, and penicillin/streptomycin. Transient transfection of HEK293, NIH3T3 and Cos7 cells was performed with FuGENE (Roche) according to manufacturer's protocols. RCS cells were transfected with calcium-phosphate technique. KMS11 cells (kindly provided by A. Neri) were maintained in RPMI supplemented with 10% fetal bovine serum. For stimulation with FGF9 (R&D), cells were serum-deprived for 12 h, and then FGF9 was added for 10 min at 100 ng/ml in the presence of Heparin (Sigma).

Immunoprecipitation and Western Blot—Transfected cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of a mixture of protease and phosphatase inhibitors. Cell lysates were clarified by centrifugation and then subjected to specific immunoprecipitation using the following antibodies: anti-HA (Roche), anti-FGFR3 C-15, anti-STAT1 E-23, and anti-FRS2 α H-91 (Santa Cruz). Immunocomplexes were collected with protein A-Sepharose (Amersham Biosciences), washed three times with radioimmune precipitation assay buffer, once with Tris-buffered saline buffer and resuspended in 2 \times gel loading buffer. STAT1 and FRS2 α proteins were separated by 7.5% SDS-PAGE, whereas FGFR3 proteins were resolved

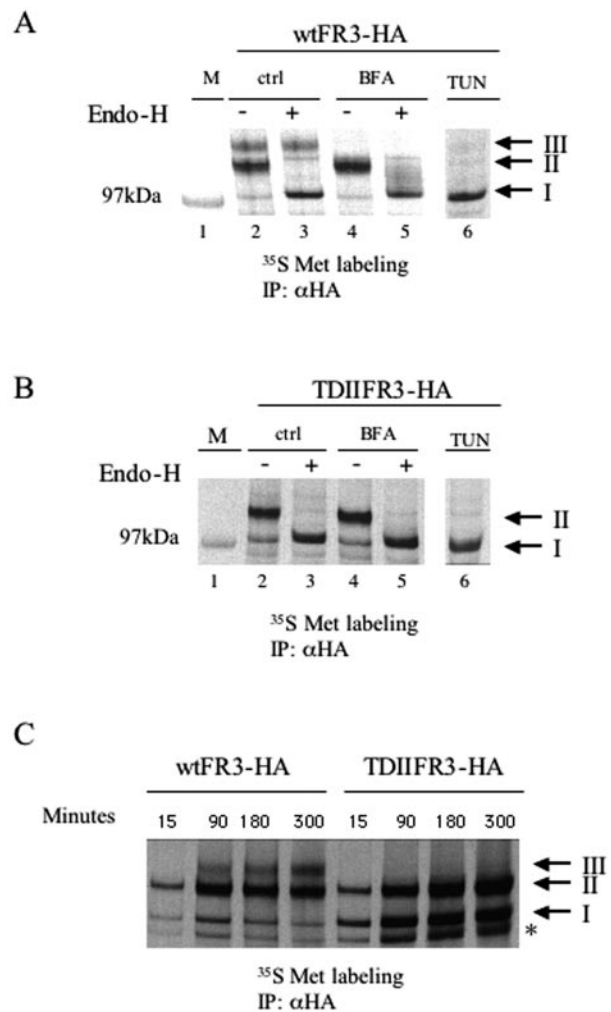


FIG. 2. Biosynthesis of wt- versus TDII-FGFR3. Metabolic labeling of HEK293 cells transfected with wt-FGFR3 or the mutant TDII-FGFR3. Cells were immunoprecipitated with anti-HA antibodies followed by autoradiography. **A**, synthesis of wt-FGFR3. Three forms are detected: form I is unglycosylated and forms II and III represent different sugar compositions. The form II is sensitive to Endo-H, whereas form III is resistant to Endo-H. BFA inhibits formation of the mature form III. Tunicamycin reduces to a unique unglycosylated 98 kDa form I. **B**, synthesis of TDII-FGFR3. Two bands are detected: unglycosylated form I and immature, Endo-H-sensitive form II. The mature form III is undetected. BFA does not alter band pattern as for wt-FGFR3. **C**, kinetic labeling of wt- and TDII-FGFR3. Cells are exposed to [³⁵S]methionine for different time intervals as indicated. At 90 min the mature form III appears in wt-FGFR3. In TDII-FGFR3, the mature form III is absent after 300 min labeling. The asterisk shows a low molecular weight band recognized only by anti-carboxyl-terminal FGFR3 antibodies. It probably belongs to a truncated form of the receptor (seen by Western blot, not shown).

