FGF receptors ubiquitylation: dependence on tyrosine kinase activity and role in downregulation

E. Monsonego-Ornan[a,b,1], R. Adar[a], E. Rom[b], A. Yayon[a,b,*]

[a] *Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel*
[b] *ProChon Biotech Ltd., Kiryat Weizmann, Bldg. 12, P.O. Box 1482, Rehovot 76114, Israel*

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Abstract **A crucial aspect of ligand-mediated receptor activation and shut-down is receptor internalization and degradation. Here we compared the ubiquitylation of either wild type or a K508A 'kinase-dead' mutant of fibroblast growth factor receptor 3 (FGFR3) with that of its naturally occurring overactive mutants, G380R as in achondroplasia, or K650E involved in thanatophoric dysplasia. Fibroblast growth factor receptors ubiquitylation was found to be directly proportional to their intrinsic tyrosine kinase activity, both of which could be blocked using kinase inhibitors. Despite excessive ubiquitylation, both overactive mutants failed to be efficiently degraded, even when challenged with ligand or overexpression of c-Cbl, a putative E3 ligase. We conclude that phosphorylation is essential for FGFR3 ubiquitylation, but is not sufficient to induce downregulation of its internalization resistant mutants.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Achondroplasia; Thanatophoric dysplasia; Tyrosine kinase receptors; E3 ligase; Internalization

1. Introduction

Fibroblast growth factor receptor 3 (FGFR3) is one of four distinct members of membrane spanning tyrosine kinases that serve as high affinity receptors for more than 20 different fibroblast growth factors (FGFs) [1]. Binding of the FGF ligand in concert with a heparan sulfate [2] induces receptor dimerization, trans-phosphorylation and activation followed by receptor internalization and downregulation. These lead to the controlled activation of specific signal transduction pathways and expression of FGF target genes, critically required during embryogenesis, tissue repair and angiogenesis [3,4].

Mutations in FGF receptors 1, 2 and 3 are known to give rise to a variety of inherited skeletal disorders [5,6]. Mutations in FGFR3 are responsible for disorders predominantly of the long bones, including achondroplasia (Ach), the most common form of human genetic dwarfism [7,8]. A N540K mutation in the proximal tyrosine kinase domain of FGFR3 is found in the milder disorder of hypochondroplasia [9], while substitutions of a cysteine for one of the residues 248, 249, 370 or 371 in the extracellular domain, or a K650E mutation in the kinase domain, give rise to the most severe and neonatal lethal, thanatophoric dysplasia (TD) types, I and II, respectively [10,11]. All these skeletal malformations represent autosomal dominant disorders characterized by disproportionately short limbs and relative macrocephally.

The predominant molecular mechanism underlying the various chondrodysplasia syndromes is constitutive, ligand-independent activation of the mutant receptors and their downstream signaling [12]. For example, TDI results from covalent disulfide linked receptor–dimers, while TDII results most often from direct activating mutations in the kinase domain of FGFR3, both of which can lead to ligand-independent, constitutive receptor autophosphorylation [10,13]. Recently we have found that the Ach mutation G380R uncouples ligand-mediated receptor activation from its downregulation, leading to excessive receptor accumulation and inappropriate FGF-mediated signaling [14]. The implication of this finding is that signal termination via receptor degradation is an essential component of FGF signal transduction, similarly to other tyrosine-kinase receptors [15]. However, questions regarding the mechanism and the reasons for the resistance of these FGFR3 mutants to downregulation have remained open.

Among the most likely candidate mechanisms of FGFR3 breakdown is via the proteosome, after tagging with ubiquitin. The 76 amino acid polypeptide, ubiquitin, is attached onto proteins by the sequential action of the enzymes E1, E2 and E3. The E1 enzyme first activates ubiquitin in an ATP-dependent manner, ubiquitin is then transferred to the active site of an ubiquitin-conjugating enzyme (E2) and then to the active site of an ubiquitin-protein ligase (E3) [16]. In the last reaction, catalyzed either by E2 with the help of E3, or directly by E3, an isopeptide bond is formed between the C-terminal glycine of ubiquitin, and the ε-amino group of a lysine residue on the target protein. The generation of multiubiquitin chains is often mediated by the repeated attachment of ubiquitin onto Lys48 of another ubiquitin or of a growing ubiquitin chain. The E3 enzymes therefore perform the important task of recognizing proteins destined for degradation by the proteosome [17]. It is now widely accepted that Cbl functions as a negative regulator of receptor tyrosine kinases (RTKs), possibly by acting as an E3 ligase as demonstrated by Cbl-induced ubquitylation [18].

