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Ubiquitination of Fibroblast Growth Factor Receptor 1 Is Required for Its Intracellular Sorting but Not for Its Endocytosis

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Endocytosis and targeting of growth factor receptors for lysosomal degradation have been associated with ubiquitination of the intracellular part of the receptors. To elucidate the role of receptor ubiquitination in internalization and sorting of fibroblast growth factor receptor (FGFR), we constructed several mutants of FGFR1 in which lysines, potential ubiquitination sites, were substituted for arginines. Substitution of all lysine residues in the intracellular part of FGFR1 resulted in inactivation of the tyrosine kinase domain of the receptor. However, several multilysine FGFR1 mutants, where up to 26 of 29 lysines in the intracellular part of the receptor were mutated, retained tyrosine kinase activity. The active multilysine mutants were poorly ubiquitinated, but internalized normally, indicating that ubiquitination of the receptor is not required for endocytosis. In contrast, degradation of the multilysine mutants was dramatically reduced as the mutants were inefficiently transported to lysosomes but rather sorted to recycling endosomes. The altered sorting resulted in sustained signaling. The duration of FGFR1 signaling seems to be tightly regulated by receptor ubiquitination and subsequent sorting to the lysosomes for degradation.

INTRODUCTION

Internalization of activated receptor tyrosine kinases (RTKs) by endocytosis followed by sorting to lysosomes and subsequent degradation of the receptors is one of the ways cells achieve signal attenuation. Endocytosis can also lead to alteration in the signaling simply by guiding the receptors to a new cellular localization (Wiley and Burke, 2001). RTKs localized on endosomes can recruit and trigger signaling molecules differently from those recruited by receptors remaining at the plasma membrane.

During recent years, the role of ubiquitin (Ub) in internalization and sorting of receptors has been intensively investigated. Ubiquitin is a small molecule of 8 kDa that becomes covalently attached to lysine residues of target proteins through the action of Ub ligases (E3 enzymes). It has been suggested that RTK ubiquitination is important both for receptor internalization and degradation in the lysosomes, but the role of ubiquitination is not entirely clear (Mukhopadhyay and Riezman, 2007).

Endocytosis and lysosomal targeting of RTKs is probably best demonstrated in the case of epidermal growth factor receptor (EGFR), which is ubiquitinated by the E3 ubiquitin ligase Cbl. Overexpression of Cbl leads to enhanced endocytosis, whereas overexpression of dominant-negative Cbl mutants or knockdown of Cbl leads to reduced endocytosis (Lill *et al*., 2000; Jiang and Sorkin, 2003; Duan *et al*., 2003; Stang *et al*., 2004; Huang *et al*., 2006). Clearly, recruitment of Cbl is required for proper endocytosis, but the question of whether ubiquitination of the receptor itself is necessary is still under investigation.

Fusion of an ubiquitin moiety to mutants of EGFR that were inefficiently internalized induced their endocytosis. This indicates that ubiquitination is sufficient to drive endocytosis (Haglund *et al*., 2003; Mosesson *et al*., 2003). In contrast, when specific lysines in the EGFR kinase domain identified as ubiquitination sites by mass spectrometry were substituted with arginines, the endocytosis of EGFR was unimpaired (Huang *et al*., 2006, 2007), indicating that ubiquitination of the receptor is not required for internalization.

After internalization, ubiquitinated receptors are recognized by Hrs and the ESCRT complexes, internalized into multivesicular bodies and eventually degraded in lysosomes (Raiborg *et al*., 2003). Sorting of EGFR to degradation in lysosomes has been reported to be mediated by recruitment of c-Cbl followed by ubiquitination of the receptor (Levkowitz *et al*., 1998). In agreement with this, lysines identified as ubiquitination sites in the kinase domain of EGFR were demonstrated to be essential for down-regulation of EGFR (Huang *et al*., 2006).

The family of high-affinity fibroblast growth factor receptors (FGFR) with tyrosine kinase activity comprises four members, designated FGFR1-4, and a number of splicing variants. The binding of fibroblast growth factors (FGFs) to FGFR results in the formation of double dimers of receptor

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and ligand into a complex that is stabilized by heparan sulfate proteoglycans. The complex formation activates the intracellular tyrosine kinase domain by autophosphorylation followed by activation of downstream signaling pathways (Schlessinger, 2004). The kinase activity plays a role in internalization of FGFR1 because a kinase negative FGFR1 mutant (K514A) exhibited a reduced rate of internalization (Sorokin *et al*., 1994). Also, tyrosine 766, one of the autophosphorylation sites of FGFR1, was found to be important for internalization because an FGFR1 Y766F mutant failed to internalize (Sorokin *et al*., 1994). Phosphorylation of Tyr 766 is essential for binding and activation of phospholipase C (PLC) γ. Surprisingly, mutation of the corresponding tyrosine in FGFR2 did not alter the rate of internalization of this receptor (Ceridono *et al*., 2005).

After internalization, endocytosed FGF/FGFR complexes reach early/sorting endosomes. From here, FGFR4 is sorted mainly to the recycling compartment, whereas FGFR1-3 are sorted mostly to degradation in the lysosomes. FGFR4 was found to be less ubiquitinated than FGFR1, and different levels of ubiquitination were proposed as a mechanism to regulate their sorting (Haugsten *et al*., 2005). A few reports have described ubiquitination of FGFRs (Mori *et al*., 1995; Monsonego-Ornan *et al*., 2002; Wong *et al*., 2002; Cho *et al*., 2004; Haugsten *et al*., 2005). Cho *et al*. (2004) reported that mutants of FGFR3 associated with skeletal disorders had a slower rate of degradation, which correlated with their lower level of ubiquitination than that of FGFR3 wild-type receptor. Furthermore, c-Cbl has been demonstrated to play a role in FGFR ubiquitination through binding to FRS2 via Grb2 (Wong *et al*., 2002; Cho *et al*., 2004).

To further investigate the role of FGFR ubiquitination in endocytosis and sorting, lysines in the intracellular part of FGFR1 were mutated to arginines. Mutant receptors that retained kinase activity but were poorly ubiquitinated were then used to elucidate the role of FGFR1 ubiquitination in receptor down-regulation.

MATERIALS AND METHODS

Antibodies and Reagents

The following primary antibodies were used: rabbit anti-FGFR1, rabbit anti-FGFR4, rabbit anti-p-PLCγ1 (Y783), mouse anti-pY99, mouse anti-ubiquitin P4D1, rabbit anti-Cbl (C-15), mouse anti-Cbl-b (G-1) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-early endosomal antigen (EEA)1 (BD Biosciences Transduction Laboratories, Lexington, KY); mouse anti-Myc Tag, clone 4A6 (Millipore, Billerica, MA); mouse anti-p-FGFR (Tyr 653/654), rabbit anti-p-FRS2 alpha (Tyr196), mouse anti-p-extracellular signal-regulated kinase (ERK)1,2 (Thr202/Tyr204) (Cell Signaling Technology, Danvers, MA); mouse anti-heat shock protein 90 kDa (Hsp90) (Assay Designs, Ann Arbor, MI); rabbit anti-ricin (Sigma-Aldrich, St. Louis, MO); and mouse anti-clathrin heavy chain (Affinity BioReagents, Golden, CO). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa 488-epidermal growth factor (EGF) and Alexa 647-transferrin (Tf) were from Invitrogen (Carlsbad, CA). FGF1 and FGF1-myc8 were prepared as described previously (Nilsen *et al*., 2007). FGF1 was iodinated by the Iodogen method according to the manufacturer's protocol (Pierce Chemical, Rockford, IL). ^{125}I-Na was from PerkinElmer Life and Analytical Sciences (Boston, MA). FGF1 was labeled with Cy3-maleimide (GE Healthcare, Chalfont St. Giles, United Kingdom) following the manufacturer's procedures. The following reagents were used: heparin, PD173074, monensin, brefeldin A, cycloheximide, 10% Formalin solution, ricin (Sigma-Aldrich); DharmaFECT transfection reagent 1, ON-TARGETplus siCONTROL siRNA (Dharmacon RNA Technologies, Lafayette, CO); Cbl-b siRNA (h), c-Cbl siRNA (h) (Santa Cruz Biotechnology); leupeptin (Peptide Institute, Osaka, Japan); FuGENE 6, DOTAP (Roche Diagnostics, Indianapolis, IN); DRAQ5 (Biostatus); SU5402 (Calbiochem, San Diego, CA); and Geneticin (G-418; Invitrogen).