by 7% Tricine gels (18). Proteins were transferred onto polyvinylidene difluoride membranes (ImmobilonP, Millipore), blocked with 5% milk in Tris-buffered saline, pH 7.5, and incubated with desired antibodies. Phosphorylated STAT1 proteins were detected by specific anti-phospho-STAT1 antibodies (Cell Signaling), whereas phosphorylated FGFR3 proteins were revealed by anti-phosphotyrosine PY99 (Santa Cruz). Phosphorylated FRS2 α proteins were detected by anti-phosphotyrosine-RC20:HRPO antibodies (Transduction Lab).

Deglycosylation—Where indicated, immunoprecipitates 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) was added directly to the recovered samples. Concerning the PNGase F (New England Biolabs) reactions, samples were diluted 1:1 with 50 mM sodium citrate, and 1% of Nonidet P-40 was added prior to adding the enzyme. Both enzymatic reactions were 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 me-

FIG. 3. TDII-FGFR3 co-localizes with calreticulin in the ER. Confocal microscopy analysis of HEK293 cells transfected with wtFGFR3-GFP or TDIIFGFR3-GFP molecules (green). Following transfection, cells were fixed and incubated with calreticulin antibodies that were revealed by rhodamine-conjugated secondary antibodies (red). Merge analysis (yellow) showed co-localization of TDII-FGFR3 with calreticulin in the ER. Bar magnification is indicated.

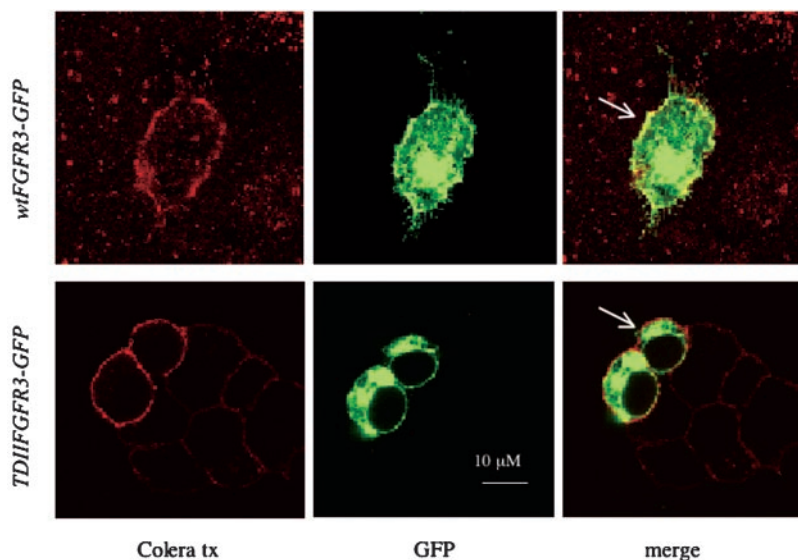
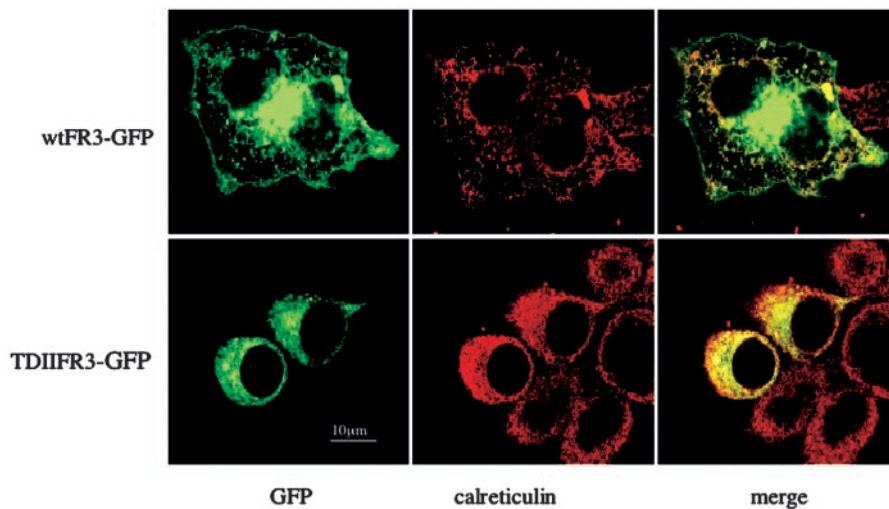