In the case of plasma membrane proteins, the function of ubiquitylation may extend beyond just tagging for degradation. In yeast, it serves to trigger the internalization of plasma membrane proteins via the endocytic pathway [19], which leads to their subsequent degradation in the vacuole. In at

*Corresponding author. Fax: (972)-8-931 8840.
E-mail address: yayon@prochon.co.il (A. Yayon).

[1] Present address: Institute of Animal Science, ARO, Volcani Center, Bet Dagan, Israel.

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least one case, that of the growth hormone receptor (GHR), ubiquitylation is required for internalization of the receptor, however, it is unlikely that ubiquitylation is the sole trigger for internalization [20,21].

So far only one study of FGFR ubiquitylation has been reported, in which it was shown that FGFR1 ubiquitylation is ligand-dependent, although to a lesser extent than for other members of the tyrosine kinase receptors super-family [22]. In the present study we have analyzed the ubiquitylation patterns of FGFR3 and its naturally occurring mutants, their dependence on kinase activity and the relationship between ubiquitylation and degradation of these receptors.

2. Materials and methods

2.1. Cell lines

Non-transformed rat chondrocytes derived from fetal calvaria (RCJ 3.1C5.18), a generous gift from Dr. J. Aubin [23], and human embryonal kidney cells expressing large T-antigen (293T). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

2.2. Expression of wild type (WT) and mutant human FGFR3

Transfections: cDNAs of the WT FGFR3, Ach (G380R), TDII (K650E) and kinase-dead (KD) (K508A) mutants in pcDNA3 expression vector (Invitrogen) and ubiquitin–HA (ubiquitin–hemagglutinin) and Cbl–HA (Cbl–hemagglutinin) were transfected into 293T cells by the calcium phosphate method. *Infections*: Retroviruses of the FGFR3 and the Ach mutant were produced in 293T cells by co-transfection of these cDNAs in a pLXSN vector with a Psi helper phage. Collection of virus-containing medium started 30 h after transfection. Medium was collected five times at 8 h intervals. The infected medium was filtered and kept at −80°C for further use. The infections were performed by incubating RCJ cells with the virus-enriched medium and 8 μg/ml of polybrene for 6 h followed by selection in G418 (0.5 μg/ml). Positive pools were screened for FGFR3 expression by SDS–PAGE and Western blots.

2.3. Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.5% Nonidet P-40, 1 mg/ml approtinin, 1 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation at $12\,000 \times g$ for 15 min. The lysates were subjected to immunoprecipitation for 16 h at 4°C with anti-FGFR3 C-terminus antibody (Santa Cruz, CA, USA); and analyzed by 6% SDS–PAGE and Western blots. Monoclonal anti-HA antibodies were a generous gift from Dr. Yaov Henis, Tel Aviv University; antibodies to ubiquitin were from Sigma. Protein bands were visualized using secondary antibodies coupled to horseradish peroxidase and the ECL kit (Amersham) according to the manufacturer's instructions. Densitometry was done using a Model GS-690 Imaging Densitometer (Bio-Rad).

3. Results

3.1. Ubiquitylation of FGFR3 and its mutants

Ubiquitylation of FGFR3 was analyzed by co-expressing the WT receptor or its mutants together with an ubiquitin–HA fusion protein and analyzing their associated HA-tag after 20, 48 and 90 h (Fig. 1). The expression levels of the WT and Ach FGFR3 reached a peak at 48 h and declined by 90 h, while the TDII mutant levels remained relatively unchanged up to 90 h (Fig. 1, top panel). The ubiquitylation pattern of the WT receptors, as detected by the HA-tag, also peaked at 48 h and thereafter declined in parallel with the receptor protein levels (Fig. 1, bottom panel). In contrast,