Plasmids

pcDNA3-hFGFR1 (Haugsten *et al*., 2005) and pcDNA3-hFGFR4 (Klingenberg *et al*., 2000) have been described previously. pERK5-myc-dominant-negative Grb2 (d.n.Grb2) and pcDNA3-myc-tagged-UbR (Stang *et al*., 2004) were generous gifts from Inger Helene Madshus (Institute of Pathology, Rikshospitalet, University of Oslo, Oslo, Norway) and Harald Stenmark (Norwegian Radium Hospital, Oslo, Norway), respectively. Vector-based small interfering RNA (siRNA) against clathrin heavy chain (Grimmer *et al*., 2005) was a generous gift from Kirsten Sandvig (Norwegian Radium Hospital, Oslo, Norway). pcDNA3-hFGFR1 was used as template for site-directed and multisite-directed QuikChange mutagenesis to generate lysine mutants following the manufacturer's procedures (Stratagene, La Jolla, CA). To generate pcDNA3 hFGFR1 29R-UbR, an XhoI site was added to the end of the coding sequence of hFGFR1 29R by site-directed QuikChange mutagenesis. UbR was amplified from pcDNA3-myc-UbR by polymerase chain reaction (PCR) with primers containing an in-frame XhoI site. UbR was then ligated into pcDNA3-hFGFR1 29R-XhoI. pET21d-FGF1-myc8 was generated by introducing eight copies of the myc-tag into an internal loop of FGF1 in pET21d-FGF1 by using standard cloning techniques. All constructs were confirmed by DNA sequencing.

Cells and Transfection

Transient expression of the different constructs was performed by transfecting cells with the plasmid DNA using FuGENE 6 transfection reagent according to the manufacturer's protocol. Cells were seeded into plates the day preceding the transfection, and experiments were performed 16–24 h after transfection. For the knockdown of clathrin, the cells were transfected with vector-based siRNA against clathrin heavy chain for 96 h. For the knockdown of c-Cbl and Cbl-b, the cells were simultaneously transfected with 25 nM of each siRNA or 50 nM scramble siRNA by using DharmaFECT transfection reagent 1 according to the manufacturer's protocol. The transfection was repeated after 48 h, and the cells were kept for additional 48 h. DOTAP liposomal transfection reagent was used according to the manufacturer's protocol to obtain U2OS cells stably expressing desired FGFR construct. Clones were selected with 1 mg/ml G-418. Clones were chosen based on their receptor expression level analyzed by immunofluorescence and immunoblotting. Both HeLa cells and U2OS cells were propagated in DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. In addition, 1 mg/ml G-418 was added to the growth media of stably transfected U2OS cells.

Laser Scanning Confocal Microscopy

Cells grown on coverslips were kept with 100 ng/ml cyanine (Cy)3-FGF1 for 1 h in HEPES medium at 4°C in the presence of 50 U/ml heparin. The cells were then washed three times in phosphate-buffered saline (PBS) and incubated further for different periods of time in DMEM at 37°C. For d.n.Grb2 experiments and Cbl siRNA experiments, the cells were incubated with Cy3-FGF1 at 37°C without prebinding at 4°C. The cells were fixed in 10% Formalin solution and mounted in Mowiol. In some cases, the cells were in addition to Cy3-FGF1 incubated with 100 ng/ml Alexa 488-EGF, 5 μg/ml Alexa 647-Tf, or 1 μg/ml ricin. Inhibitors were added 20 min before the experiment and kept throughout the experiment. When antibodies were used to visualize structures within the cell, the cells were permeabilized with 0.05% saponin or 0.1% Triton X-100 and incubated with the primary antibody for 20 min, washed, and then incubated with the secondary antibody coupled to a fluorophore for 20 min before mounting in Mowiol. The cells were examined with an LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany). Images were prepared with LSM Image Browser version 3.2 (Carl Zeiss) and CorelDRAW11 (Corel, Fremont, CA). Quantification of colocalization was performed as described previously (Haugsten *et al*., 2005). For quantification of Cy3-FGF1 uptake, cells randomly located on the coverslips were scanned at fixed intensity settings below pixel-saturation, and the total cellular intensity was determined using the histogram function in the LSM 510 software (Carl Zeiss). All pixel values above the background level were quantified.

Ubiquitination Assay

U2OS cells stably expressing various FGFR1 constructs were serum starved for 1 h in DMEM before further incubation with 100 ng/ml FGF1 and 10 U/ml heparin for 10 min. The cells were immediately lysed in lysis buffer containing 2 mM *N*-ethylmaleimide (0.1 M NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1 mM EDTA supplemented with protease inhibitors, and phosphatase inhibitors, pH 7.4), and the lysate was added to Sepharose beads incubated with anti-FGFR1 antibody. After tumbling for 1 h at 4°C, the beads were washed three times in 0.1% Tween 20 in PBS (supplemented with 2 mM *N*-ethylmaleimide, protease inhibitors, and phosphatase inhibitors) and once in 1:10 diluted PBS. The proteins that remained bound to the Sepharose beads were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and immunoblotting using anti-ubiquitin antibodies. After stripping, the membrane was reprobed with the anti-FGFR1 antibodies. The chemiluminescent signal was detected on film or scanned using the Chemigenious Bio Imaging system (Syngene, Frederick, MD). ImageQuant version 5 (GE Healthcare) was used for quantification of the intensity of bands of interest.

Internalization of ^{125}I-FGF1 and Saturation Binding Experiments

Internalization experiments were performed on confluent cells growing on 12-well gelatinized microtiter plates incubated for indicated times (5, 10, and

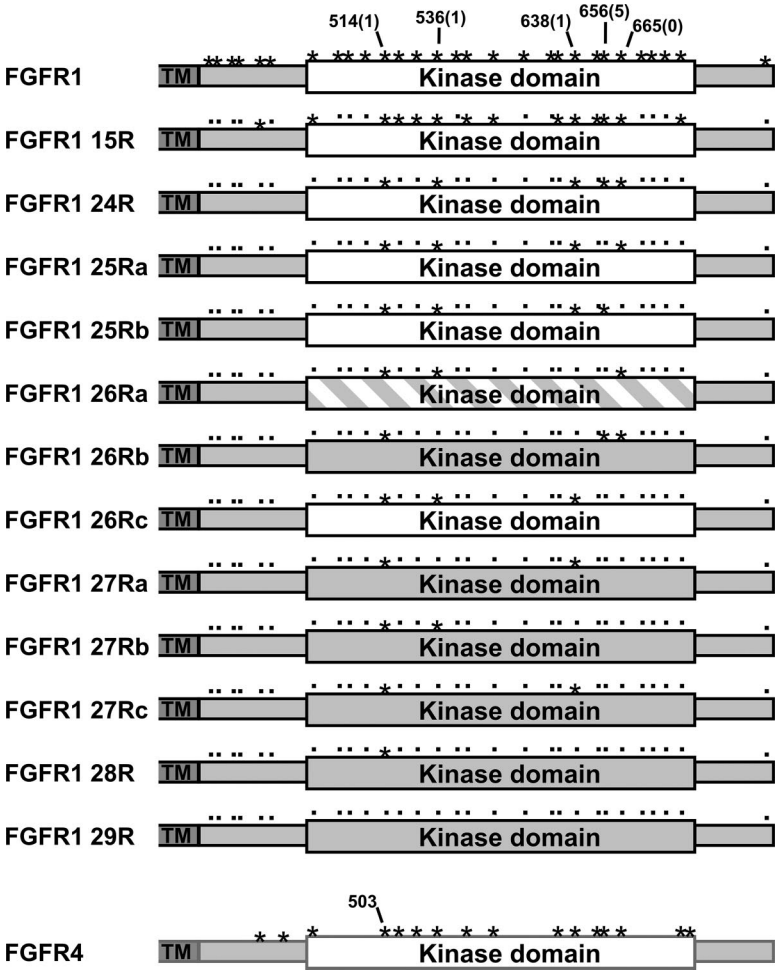

Figure 1. Schematic presentation of the intracellular part of wild-type and mutant FGFRs. An asterisk indicates a lysine, and a dot indicates a lysine mutated to an arginine. A kinase domain in white indicates an active receptor, a kinase domain in gray an inactive receptor, and a striped kinase domain indicates a semiactive receptor. Important residues are labeled with their number. Numbers in brackets indicate the FSA of the residue (Mohammadi *et al*., 1996) in a scale from 0 to 10. The lower the value the less solvent-exposed the residue. TM, transmembrane region.