FIG. 4. FGFR3 with the TDII mutation is not processed on the cell membrane. Confocal microscopy analysis of HEK293 cells transfected with wtFGFR3-GFP or TDIIFGFR3-GFP molecules (green). At 48 h following transfection, cells were fixed and incubated with fluorescent-conjugated cholera toxin B peptides (red). Merge analysis showed the presence of the wt-FGFR3 on the cell surface. On the contrary, the TDIIFGFR3-GFP molecules were not present on the cell surface as indicated by arrows. Bar magnification is indicated.

dium medium (ICN) for one hour prior to labeling with 200 $\mu\text{Ci/ml}$ of [^{35}S]methionine/cysteine (Pro-mix, Amersham Biosciences). Where indicated, cells were labeled in the presence of 5 $\mu\text{g/ml}$ brefeldin A (BFA) or 5 $\mu\text{g/ml}$ tunicamycin.

Immunocytochemistry—Cells were grown on glass coverslips surfaced with 10 $\mu\text{g/ml}$ of polylysine and transfected with wt and TDII GFP-conjugated constructs for 24, 48, or 72 h. Cells were then rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde, permeabilized with 0.4% of Triton X-100, and incubated with anti-calreticulin antibodies diluted 1:100 for 1 h at room temperature. The specific labeling was revealed by using secondary rhodamine-conjugated antibodies (Alexa 594, Molecular Probes). Cell membranes were visualized with fluorescent conjugates cholera toxin B subunit from Molecular Probes. To detect apoptosis, we used antibodies directed to the cleaved p85-poly(ADP)ribose polymerase (PARP) (19) (Promega) at 1:100 dilution. Analyses were performed with confocal microscopy (Zeiss).

RESULTS

Biosynthesis of wt-FGFR3—To study the biosynthesis of the FGFR3, a double HA epitope-sequence was inserted at the 3' end of murine wt-FGFR3 cDNA. HEK293 cells were transfected with wt-FGFR3 and several G418-resistant cell clones were isolated. The FGFR3 biosynthetic pattern was studied upon immunoprecipitation and Western blot with anti-HA antibodies. Two specific bands of ~ 120 (II) and 130 (III) kDa, were observed in the wt-FGFR3 (Fig. 1, lane 4) as detected by anti-HA antibodies and according to what was previously described (16). Enzymatic digestion with PNGase F caused the disap-

pearance of both 120 and 130 kDa species that were reduced to an intense 98-kDa band, thus showing that the higher molecular mass species are N-linked glycosylated isoforms (Fig. 1, lane 6). Differently, Endo-H treatment resulted in the disappearance only of the intermediate 120 kDa species, therefore indicating their presence as high-mannose glycomers still residing into the ER/cis-Golgi compartments (Fig. 1, lane 5). An identical protein profile was detected for the endogenous FGFR3 in the multiple myeloma KMS11 human cells that present the G373C FGFR3 mutant (20) (Fig. 1, lanes 1–3). Endogenous wt-FGFR3 from RCS chondrocytic cells presented the same pattern (not shown). The identity of the FGFR3 protein profiles ruled out any interference by the HA epitope on the normal processing of the FGFR3-HA protein.