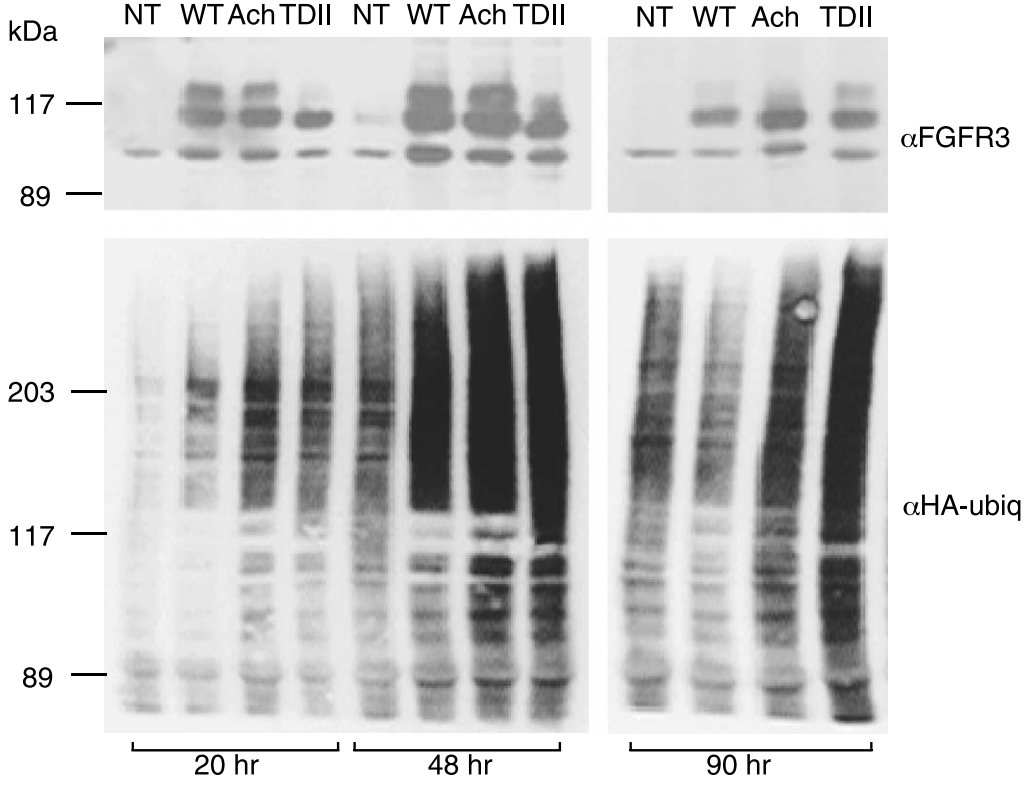

Fig. 1. Time-dependent expression and ubiquitylation of WT, Ach (G380R) and TDII (K650E) FGFR3. 293T cells were transiently transfected with the WT, Ach and TDII mutant receptors, together with the ubiquitin–HA construct. After the indicated time periods the cells were lysed, immunoprecipitated with anti-FGFR3 C-terminus antibodies and probed on an immunoblot with antibodies to either the kinase domain of FGFR3 (αFGFR3) or anti-HA (αHA–ubiq). NT, transfected only with HA–ubiquitin (without FGFR3).