15 min) at 37°C with 5 or 10 ng of ^{125}I-FGF1 in HEPES medium containing 0.2% gelatin and 20 U/ml heparin. The cells were then washed twice with ice-cold HEPES medium and once in 1 M NaCl in PBS. Surface-bound ^{125}I-FGF1 was collected after removal with high salt/low pH buffer (2 M NaCl and 20 mM NaAc, pH 4.0) and finally, internalized ^{125}I-FGF1 was collected after solubilization of cells in 1 M KOH. Radioactivity was measured with a gamma-counter, and the ratio of internalized to surface-localized ^{125}I-FGF1 was plotted as function of time. When inhibitors were used, the cells were starved for 24 h, and the inhibitors were added 20 min before experiments and kept throughout the experiment. ^{125}I-FGF1 saturation binding experiments were performed essentially as previously published experiments (Munoz *et al*., 1997). The cells were incubated for 2 h at 4°C in HEPES medium containing 40 U/ml heparin, 0.2% gelatin, and increasing concentrations of ^{125}I-FGF1. Then, the cells were washed twice with ice-cold HEPES containing 10 U/ml heparin and once in PBS containing 10 U/ml heparin and once in 1 M NaCl in PBS. Cells were lysed in 1 M KOH, and the solubilized radioactivity was measured with a gamma-counter. The data were fitted to binding curves.

Western Blot Analysis of FGFR Activation and Signal Transduction

U2OS cells, stably expressing various types of FGF receptors, were serum-starved for 24 h in DMEM. The medium was changed for fresh DMEM, and serum starvation was continued for 1 h more. In some cases, 15 min before the end of serum starvation, the medium was supplemented with the indicated inhibitors. Next, cells were left untreated or treated with 100 ng/ml FGF1 and 10 U/ml heparin for the indicated time periods. Cells were lysed in lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, and 1 mM EDTA supplemented with protease inhibitors and in some cases phosphatase inhibitors, pH 7.4), and cellular material was analyzed by SDS-PAGE and immunoblotting using anti-p-FGFR antibodies (mouse). After stripping, the membrane was reprobed with the desired antibody in the following order: anti-p-FRS2α (rabbit), anti-p-PLCγ1 (rabbit), anti-p-ERK1,2 (mouse), anti-FGFR1 (or anti-FGFR4) (rabbit), and anti-Hsp90 (mouse). The chemiluminescent signal was detected on film or scanned using the Chemigenious Bio Imaging system (Syngene). ImageQuant version 5 was used for quantification of the intensity of bands of interest.

RESULTS

Characterization of FGFR1 Lysine Mutants

To elucidate the role of receptor ubiquitination in FGFR internalization and intracellular sorting, several FGFR1 mutants, in which lysines in the intracellular part of the receptor (indicated with asterisks) were substituted for arginines (indicated with dots), were constructed (Figure 1). Lysine but not arginine residues are possible ubiquitination sites, and the intracellular part of FGFR1 contains 29 lysine residues, whereas FGFR4 contains 16 lysine residues (Haugsten *et al*., 2005). We first constructed a mutant FGFR1 that was more FGFR4-like with respect to lysine content. An alignment of the amino acid sequence of the two receptors was constructed and used to identify lysines present in FGFR1 but not in FGFR4. These lysines were changed to arginines to produce the mutant FGFR1 15R. Furthermore, several FGFR1 multilysine mutants were constructed (Figure 1). In FGFR1 29R, all 29 lysines in the intracellular part were substituted for arginines. Lysine 514 in the intracellular part of FGFR1 and the corresponding lysine 503 in FGFR4 are known to be crucial for receptor kinase activity. To obtain an active FGFR1, a series of mutants were made where lysine 514 was conserved. Selected lysines, based on their conser-

vation through the FGF receptor family and their low fractional solvent accessibility (FSA) value (Mohammadi *et al.*, 1996), were left unchanged, whereas the remaining lysines in the intracellular part of the receptor were substituted for arginines (Figure 1). These mutants were then tested further.

The FGFR1 lysine mutants were expressed by transient transfection in HeLa cells and analyzed by confocal microscopy. Heparin was added to the experiments to avoid FGF1 binding to cell-surface heparan proteoglycans and to facilitate binding of FGF to the high-affinity FGFRs. When HeLa cells transiently transfected with the different constructs were treated with Cy3-labeled FGF1 (Cy3-FGF1) at 4°C in the presence of heparin, fixed, and then analyzed in the confocal microscope, cell surface staining of Cy3-FGF1 was detected. This indicates that the different FGFR1 mutants are presented at the cell surface and that they bind FGF1 (Figure 2A, only shown for some of the constructs). No binding was detected in untransfected cells.

When FGF1 is bound to the wild-type receptor, further incubation at 37°C allows the complex to be internalized and the fluorescent growth factor to occur as intracellular dots, indicating uptake into vesicles (Figure 2B). HeLa cells transiently transfected with the different FGFR constructs were incubated at 37°C for 30 min with Cy3-FGF1 and heparin, fixed, and examined in the confocal microscope. In cells transfected with FGFR1 29R, Cy3-FGF1 surface staining was still pronounced, and no or only a few red intracellular dots were observed, indicating that the FGF1 bound to FGFR1 29R is largely not internalized. The same was observed for FGFR1 26Rb, -27Ra, -27Rb, -27Rc, and 28R (data not shown). Intracellular dots together with some surface staining was observed in HeLa cells transfected with FGFR1 25Ra and 26Rc, whereas Cy3-FGF1 was observed mainly intracellularly in FGFR1-, FGFR1 15R-, or FGFR4-transfected cells. The sustained surface staining of cells transfected with FGFR1 25Ra or 26Rc suggest that these receptors could be endocytosed more slowly than wild-type receptors or that they are recycled together with the ligand back to the cell surface.

To test whether the different FGFR constructs are activated upon ligand binding, the same cells as described above were stained with anti-phosphotyrosine 99 antibody (pY99) (Figure 2B). This antibody specifically detects phosphorylated tyrosine residues and only stains cells that express an active receptor construct upon FGF treatment. To detect both transfected and untransfected cells, all cells were labeled with DRAQ5 (a fluorescent DNA staining dye). In FGFR1 29R, no anti-pY99 staining was visualized, indicating that the receptor is inactive. This is in agreement with the importance of lysine 514 for kinase receptor activity. Several lysines in the intracellular part of FGFR1 turned out to be necessary for receptor signaling activity because no anti-pY99 staining was observed in cells transfected with FGFR1 26Rb, -27Ra, -27Rb, -27Rc, or -28R (data not shown). For all other receptor mutants, staining with anti-pY99 antibody was detected, indicating that these mutants are active. The active receptor mutants are indicated in Figure 1 with the kinase domain in white, and inactive mutants are indicated with the kinase domain in gray.