Biosynthetic Pattern of TDII-FGFR3—A comparative analysis between wt and mutated TDII-FGFR3 biosynthesis was performed by transient transfection of the FGFR3 HA-tagged molecules into HEK293 cells followed by metabolic labeling with [^{35}S]methionine/cysteine. wt-FGFR3 exhibited the three isoforms described above (Fig. 2A, lane 2). Surprisingly, only the 98-kDa and a predominant 120-kDa bands (I and II) were detected in TDII (Fig. 2B, lane 2). To determine whether the different pattern between wt and TDII receptors was ascribed to an anomalous glycosylation or to uncompleted maturation of the TDII receptor, analysis with specific enzymes and drugs were performed. Because sugar composition of glycoprotein

reflects their location in the secretory pathway (21), Endo-H was considered as diagnostic for the position of the band II intermediates. As already mentioned for wt-FGFR3, also the 120-kDa TDII proteins were Endo-H-sensitive, thus confirming their nature as immature intermediates located within the ER/cis-Golgi compartments (Fig. 2, A and B, lanes 3). We further confirmed the location of the 120-kDa TDII-FGFR3 by performing experiments with the fungal drug BFA, which enhances retrograde traffic from Golgi to ER (22). BFA inhibits the formation of the mature form (III) of wt-FGFR3 (Fig. 2A, lanes 4 and 5). In TDII, BFA does not affect the protein profile thus confirming that the 120-kDa species reside in the ER (Fig. 2B, lanes 4 and 5). Inhibition of glycosylation by tunicamycin blocks wt and TDII biosynthesis at the immature unglycosylated band I (Fig. 2, A and B, lanes 6). We further investigated the possibility that the predominance of the band II in TDII was due to a peculiarity of the HEK293 cells. For this purpose, transfection experiments were performed in NIH3T3 as well in Cos7 cells. In both cell types, TDII-FGFR3 biosynthetic pattern was characterized by the absence of the mature form and by Endo-H-sensitive intermediate glycomers (not shown). In addition, considering that chondrocytes are the cells mostly affected by FGFR3 mutation in the growth plate, we tested whether TDII-FGFR3 showed the same biosynthetic pattern in the RCS cells. As expected, only the 120-kDa form was observed in RCS cells transfected with TDII-FGFR3 molecules (not shown). A kinetic of [³⁵S]methionine/cysteine incorporation was performed to test whether TDII-FGFR3 required a longer time to be processed into the mature form. After 5 h of labeling, the mature 130-kDa forms in TDII were still undetected, whereas the wt mature forms were clearly detected after 90 min of labeling (Fig. 2C). In addition, after 15 min of labeling, we consistently observed a comparable 1:1 ratio between the 98 and the 120-kDa species in TDII (Fig. 2C). This suggests a possible delay in the processing of the native isoforms into the following maturation step, whereas, in the wt-FGFR3, the native forms were rapidly glycosylated (Fig. 2C).

Intracellular Localization of TDII Mutant Receptor—To visualize the localization of the TDII intermediates within the cell, the GFP protein was fused at the C terminus of wt and TDII receptors. 48 h after transfection, HEK293 cells were fixed and stained with antibodies that recognize calreticulin, which is the major calcium-binding protein found in the ER (23). Merging analysis by confocal microscopy showed that TDII-GFP co-localize with calreticulin in the ER (Fig. 3). The same result was obtained when cells were analyzed at 72 h post transfection (not shown). Furthermore, by staining the cells with rhodamine-conjugated cholera toxin peptides, which specifically recognize membrane lipid raft, we were able to confirm the absence of TDII-FGFR3 from the cell surface (Fig. 4). On the contrary, the wt-FGFR3-GFP was clearly exposed on the cell surface (Fig. 4).