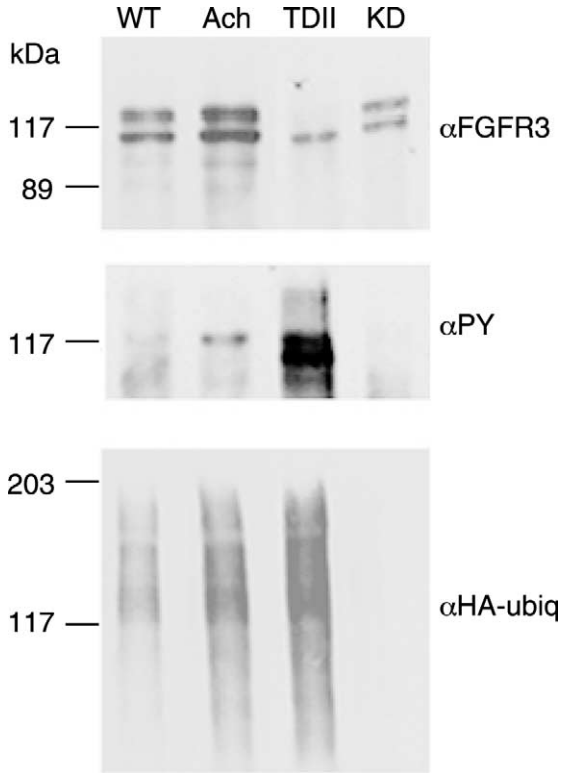

Fig. 2. Tyrosine phosphorylation and ubiquitylation of WT and mutant FGFR3. 293T cells were transiently co-transfected with the WT, G380R Ach, K650E TDII and K508A KD mutant receptors, together with the ubiquitin–HA construct, for 48 h. The transfected cells were lysed, immunoprecipitated with anti-FGFR3 C-terminus antibodies and probed on an immunoblot with antibodies to the kinase domain of FGFR3 (αFGFR3, top), anti-phosphotyrosine (αPY, middle) or anti-HA (αHA–ubiq, bottom).

the Ach and TDII mutant receptors remained highly ubiquitylated even at 90 h. Some ubiquitylation was also detected in non-transfected cells and is attributable to endogenous FGFR3 expression in 293T cells. The ubiquitylated proteins, corresponding to intact receptors and their degradation products bearing ubiquitin chains of different lengths, typically appear as ladders in SDS–PAGE due to their anomalous mobility.

3.2. *Ubiquitylation of FGFR3 correlates with its kinase activity*

We asked whether the high ubiquitylation of the two overactive mutant receptors was related to their increased tyrosine kinase activity. To this end, 293T cells expressing variously active mutants of FGFR3 together with the ubiquitin–HA construct were analyzed for expression, tyrosine phosphorylation as well as FGFR3 ubiquitylation (Fig. 2). The Ach mutant was more ubiquitylated than the WT, but less than the TDII mutant, while the KD K508A mutant failed to undergo phosphorylation and did not appear to be ubiquitylated at all (Fig. 2, bottom panel). The relative ubiquitylation levels followed the order TDII > Ach > WT > KD and paralleled the observed tyrosine phosphorylation levels (Fig. 2, middle panel). The detected phosphorylation levels are also in agreement with the expected relative activity of these receptors and the severity of the corresponding disorders [24,25]. It is notable that all these receptors showed tyrosine phosphorylation even though the cells were not stimulated with exogenous FGF. This activity could conceivably result from the high levels of receptor expression in the cells that might promote their spontaneous dimerization and phosphorylation, or due to some FGFs secreted into the medium by the same cells [26].

The addition of saturating amounts of FGF9 to produce maximal FGFR3 stimulation caused only a mild increase in the ubiquitylation of the WT receptor within 30 min when taking into account the corresponding receptor levels (Fig. 3A). Furthermore, 30 min of FGF9 exposure did not noticeably change the ubiquitylation levels of the Ach or TDII mutants (Fig. 3A, lower panel). Since the effect of the ligand after 30 min was only moderate, we further studied longer activation periods enabling processes which require new protein synthesis. Six hours of FGF9 exposure indeed produced a more notable increase in the ubiquitylation of WT and Ach receptors (Fig. 3B). The TDII mutant increase in ubiquitylation is less pronounced, due to its very high constitutive basal kinase activity. An inhibitor of FGFR3 tyrosine kinase, SU 5402 [27], significantly inhibited the ubiquitylation of all of the receptor types (Fig. 3B). Taken together, these results indicate a good correlation between RTK activity and ubiquitylation level, nevertheless only after prolonged activation.

To test the possibility that c-Cbl, the E3 ubiquitin ligase of the EGF receptor, may also be associated with FGFR3 or its mutants, we performed co-transfections of FGFR3 cDNA with a c-Cbl–HA construct. Following immunoprecipitation and Western blot analysis, no direct association between these two proteins could be observed (Fig. 4). Furthermore, overexpression of c-Cbl did not affect the expression of FGFR3 or its ubiquitylation, in contrast to similar experiments reported for the EGF receptor [28]. Interestingly, a correlation was found between the expression of c-Cbl and the activity of FGFR3. Expression of the overactive mutant receptors yielded higher levels of c-Cbl, with the highest level of c-Cbl detected in cells expressing the TDII mutant (not shown).