The activity of some of the lysine mutants was more closely characterized in stably transfected U2OS cells. Stably transfected clones were selected based on their level of receptor expression (data not shown). Clones expressing indicated receptor construct were serum starved and then left untreated or treated with FGF1 and heparin, in the absence and presence of PD173074, before lysis and immunoblotting analysis (Figure 3). PD173074 is an inhibitor of the FGFR tyrosine kinase. Treatment with FGF1 induced phosphorylation of the receptor and downstream signaling molecules such as PLCγ1, FRS2α, and ERK1,2, whereas addition of PD173074, blocked their activation. PD173074 blocked sig-

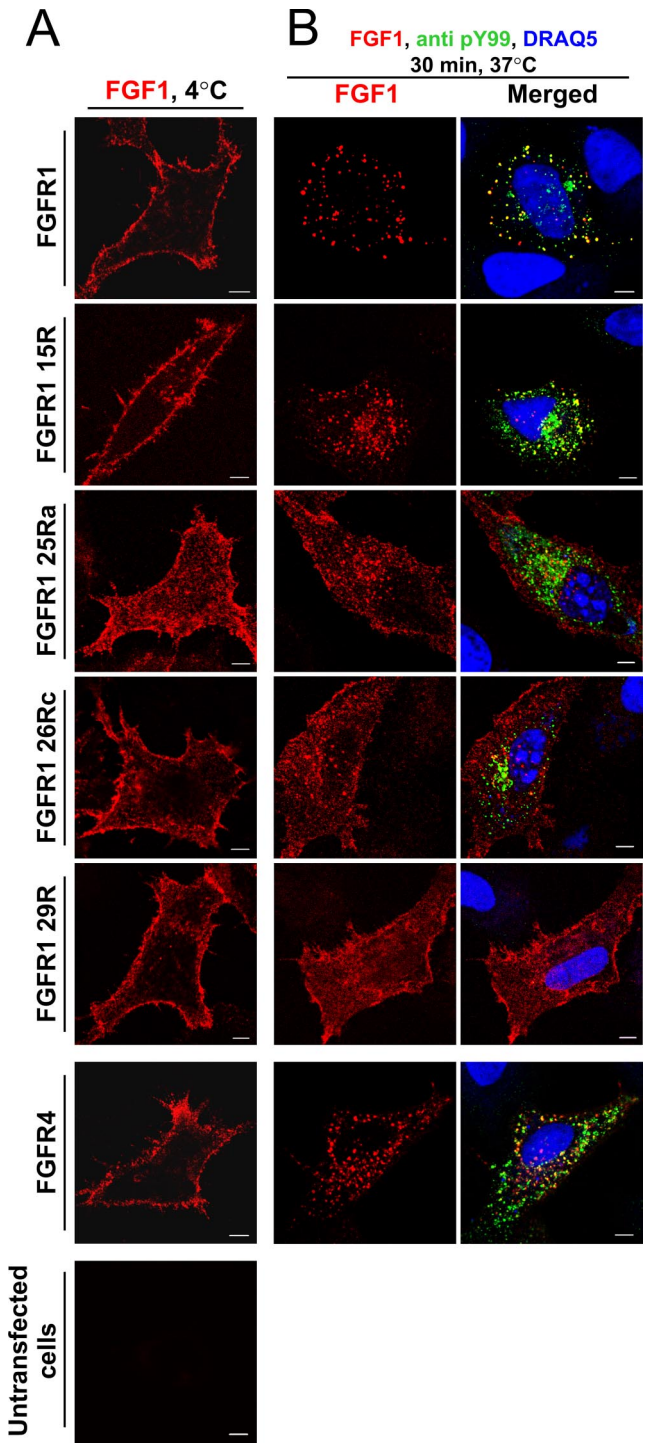

Figure 2. FGF1 binding, internalization, and activation of wild-type and mutant FGFRs. HeLa cells transiently transfected with the indicated receptor construct were treated with Cy3-FGF1 and 50 U/ml heparin for 1 h at 4°C and either fixed immediately (A) or incubated for 30 min at 37°C before fixation (B). Cells incubated at 37°C were stained with anti-phosphotyrosine 99 antibody (anti-pY99) and DRAQ5. Cells were examined by confocal microscopy. Bar, 5 μm.

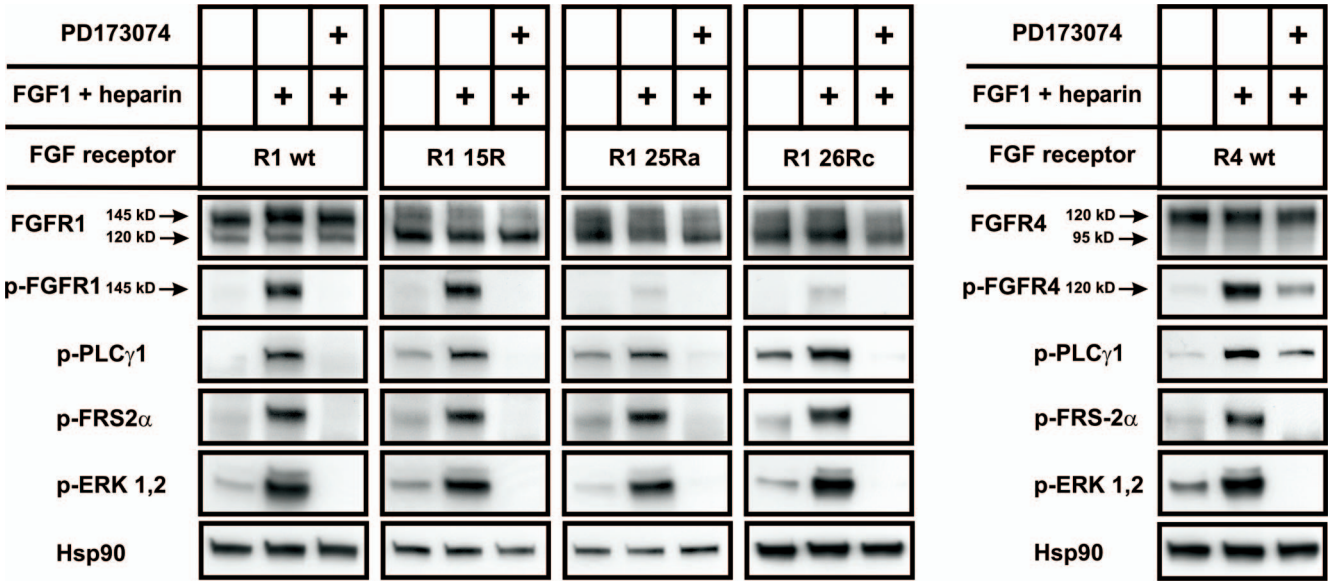

Figure 3. Activity of wild-type and mutant FGFRs. U2OS cells, stably expressing FGFRs as indicated, were serum starved for 24 h and then left untreated or treated with 100 ng/ml FGF1 and 10 U/ml heparin, in the absence or presence of 50 nM PD173074. Cells were lysed, and the cellular material was analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. A p in front of the name of the antibody indicates that it recognizes the phosphorylated form of the protein.

naling from FGFR4 somewhat less efficiently than signaling from FGFR1. The bands indicating phosphorylated FGFR1 25Ra and FGFR1 26Rc were weaker than the band corresponding to phosphorylated wild-type receptor. These receptors might be less active than FGFR1 but potent enough to activate the downstream signaling molecules. It is also possible that the antibody that recognizes phosphorylated FGFR1 has a lower affinity for FGFR1 25Ra and FGFR1 26Rc. The antibody is raised against a synthetic phospho-peptide corresponding to residues surrounding tyrosine 653/654. Two lysines in this region are substituted for arginines in FGFR1 25Ra and 26Rc. Antibodies against total FGFR1 revealed two bands corresponding to proteins of 120 and 145 kDa, probably representing different forms of glycosylated FGFR1. Biotinylation of cell surface proteins followed by a pull-down of biotinylated proteins, and Western blot analysis revealed that only the upper band, 145-kDa form of the receptor, was present at the cell surface (data not shown). Only the 145-kDa form was detected with the phospho-FGFR antibody, indicating that only the receptors present at the cell surface are activated by FGF. Immunoblotting with anti-Hsp90 was included to confirm equal loading.

Ubiquitination of the different FGFR constructs was analyzed by Western blotting of FGFR immunoprecipitates from U2OS cells stably expressing the different receptor constructs upon FGF1 stimulation. Ubiquitination was detected as a smear of bands migrating more slowly than the nonubiquitinated receptors (Figure 4A). FGFR1 was strongly ubiquitinated, whereas FGFR1 15R was considerably less ubiquitinated than wild-type receptor. The multilysine mutants FGFR1 25Ra and 26Rc displayed a smear of bands similar to the background levels in untransfected cells. The intensity of the smear of bands was quantified and normalized to total receptor level, and Figure 4B shows the mean from three independent experiments with FGFR1 ubiquitination set to 100%. Ubiquitination of FGFR1 15R was reduced to ~40%, and ubiquitination of FGFR1 25Ra and FGFR1 26Rc was reduced to 3–4%. Thus, the level of receptor ubiquitination correlated with the number of lysines in the intracellular part of the receptor.