STAT1 but Not FRS2 α Is Activated in the Presence of the Intermediate TDII-phosphorylated Glycomers—As previously reported (13), the human K650E mutation strongly activates FGFR3, which, in turn, activates STAT1. We have investigated whether the intracellular intermediate TDII receptor was phosphorylated and, most importantly, whether STAT1 was activated in cells transiently transfected with TDII-FGFR3. For this purpose, cell lysates from HEK293 transfected with the wt- or TDII-FGFR3 were first immunoprecipitated with anti-HA and subsequently with anti-STAT1 antibodies. The collected immunocomplexes were then analyzed by immunoblot with anti-phosphotyrosine and anti-phospho-STAT1 antibodies. Interestingly, the immature 120-kDa TDII-FGFR3 glycomers were phosphorylated (Fig. 5A). Accordingly, STAT1 was

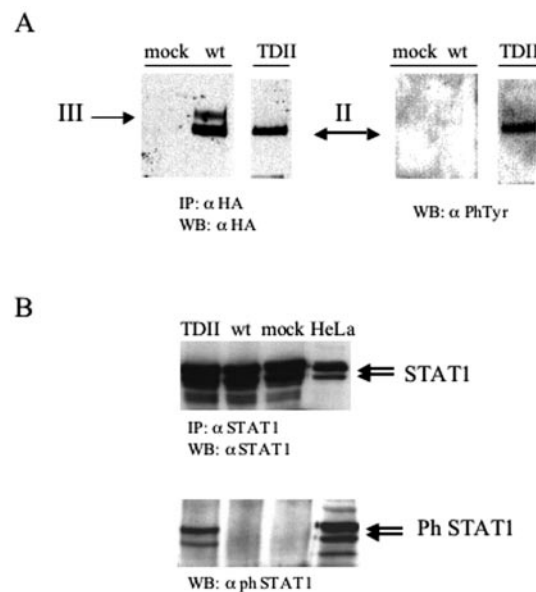


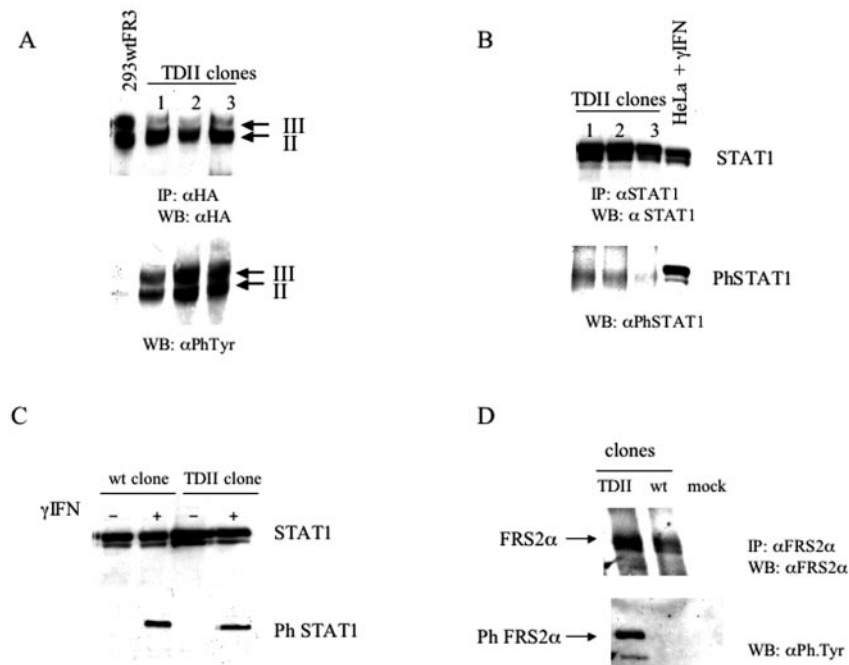
FIG. 5. STAT1 is activated in the presence of phosphorylated TDII-FGFR3 immature forms. A, wt-FGFR3 presents forms II and III, whereas TDII-FGFR3 shows only the intermediate forms II. Anti-phosphotyrosine antibodies recognize the intermediate forms (II) in TDII-FGFR3 but not of wt-FGFR3. B, cells transfected with TDII or wt-FGFR3 or not transfected (mock) were immunoprecipitated with anti-STAT1 antibodies. HeLa cells were stimulated with γ IFN as positive control for STAT1 activation. As shown, STAT1 appeared phosphorylated only in TDII-FGFR3 but not in wt-FGFR3 cells. Antibodies used for IP (immunoprecipitation) or WB (Western blot) are indicated.