3.3. *The degradation pathway of FGFR3 – proteosomes versus lysosomes*

A number of plasma membrane proteins that are ubiquitylated appear to be degraded through both the proteosomal and lysosomal pathways [29]. In an attempt to identify the preferred degradation pathway of FGFR3 following its activation, we used chloroquine and MG-132, inhibitors of lysosomal and proteosomal degradation, respectively. In these experiments (Fig. 5) we employed the RCJ chondrocyte cell line model [30]. RCJ cells expressing either WT or the Ach mutant FGFR3 were exposed to FGF9 for 1 h, in the presence of cycloheximide, to block protein synthesis, with or without the addition of chloroquine or MG-132. As expected, addition of FGF9 caused a dramatic decrease in the level of the WT receptor (Fig. 5, top panel). Both chloroquine and MG-132 prevented most of this effect, indicating that degradation of FGFR3 involves both the proteosomal and lysosomal pathways. FGF9-induced degradation of the Ach mutant FGFR3 was, as expected, less marked than for the WT, but was also prevented by both chloroquine and MG-132. The total ubiquitylation of these receptors, analyzed with anti-ubiquitin antibodies (Fig. 5, bottom panel), appeared unchanged following FGF9 stimulation. However, when compared to the total receptor protein level, which decreased after FGF9 exposure, the stoichiometric ratio of ubiquitin/FGFR3 actually increased, particularly in the case of the WT protein. This