Internalization of FGFR1 Lysine Mutants

The rate of internalization of the different mutants was examined in the stably transfected U2OS cells and measured as the ratio of endocytosed to surface-bound ^{125}I-FGF1 at different times. The results were confirmed in two clones for each construct (data shown only for one clone), and nonsaturating amounts of ^{125}I-FGF1 were used. Saturation of FGF binding sites at the cell surface was predicted from ^{125}I-FGF1 binding experiments (data not shown). In accordance with previously reported data (Sorokin *et al.*, 1994), internalization of FGF1 by FGFR1 in the presence or absence of receptor kinase inhibitors SU5402 or PD173974 showed that tyrosine kinase activity of the receptor was required for a maximal rate of internalization (Figure 5A). Surprisingly, U2OS cells expressing FGFR1 25Ra or 26Rc endocytosed ^{125}I-FGF1 at a rate similar to FGFR1-expressing cells (Figure 5B). Furthermore, FGFR1 29R was hardly internalized. Figure 5C presents the mean of the slope from five independent experiments.

To test whether the surface staining in Figure 2B in FGFR1 25Ra- and FGFR1 26Rc-transfected cells was due to recycling or inefficient endocytosis, the cells were treated with monensin. Monensin is a monovalent carboxylic ionophore that has been shown to block recycling of several receptors (Basu *et al.*, 1981; Felder *et al.*, 1990). After treatment with 50 μM monensin, Cy3-FGF1 surface staining in HeLa cells transiently transfected with FGFR1 25Ra disappeared (Figure 5D). This indicates that the Cy3-FGF1 cell surface staining in untreated cells is rather a result of recycling of the receptor and the ligand back to the cell surface than inefficient endocytosis. This is in agreement with the data presented in Figure 5B. Treatment with monensin did not alter the rate of internalization of radiolabeled FGF1 in U2OS cells stably expressing FGFR1 (data not shown).

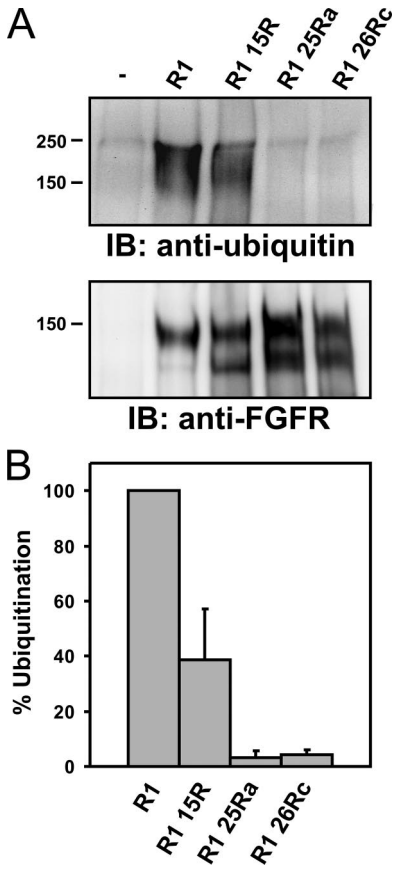

Figure 4. Ubiquitination of wild-type and mutant FGFRs. (A) U2OS cells, stably expressing FGFRs as indicated, were serum starved for 24 h and then left untreated or treated with 100 ng/ml FGF1 and 10 U/ml heparin for 10 min at 37°C. After lysis, solubilized receptor proteins were immunoprecipitated and analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. (B) The amount of ubiquitinated receptor normalized to the total FGFR was expressed as a percentage with the ubiquitination of wild-type FGFR1 set to 100. The mean of three independent experiments are shown. Error bars denote the SE.

Furthermore, to test whether ubiquitination of FGFR1 29R could induce its internalization, an ubiquitin moiety was fused in-frame to the cytoplasmic tail of FGFR1 29R (FGFR1 29R-UbR) (Figure 5E). The two last glycines in the ubiquitin moiety were deleted to prevent conjugation to other proteins (Raiborg *et al*., 2002; Haglund *et al*., 2003; Mosesson *et al*., 2003). HeLa cells transiently transfected with FGFR1, FGFR1 29R, or FGFR1 29R-UbR were incubated with Cy3-FGF1 for 2 h at 37°C, fixed, and examined by confocal microscopy. Mostly cell surface staining was observed for cells transfected with FGFR1 29R and FGFR1 29R-UbR, whereas only intracellular staining was observed in FGFR1 wild-type–transfected cells (Figure 5E). Clearly, fusing an ubiquitin moiety to the cytoplasmic tail of FGFR1 29R did not induce its internalization. Thus, the abolished kinase activity, and not the lack of ubiquitination, seems to keep FGFR1 29R at the cell surface.

It is not entirely clear which pathways and proteins are involved in internalization of FGFRs. Both clathrin and clathrin-independent pathways have been suggested to play a role in FGFR internalization (Gleizes *et al*., 1996; Marchese *et al*., 1998; Citores *et al*., 1999; Belleudi *et al*., 2007). To investigate whether wild-type FGFR1 and the lysine mutants use the same pathway for internalization, we performed knockdown experiments with vector-based siRNA targeted against clathrin heavy chain (Grimmer *et al*., 2005) in U2OS cells stably expressing the different receptors. Confocal analysis showed that in cells expressing FGFR1 and where clathrin heavy chain was knocked down by siRNA (labeled with an asterisk), FGF1 was prevalently localized to the cell surface after 30 min of endocytosis (Figure 6A). Also, the uptake of Tf, which is known to be clathrin-dependent was blocked in siRNA-expressing cells (Figure 6A, top), whereas internalization of ricin, which is not dependent on clathrin, was normal (Figure 6A, bottom). Also, in cells expressing the FGFR1 15R, -25Ra, or -26Rc mutants and depleted for clathrin heavy chain, FGF1 is hardly endocytosed (Figure 6B). These results suggest that both FGFR1 wild-type and the multilysine mutants use the same clathrin-dependent endocytic pathway for internalization in U2OS cells.

A protein involved in clathrin-dependent endocytosis of EGFR is Grb2 (Jiang *et al*., 2003). Grb2 has also been suggested to play a role in FGFR endocytosis by binding to the receptor via FRS2α and to recruit the ubiquitin ligase Cbl to the receptor. Knockdown of FRS2α partially inhibited internalization of FGFR (Wong *et al*., 2002). To investigate whether internalization of FGFR1 and the different multilysine mutants is dependent on Grb2, we used a myc-tagged d.n.Grb2 in which the N-terminal and C-terminal Src homology 3 domains are not functional (Stang *et al*., 2004). U2OS cells transiently transfected with the d.n.Grb2 and stably expressing wild-type FGFR1 or the lysine mutants were allowed to internalize Cy3-FGF1 for 20 min before fixation and staining with anti-myc antibody to indicate d.n.Grb2-transfected cells. The cells were then analyzed by confocal microscopy. Expression of d.n.Grb2 resulted in reduced internalization of Cy3-FGF1, indicated by fewer intracellular dots in cells expressing d.n.Grb2 construct than in cells not expressing the d.n.Grb2 (Figure 7A). Furthermore, quantification of the intensity of Cy3-FGF1 staining in cells expressing d.n.Grb2 compared with nonexpressing cells revealed that the Cy3-FGF1 uptake was reduced to ~50–60% in d.n.Grb2-expressing cells (Figure 7B). Expression of d.n.Grb2 had similar effects on internalization of the wild-type FGFR1 and the lysine mutants. Cy3-FGF1 internalization was reduced to ~54% in FGFR1-expressing cells, ~62% in FGFR1 15R-expressing cells, and ~59% in FGFR1 25Ra- and FGFR1 26Rc-expressing cells. In sum, these results demonstrate that both wild-type FGFR1 and lysine mutants are internalized through a clathrin-dependent pathway and that their internalization is to a similar extent dependent on Grb2.