phosphorylated in TDII but not in wt-FGFR3 cells (Fig. 5B). In addition, when TDII cells were treated with BFA for 5 h, STAT1 was still detected in its phosphorylated form, thus confirming that such an activation occurred even if the traffic out the ER was inhibited (not shown). Furthermore, we have investigated whether the docking protein FRS2 α , which is anchored on the cell membrane, could be activated by TDII intermediates. We did not detect phosphorylated FRS2 α in the TDII cells (not shown).

STAT1 Activation Is Abrogated in Stable Cell Clones Selected to Express TDII-FGFR3—We obtained stable TDII-FGFR3 cell clones, although the number of the positive TDII colonies was ~50% less compared with the number of wt-FGFR3 selected cell clones.

Analysis of FGFR3 showed that the 120-kDa intermediate glycomers were highly represented in all TDII cell clones although, to a lesser extent, the mature 130-kDa form was also present (Fig. 6A). Of note, in independent clones showing different levels of FGFR3 expression, the intermediate 120-kDa forms were always favored. Both forms of the receptor were constitutively phosphorylated. Interestingly, the phosphorylation level of 120 and 130 kDa bands were comparable in intensity, suggesting a higher degree of phosphorylation of the mature forms versus the intermediates (Fig. 6A). Alternatively, the phosphorylated 130-kDa mature proteins include endogenous co-immunoprecipitated FGFR3. Remarkably, in all the cell clones analyzed, we were unable to detect phosphorylated STAT1 protein (Fig. 6B). To determine whether failure in STAT1 activation was due to a cellular defect in the activation of the STAT1 pathway, cells were stimulated with γ interferon. STAT1 was phosphorylated in wt and TDII clones as well as in the parental cells treated with γ interferon (Fig. 6C). We deduced that only the cells that had lost the ability to activate STAT1 through the TDII mutant would be favorably selected to become permissive for the TDII-FGFR3. This indicates that the selection process favored cells defective of a specific factor(s)

FIG. 6. Analysis of TDII proteins in stable TDII-HEK293 cell clones. *A*, both immature (II) and mature (III) species were expressed in TDII stable cell lines. Although the TDII intermediate isoforms (II) were predominant, anti-phosphotyrosine antibodies showed a comparable degree of phosphorylation for both II and III species (shown in the three independent clones 1, 2, and 3). In the wt stable cell lines species II and III were equally represented and not phosphorylated. *B*, STAT1 was not phosphorylated in TDII-FGFR3 clones despite phosphorylation of TDII receptors. *C*, γ interferon induces phosphorylation of STAT1 in TDII and wt clones, thus showing the integrity of that pathway of activation. *D*, FRS2 α is constitutively activated in the TDII but not in wt clones. Antibodies used for IP (immunoprecipitation) and WB (Western blot) are indicated.



linked to TDII-FGFR3 and playing a major role in mediating STAT1 activation. The situation was different for FRS2 α docking protein. Indeed, FRS2 α was present as a constitutively phosphorylated protein only in the TDII cell clones (Fig. 6D). Finally, wt and TDII cell clones were treated with FGF9, a specific ligand for FGFR3. This stimulation resulted in the phosphorylation of FRS2 α in the wt clones, whereas in TDII the FRS2 α remained unchanged in its constitutively phosphorylated form. Differently, FGF9 did not induce STAT1 phosphorylation in both wt and TDII cell clones (not shown).