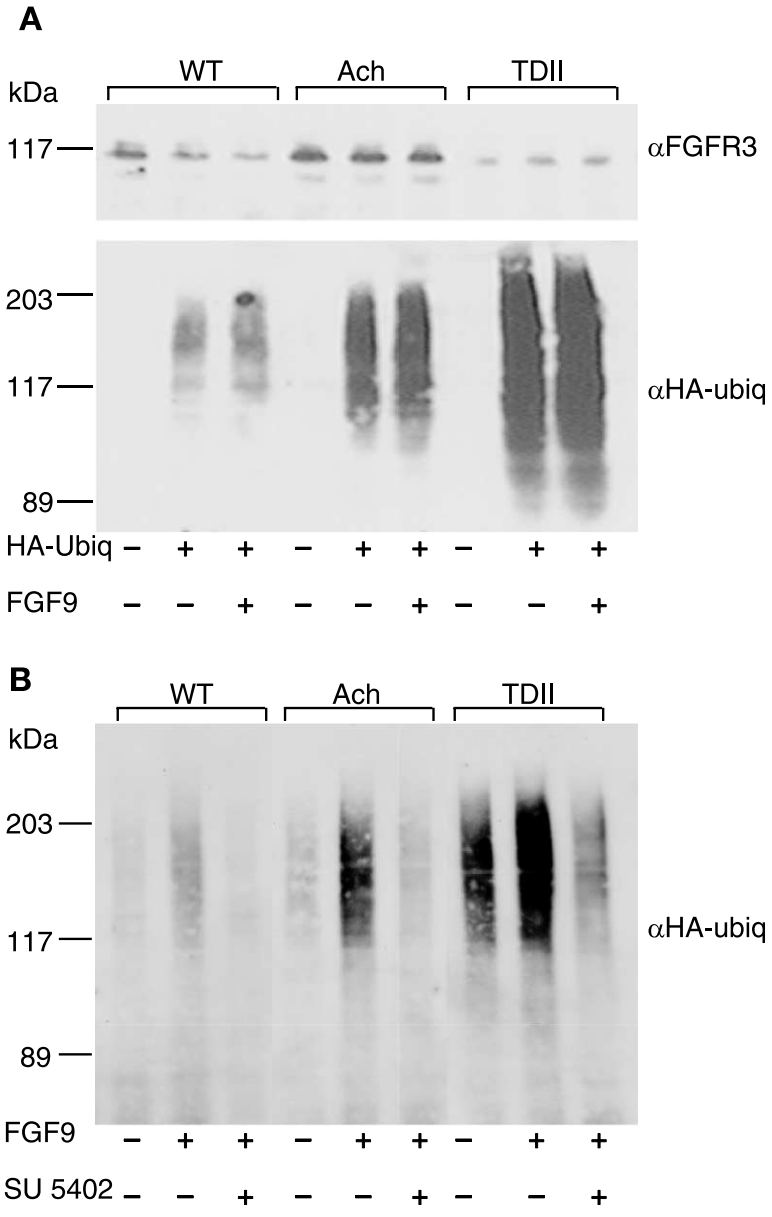

Fig. 3. The effect of FGF9 stimulation and of a tyrosine kinase inhibitor on ubiquitylation of WT and mutant FGFR3. A: 293T cells were transiently transfected with the WT, Ach or TDII receptors, either without or together with the ubiquitin–HA construct (HA–ubiq). Forty-eight hours later and following incubation with or without FGF9 for another 30 min, the cells were lysed, immunoprecipitated with anti-FGFR3 C-terminus antibodies and probed on an immunoblot with antibodies to either the kinase domain of FGFR3 (αFGFR3) or anti-HA (αHA–ubiq). B: 293T cells co-expressing FGFR3 and ubiquitin–HA for 48 h were incubated with or without FGF9 for 6 h in the absence and presence of the kinase inhibitor SU 5402 and analyzed for ubiquitin–HA (αHA–ubiq) as above.

confirms the observation of ligand-activated enhancement of receptor ubiquitylation. Blocking receptor degradation by chloroquine or MG-132 caused an accumulation of both WT and Ach mutant ubiquitylated receptors (Fig. 5, bottom panel).

4. Discussion

We have previously found that a naturally occurring mutation in FGFR3, in which glycine 380 is substituted by arginine (G380R) in the trans-membrane domain of the receptor, results in a specific defect in internalization, leading to stabilization and accumulation of the mutant receptor at the cell surface. The direct consequence of this impaired internalization is uncontrolled and prolonged ligand-dependent activation of this receptor in chondrocytes [14]. This is thought to lead to the observed inhibition of chondrocyte maturation and terminal differentiation which is the underlying cause of the most common form of human dwarfism, Ach. The aim of this work was to investigate the basis of the defective downregulation of this and other mutant FGF receptors.

The primary focus in this study was on FGFR3 ubiquitylation, since this process is known to be directly involved in the internalization of membrane proteins in yeast and mammalian cells [31] as well as in the downregulation of various membrane receptors, transporters and channels [15]. In most cases, ubiquitylation of these plasma membrane proteins leads to their internalization followed by targeting to the lysosome

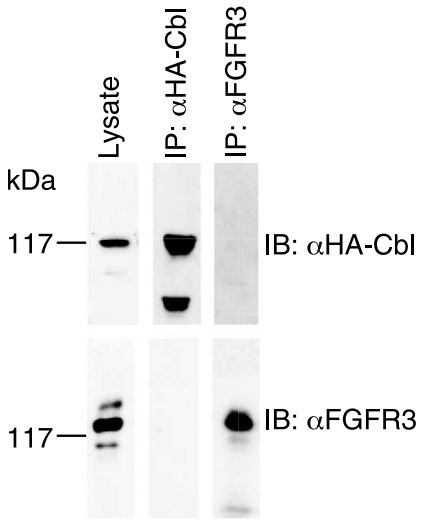

Fig. 4. Co-immunoprecipitation of FGFR3 and c-Cbl–HA. 293T cells were transiently co-transfected with the WT FGFR3 and c-Cbl–HA. Two days later and following incubation with FGF9 (50 ng/ml) for 30 min, the cells were lysed, and immunoprecipitated (IP) with either anti-FGFR3 antibodies (αFGFR3) or anti-HA (αHA–Cbl). The lysates and the IPs were probed on an immunoblot with antibodies to either the kinase domain of FGFR3 (αFGFR3) or anti-HA (αHA–Cbl).

(or vacuole in yeast) for degradation [29]. At least eight plasma membrane proteins in animal cells have been shown to be ubiquitylated at the cell surface. Among these are the GHR, the T cell receptor and several growth factor RTKs, such as the epidermal growth factor receptor, platelet-derived growth factor receptor (PDGFR) the colony stimulating factor-1 receptor (CSF1) [19,32–34] as well as FGFR1 [22].