To address the question whether the internalization of the multilysine mutants is dependent on Cbl, siRNA studies, in which both c-Cbl and Cbl-b were knocked down simultaneously, were performed (Supplemental Figure 1). Despite an efficient knockdown (reduction of c-Cbl and Cbl-b confirmed by Western blotting; Supplemental Figure 1A), we were not able to observe significant differences in the uptake of FGF1 in c-Cbl– and Cbl-b–depleted cells compared with cells transfected with scramble siRNA. Furthermore, the results are similar for wild-type FGFR and the multilysine mutants. We speculate that residual Cbl remaining in the cells could be sufficient to mediate its action or that Cbl-independent mechanisms of FGFR1 internalization through clathrin coated pits exist.

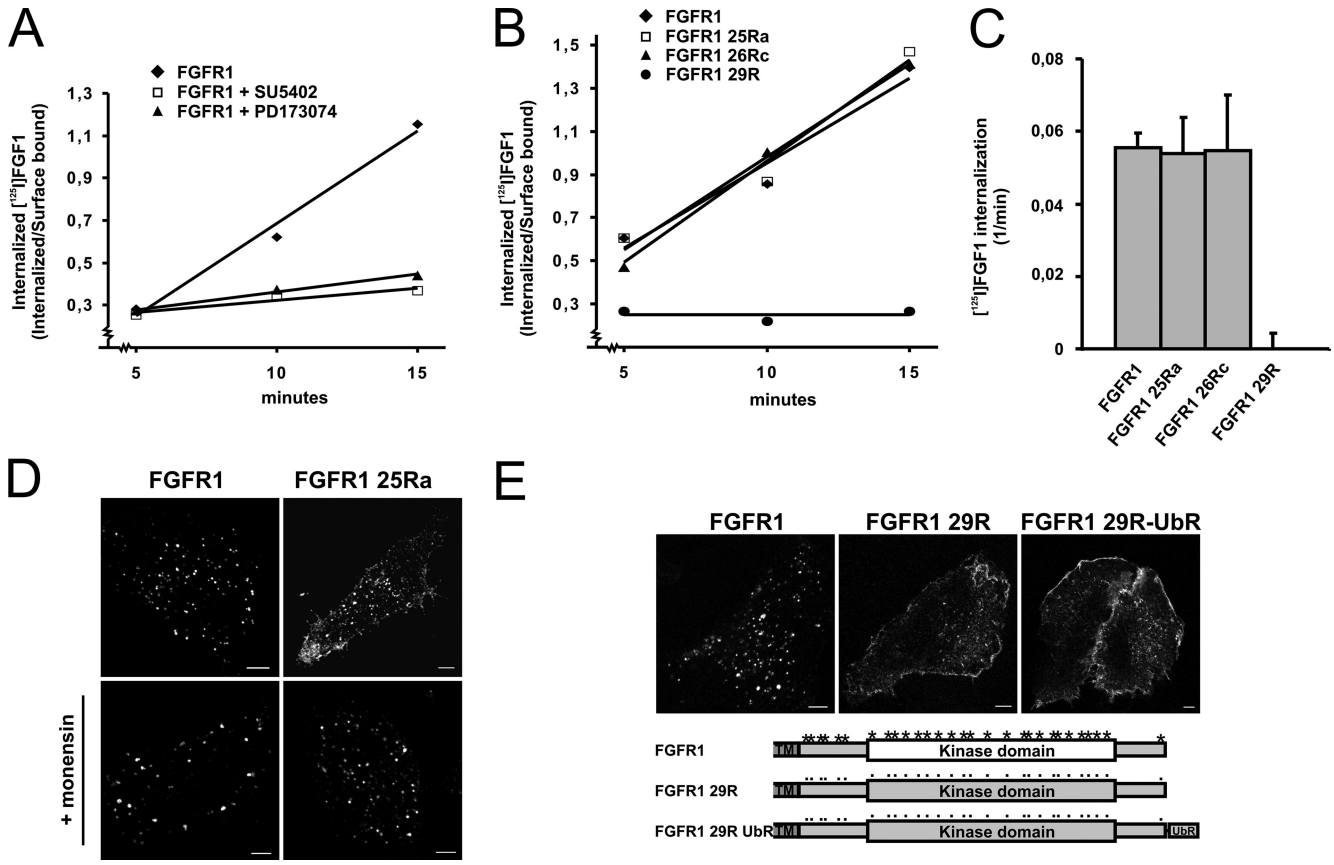

Figure 5. Endocytosis of wild-type and mutant FGFRs. (A) Endocytosis of wild-type FGFR in the presence of FGFR kinase inhibitors. U2OS cells stably expressing FGFR1 were grown on gelatinized plates and either left untreated or treated with 15 μM SU5402 or 50 nM PD173074 20 min before the experiment. The kinase inhibitors were kept throughout the experiment. The cells were then incubated in HEPES medium with 5–10 ng/ml ^{125}I-FGF1, 20 U/ml heparin, and 0.2% gelatin at 37°C for indicated times. Internalized and surface-bound ^{125}I-FGF1 were separated as described in *Materials and Methods*, and the ratio was plotted as a function of time. The graph shows one representative experiment. (B) Endocytosis of wild-type and mutant FGFRs. U2OS cells stably expressing FGFR1, FGFR1 25Ra, or FGFR1 26Rc were treated as described above. The graph shows one representative experiment. (C) The mean of the slope from five independent experiments as described in B for U2OS cells expressing FGFR1, FGFR1 25Ra, FGFR1 26Rc, or FGFR1 29R are shown. Error bars denote the SE. (D) The effect of monensin on FGFR1 25Ra sorting. HeLa cells were transiently transfected with the indicated receptors and kept at 4°C with Cy3-FGF1 and 50 U/ml heparin for 1 h and incubated further for 1 h at 37°C before fixation. Half of the cells were treated with 50 μM monensin added 20 min before incubation at 4°C and kept throughout the experiment. Cy3-FGF1 staining was visualized by confocal microscopy. Bar, 5 μm. (E) The effect of ubiquitin conjugated to FGFR1 29R on FGF1 internalization. HeLa cells transiently transfected with the indicated construct was incubated with Cy3-FGF1 and 50 U/ml heparin for 2 h at 37°C, fixed, and examined by confocal microscopy. Bar, 5 μm.

Intracellular Sorting of FGFR1 Lysine Mutants

The intracellular pathways taken by the different receptor mutants were further characterized in the confocal microscope by using markers for various intracellular compartments. Transiently transfected HeLa cells were allowed to bind Cy3-FGF1 before incubation at 37°C for different time periods. As shown in Figure 8A, top, incubation for 15 min at 37°C resulted in significant overlap of Cy3-FGF1 and EEA1, a protein associated with early/sorting endosomes (Mu *et al.*, 1995). In all the active lysine mutants, the endocytosed material accumulated in sorting endosomes after 15 min. After a 2-h chase in the presence of leupeptin to inhibit degradation in the lysosomes, the major part of the internalized Cy3-FGF1 no longer colocalized with EEA1 (Figure 8A, middle). However, quantification of colocalization between EEA1 and Cy3-FGF1 revealed a small increase in colocalization in cells transfected with FGFR1 25Ra, FGFR1 26Rc, or FGFR4 compared with FGFR1 (Figure 8B). Thus, the receptors are internalized to early endosomes from where they can be delivered to other intracellular compartments or possibly recycled back to the cell surface.