Apoptosis in TDII-FGFR3 Cells—To determine whether TDII-FGFR3 triggered apoptosis, cells were transfected with the wt or the TDII-GFP receptor constructs. At 72 h cells were fixed, incubated with antibodies that recognize only the cleaved p85-PARP, followed by rhodamine-conjugated secondary antibodies. As shown in Fig. 7, apoptosis was observed in ~40% of TDII cells as measured by the number of cells showing double fluorescence.

DISCUSSION

TDII mutation in FGFR3 strongly activates receptor signaling, as measured by tyrosine kinase activity and induction of cell proliferation in several cell lines (e.g. BaF3, NIH3T3) (24). However, the TDII disease is characterized by disrupted proliferation of chondrocytes in the growth plate, and ultimately the clinical severity parallels the profound kinase activation of the receptor (25). This paradox was reconciled by demonstrating that FGFR3 signaling can operate along two different pathways. One, the Ras-MAPK (mitogen-activated protein kinase) pathway, leads to cell proliferation (26), whereas the other pathway, operating through STAT1, induces cell cycle inhibition (27). Therefore, we have reasoned that the intrinsic tyrosine kinase activity of the TDII receptor, *per se*, was not sufficient to justify the devastating effects observed in TDII. Our first approach in this study was to determine whether biosynthesis of the TDII-FGFR3 presented anomalies. For this purpose we have generated FGFR3 (mutant and wt) tagged with the HA epitope. This allowed us to follow the biosynthesis of the receptor upon transfection in HEK293 cells. At first we ruled out any interference by HA in relation to both synthesis and activation of the FGFR3 by comparing the biosynthetic pattern of wt-FGFR3-HA with endogenous FGFR3 from different cell lines.

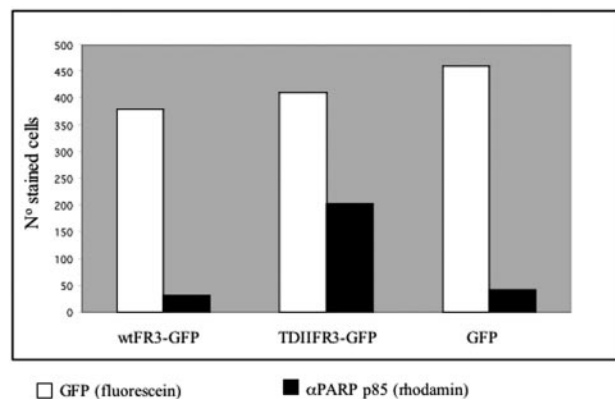


FIG. 7. Apoptosis in TDII-FGFR3-GFP cells. HEK293 cells were transfected with wt or TDII-FGFR3-GFP molecules or GFP alone. 72 h later cells were fixed and incubated with anti-p85-PARP antibodies as indicators of apoptosis. Rhodamine-conjugated secondary antibodies were used. Several fields were examined with confocal microscopy. Green (GFP) and red (cleaved PARP) cells were counted, and only cells with the double stain were considered apoptotic. The data showed here represent three independent transfection experiments that gave similar results.