Ligation of ubiquitin to the protein and tagging it to degradation in the proteosome system was suggested to occur via two different pathways. Ubiquitylation may serve as a signal for internalization, which is followed by protein degradation, implying that membrane proteins undergo ubiquitylation even before entering the early endosome [35]. The second hypothesis suggested first for the EGF system supports a concept in which receptor internalization does not require ubiquitin but occurs by recruitment of various additional components that form the clathrin-coated vesicle. Uncoating of the vesicle then serves to form the early endosome, in which the receptors are sorted either for degradation by ubiquitin conjugation (mediated by c-Cbl), or for recycling back to the membrane by another tag (mediated by v-Cbl) [36].

Ubiquitylation of FGFR1 [22], was shown to be ligand-dependent, although to a lesser extent than other members of the RTK super-family. We have confirmed this for FGFR3 by showing FGF9-induced ubiquitylation of both the WT and

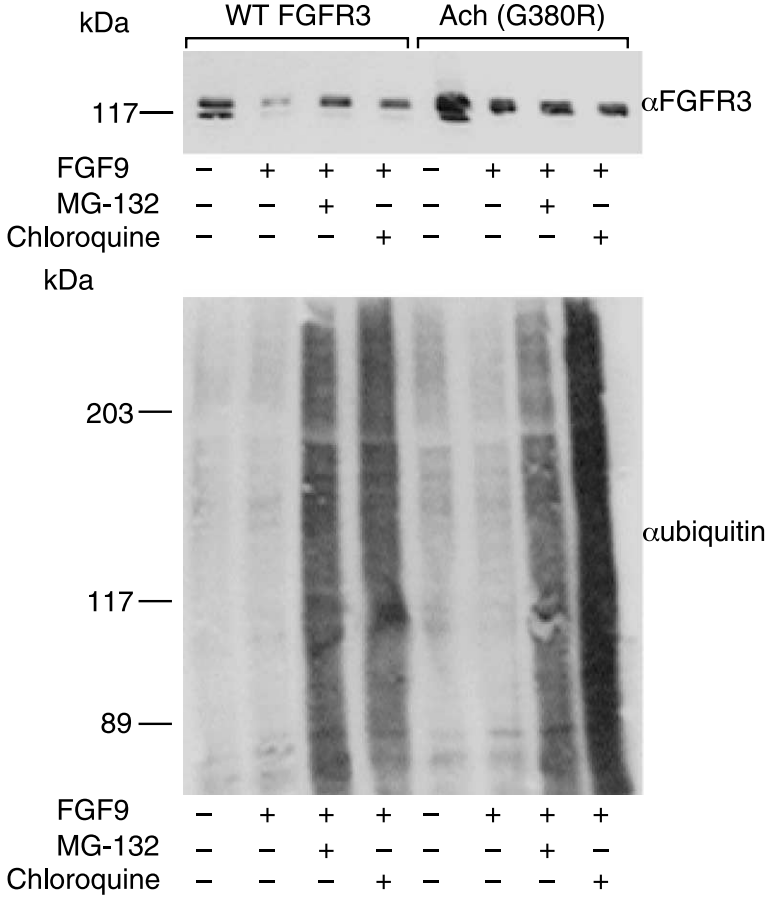

Fig. 5. Effect of proteosome and lysosome inhibitors on FGFR3 degradation in chondrocytes. RCJ cells expressing the WT or the Ach mutant FGFR3 were incubated with or without FGF9 (50 ng/ml) for 1 h, in the absence and presence of MG-132 (50 μM) or chloroquine (500 μM), as indicated. Protein synthesis was blocked by co-incubation with 10 μg/ml of cycloheximide (Chx). Cell lysates were immunoprecipitated with anti-FGFR3 antibodies and probed on an immunoblot with antibodies against the kinase domain of FGFR3 (upper panel, 'αFGFR3') or monoclonal antibodies against ubiquitin (lower panel, 'α-ubiq').