To further follow the endocytic pathway of the different FGF1/FGFR complexes, transiently transfected HeLa cells were incubated with Alexa 488-labeled epidermal growth factor (Alexa 488-EGF) and Alexa 647-labeled transferrin (Alexa 647-Tf) in addition to Cy3-FGF1, heparin, and leupeptin. EGF progress to lysosomes upon internalization, whereas Tf is known to be recycled from early/sorting endosomes via the endocytic recycling compartment back to the cell surface. Cy3-FGF1 localized to late endosomes/lysosomes will look like yellow dots in the confocal images, whereas Cy3-FGF1 localized to the endocytic recycling compartment will look like purple dots. In agreement with a previous report (Haugsten *et al.*, 2005), Cy3-FGF1 endocytosed by FGFR1 showed extensive overlap with Alexa 488-EGF, indicating that the major part of internalized FGF1/FGFR1 complexes accumulates in lysosomes, whereas FGF1 endocytosed by FGFR4 showed a considerable overlap with transferrin, indicating that a large part of the internalized FGF1/FGFR4 complexes accumulate in the endocytic recycling compartment (Figure 8A, bottom). In FGFR1 25Ra and -26Rc, most of the internalized Cy3-FGF1 showed up as

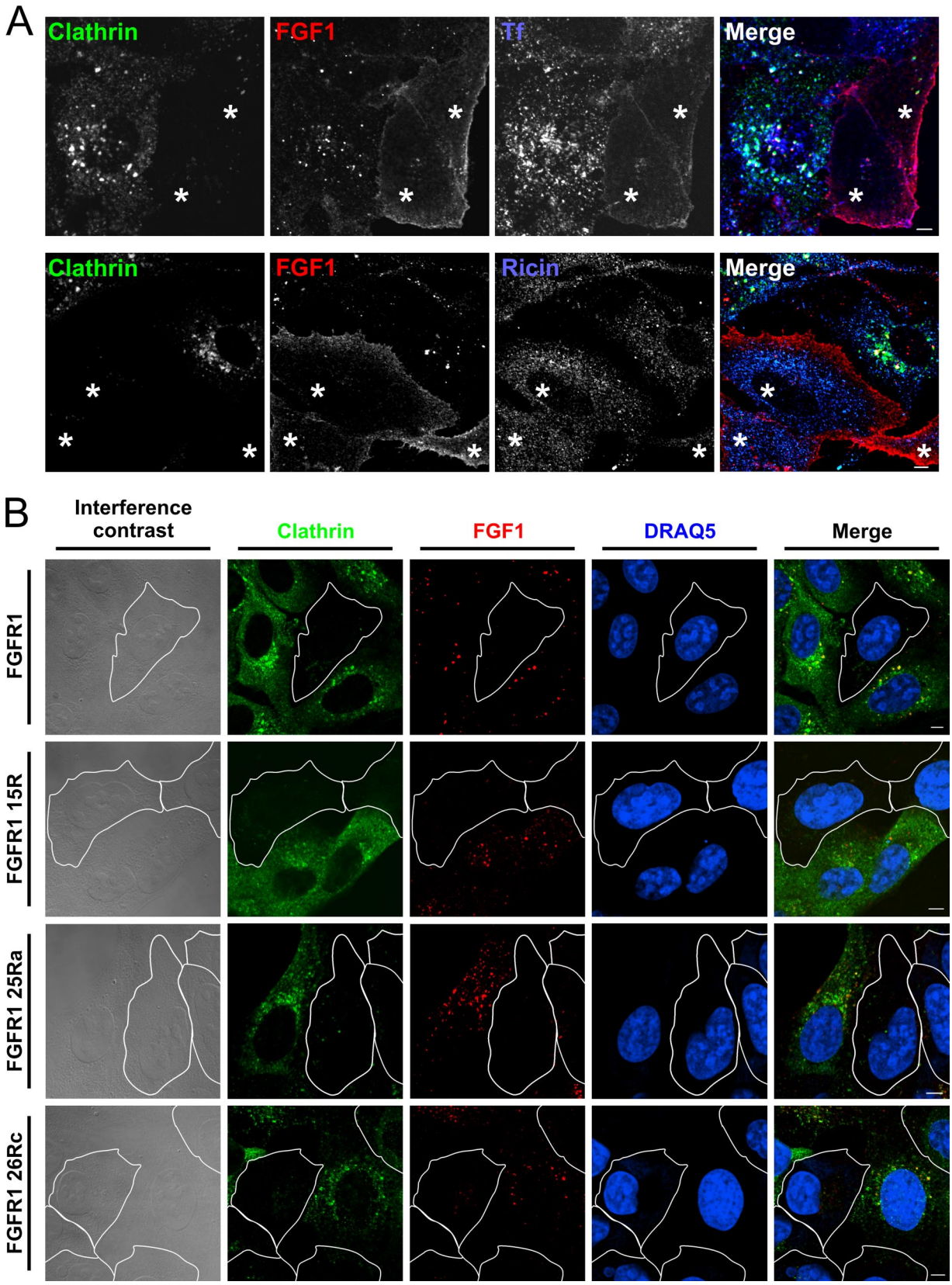

Figure 6. The effect of clathrin heavy chain depletion on FGF1 internalization. (A) U2OS cells, stably expressing FGFR1, were subjected to vector-based siRNA against clathrin heavy chain for 4 d to knock down clathrin. The cells were then kept for 1 h at 4°C with FGF1-myc8, 50 U/ml heparin, and Alexa 647-Tf or ricin and then incubated at 37°C for 30 min before fixation. The cells were stained with anti-clathrin heavy chain antibody, anti-myc antibody, and DRAQ5. Cells treated with ricin were also stained with anti-ricin antibody. The cells were examined with confocal microscopy. FGF1-myc8 was used to facilitate visualization of the cell surface by increasing the fluorescent signal.

purple dots, indicating colocalization with Tf in endocytic recycling compartment, similarly to FGFR4. FGFR1 15R, which resembles FGFR4 in number of lysines, showed characteristics intermediate between FGFR1 and FGFR4. Both purple and yellow staining was detected, indicating that the receptor was localized in lysosomes and in the endocytic recycling compartment. Quantification of the degree of colocalization between Cy3-FGF1 and the two different markers confirmed the altered sorting of the lysine mutants (Figure 8C). We conclude that ubiquitination of lysines in the intracellular part of the receptor is necessary for proper sorting of FGFR1 to lysosomes.

Duration of Signaling from FGFR1 Lysine Mutants

Several mechanisms are involved in attenuation of signaling from activated receptors. One of them is the degradation of the receptors in lysosomes. To test whether the altered sorting of the receptors influenced their signaling activities, receptor phosphorylation was analyzed in the stably transfected U2OS cells.

U2OS cells transfected with FGFR1 or FGFR4 were serum starved, and then they were treated with FGF1 in the presence or absence of brefeldin A for the indicated times before lysis and immunoblotting analysis (Figure 9A). Brefeldin A disrupts the Golgi apparatus in such a way that newly synthesized plasma membrane proteins are not transported through the Golgi and to the cell surface. Brefeldin A was included to study degradation of the receptors already present at the cell surface without interference from newly synthesized receptors. As seen in Figure 9A, the band corresponding to FGFR4 was much more persistent over time than that corresponding to FGFR1 with and without addition of brefeldin A, indicating that FGFR1 was degraded more rapidly than FGFR4. This is in accordance with data published previously (Haugsten *et al.*, 2005).

When only the receptors present at the cell surface at the beginning of the experiment were studied, by addition of brefeldin A, the bands corresponding to FGFR1 and phosphorylated FGFR1 disappeared during the first 2 h of incubation with FGF1 (Figure 9A, top right). Thus, the signaling activity of FGFR1 is turned off at a rate that correlates with its localization in late endosomes/lysosomes. In the absence of brefeldin A, the band corresponding to FGFR1 and phosphorylated FGFR1 became weaker over time, but it increased again after 4 h (Figure 9A, top left). This prolonged signaling is probably due to the appearance of new receptors at the cell surface. In FGFR4, its slow degradation led to sustained receptor signaling irrespective of treatment with brefeldin A (Figure 9A, middle). However, in the absence of brefeldin A, the amount of phosphorylated receptors seemed to increase over time. The slow degradation and sustained activation of FGFR4 is in accordance with the view that the receptor is mostly recycled upon internalization.