Interestingly, we observed that the K644E substitution hampers the intermediate glycosylated 120 kDa forms to exit the ER. This is based on the following observations: first, the mature 130-kDa form was not detected; second, the TDII 120-kDa intermediates were sensitive to Endo-H, which is specific for high-mannose sugars on glycoproteins resident in the ER/cis-Golgi compartments. Indeed, Endo-H is considered as diagnostic for the position of a glycoprotein in the secretory pathway. The same results were obtained when TDII-FGFR3 was transiently transfected into NIH3T3 or chondrocytic RCS cells, thus confirming that accumulation of the immature TDII form was due to a property of the mutated protein rather than to a defect of HEK293 cell surveillance on protein synthesis. Furthermore, the results indicate that the switch from a basic 644 lysine to the negatively charged glutamic acid, rather than the tissue specificity, plays a pivotal role in the retention of TDII receptor within the ER. In addition, the molecular weight of the protein species and the profiles obtained with specific enzymes do not indicate a defect in the glycosylation of TDII-FGFR3.

Most importantly, we show that the immature 120-kDa forms of TDII receptor were phosphorylated. Furthermore, STAT1 was activated upon TDII transfection, and this coincided with a certain level of apoptosis, in line with a previous report (15). An important, yet unanswered, question is whether TDII-FGFR3 is retained in the ER in a monomeric or dimeric form. Nonetheless, no traces of the endogenous mature 130-kDa FGFR3 were observed after five hours of metabolic labeling followed by immunoprecipitation with anti-HA antibodies. We can not exclude, however, the possibility that the endogenous 120-kDa immature FGFR3 forms dimerize with TDII and therefore are retained in the ER. However, it has been previously shown (28) that *Xenopus* FGFR2 with the corresponding K652E mutation does not require dimerization for signaling. Further evidence that confirms the signaling by TDII-FGFR3 from the ER was obtained with BFA. Indeed, by blocking the traffic out of the ER, STAT1 was still activated upon transfection.

A different situation was recorded in the stable TDII-FGFR3 cell clones in which both 120 and 130 kDa receptor forms were present in their phosphorylated state. At present, we cannot yet explain why the p130 form is present in stable TDII cell clones, however, we can speculate on two possibilities. The first one is that the defect in p130 processing may be bypassed, and over time some mature forms accumulate in stable transfectants. The second hypothesis, which is under study, is that a factor, missing in the stable TDII clones, interacts with the 120-kDa receptor forms in the ER, thus inhibiting, somehow, the regular TDII-FGFR3 processing. It remains to be determined whether STAT1 plays a direct role in such a mechanism. Indeed, surprisingly, it was not detected in its active configuration in stable TDII cells. The fact that STAT1 is activated only by the immature TDII-FGFR3 but not upon stimulation of stable clones with FGF9 supports the hypothesis that STAT1 plays a critical role in the TD (13, 15). In line with the above observations are the data obtained with human KMS11 and OPM-2 myeloma cells in which with a strong auto-phosphorylation of FGFR3, carrying the G373C and K650E mutations, respectively, there is not correspondent STAT1 activation (29). Treatment of stable TDII cell lines with the γ interferon showed activation of STAT1, thus indicating that this activation cascade was not affected and that STAT1 itself has not lost the ability to be phosphorylated. We hypothesize that the selection process favored cells in which some critical protein(s) involved in the FGFR3 signaling was missing, thus leaning toward an oncogenic potential. To verify if a switch in the signaling occurred in TDII clones, phosphorylation of FRS2 α protein was studied. Interestingly, we have not found phosphorylated FRS2 α upon transfection of the TDII molecules. On the contrary, we detected phosphorylation of FRS2 α in all the stable TDII cell clones analyzed. Considering that FRS2 α is a

myristylated protein, which is anchored on the cytoplasmic membrane, the failure in its activation represents further evidence that TDII-FGFR3 signals from the ER. Moreover, the fact that FRS2 α fails to be activated upon TDII transfection indicates that the TDII receptor does not phosphorylate the endogenous FGFR3.

In conclusion, we show that phosphorylation *per se* may not be sufficient to explain the severity of the disease. Indeed, we suggest that critical roles are played by phosphorylated immature receptor glycomers that can activate specific substrate(s) from within the cell.

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