Ach mutant receptor forms. In addition, we have shown that the ubiquitylation levels of FGFR3 are dependent on its tyrosine kinase activity by several means: (i) low or non-detectable levels of ubiquitylation of the KD mutant receptor; (ii) blocking ubiquitylation upon treatment with kinase inhibitor; and (iii) proportionally high ubiquitylation levels are found in the case of the overactive Ach and TDII mutants. We conclude that tyrosine phosphorylation of FGFR3 is essential and acts as a trigger for its ubiquitylation. This is in line with proposals that phosphorylation and the exposure of the phosphorylation site facilitate the access of the ubiquitin conjugation machinery to the receptor [20,29,35]. However, tyrosine phosphorylation of FGFR3 by itself is not sufficient to induce ubiquitylation, as concluded from the duration required to induce the process. Only long exposure (6 h or more) of the cells to FGF induced ligand-dependent ubiquitylation, suggesting that the activation of the receptor promotes the transcription of one or more proteins required for its ubiquitylation, as was shown for P53/MDM2 [37]. Thirty minutes of exposure of cells to FGF, although is sufficient to induce phosphorylation, downstream signaling and downregulation of the receptor, is yet not enough to accomplish significant ubiquitylation of this receptor.

In the case of WT FGFR3, ubiquitylation induced by FGF9 or by high levels of ubiquitin expression leads to its downregulation, presumably via degradation. However, this does not hold for the Ach or TDII mutants, whose levels are not noticeably altered in spite of their robust ubiquitylation, which is commensurate with their activity. Therefore, the failure of these mutant receptors to be downregulated cannot be attributed to a defect in ubiquitylation. Also, when these results are considered together with the inefficient internalization of the Ach FGFR3 mutant [14], they indicate that, at least in this case, ubiquitylation may occur independently of internalization.

The ubiquitylation process involves the activities of E1, E2 and E3. In the latest reaction by an E3, ubiquitin is attached to lysine residues on the protein and later is elongated by repeated attachment of ubiquitin to form multiubiquitin chains. One such E3 for membrane-associated receptors is c-Cbl, a tyrosine kinase regulator for EGF, PDGF and CSF1 receptors [30,38–40]. In a most recent study it was demonstrated that FGFR1 signaling is attenuated via an indirect association of Cbl with FRS2α [41]. This was suggested to occur by the formation of a ternary-complex whereby Grb2 is bound to FRSα by means of its SH2 domain and to Cbl by means to its two SH3 domains. We could not observe any direct association between FGFR3 and c-Cbl, most likely due to the multiple protein and low affinity nature of these interactions. This is in contrast to other RTKs such as EGF, CSF1 or PDGF receptors which directly bind Cbl [18,38,39]. While overexpression of c-Cbl did not, in our hands, affect the expression of FGFR3 or its ubiquitylation (not shown), the observation that overactive mutants, such as the K650E FGFR3, induce high expression levels of c-Cbl do suggest for some feedback interaction between c-Cbl and the activity of FGFR3.

The degradation of several mammalian receptors that are known to undergo ligand-stimulated ubiquitylation appears to be impaired by inhibitors of proteosome-mediated breakdown of ubiquitylated proteins, as well as by agents that block lysosomal degradation. This has been reported for the PDGFR and the met tyrosine kinase receptor [33,42]. It is possible that a part of these receptors is degraded by the proteosome while another fraction is degraded in the lysosome [43]. Yet another possibility is that proteosome-dependent degradation of a protein other than the receptor might be required for efficient targeting and transport to the lysosome [44]. The degradation of FGFR3 seems to involve both the proteosomal and lysosomal pathways, since the proteosomal and lysosomal inhibitors MG-132 and chloroquine blocked ligand-dependent degradation of the WT receptor. These findings were less obvious for the Ach mutant receptor whose degradation was less pronounced. Nonetheless, the fact that inhibition of WT and Ach receptor breakdown causes accumulation of receptor-associated ubiquitin in both FGFR3 forms to above control levels indicates that ubiquitylated receptors are continuously removed by degradation and that the degradation pathways of the WT and Ach FGFR3s are similar if not identical.

The ubiquitylation mechanism comprises an extra control level on the activity and expression of receptors on the cell surface, which is particularly important for FGFR3 activity in the epiphyseal growth plates of long bones. Since ubiquitylation in itself is evidently necessary, but not sufficient for normal internalization and degradation of FGFR3, it is likely that other mechanisms may be involved. Ligand-activated FGFR3 interacted with a multitude of signaling molecules and adaptor proteins, including among others, Grb, Jak, PI3K, Src and SNT/FRS2 [45,46]. One possibility to be investigated is that human pathology associated mutant receptors fail to interact with one or more of these proteins responsible also for their internalization.

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