To test whether the altered trafficking of the different FGFR1 lysine mutants affected their degradation and signaling, similar experiments as described above were carried out

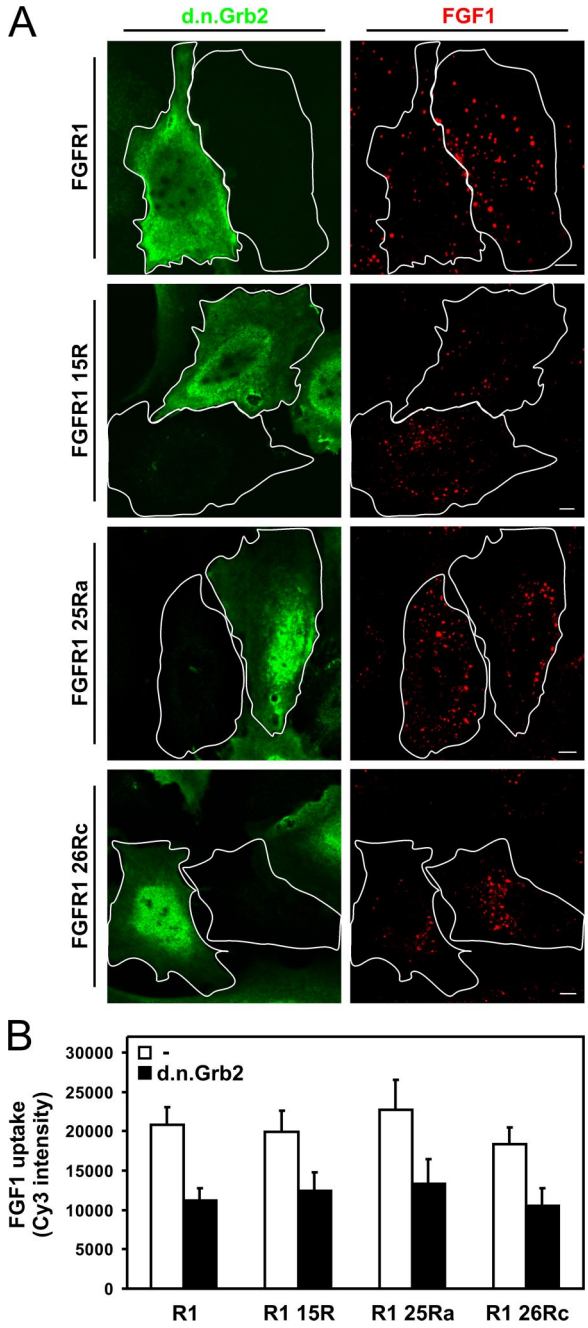

Figure 7. The effect of d.n.Grb2 expression on FGF1 internalization. (A) U2OS cells stably expressing indicated FGFRs were transfected with myc-d.n.Grb2 and incubated with Cy3-FGF1 and 50 U/ml heparin at 37°C for 20 min. The cells were then fixed and stained with anti-myc antibody. The cells were examined with confocal microscopy. d.n.Grb2-expressing cells are outlined. Bar, 5 μm. (B) The uptake of Cy3-FGF1 was measured as Cy3 intensity in untransfected (□) or myc-d.n.Grb2–transfected (■) U2OS cells stably expressing indicated FGFR constructs treated as described in A. The cells were also stained with anti-FGFR1 antibody. Cells expressing similar amounts of FGFRs were selected for quantification based on their intensity of anti-FGFR1 antibody staining, and 15–30 cells were quantified in each case. Confocal scanning was performed with identical settings. The mean intensity in each case is presented in the histogram, and error bars denote the SE.

Figure 6 (cont). The asterisks point to siRNA-transfected cells. Bar, 5 μm. (B) U2OS cells, stably expressing FGFRs as indicated, were subjected to vector-based siRNA against clathrin heavy chain for 4 d to knock down clathrin. The cells were then kept for 1 h at 4°C with Cy3-FGF1 and 50 U/ml heparin and then incubated at 37°C for 30 min before fixation. The cells were stained with anti-clathrin heavy chain antibody and DRAQ5 and examined with confocal microscopy. siRNA-expressing cells are outlined. Bar, 5 μm.

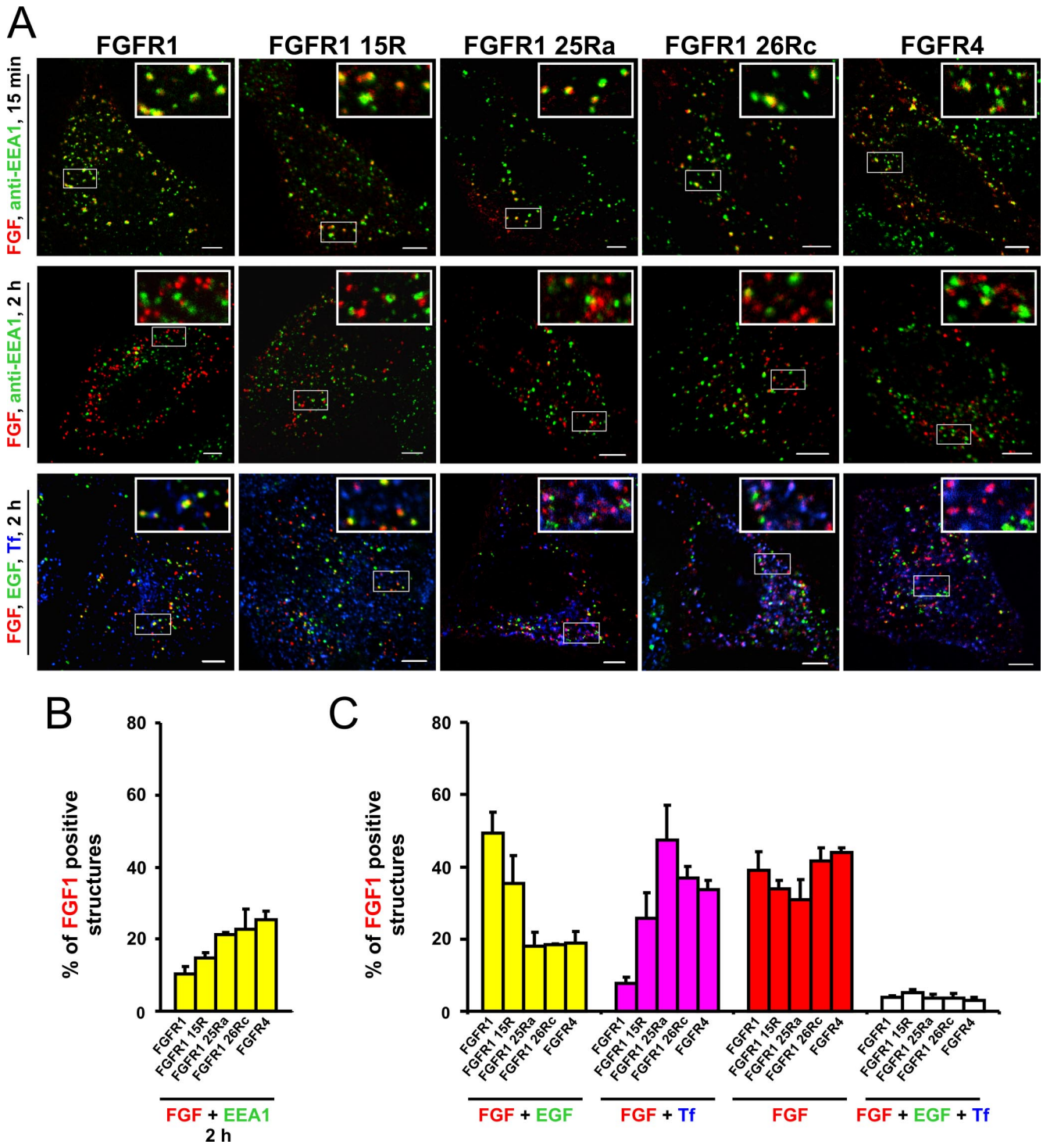

Figure 8. Sorting of wild-type and mutant FGFRs. (A) HeLa cells transiently transfected with the indicated construct were kept at 4°C with Cy3-FGF1 and 50 U/ml heparin for 1 h and then incubated at 37°C for 15 min or 2 h before fixation. The cells were stained with anti-EEA1 antibody (two top panels). Cells presented in the bottom panel were kept at 4°C with Alexa 488-EGF in addition to Cy3-FGF1 and heparin and incubated for 2 h at 37°C. Alexa 647-Tf was added after 90 min at 37°C. The cells were examined by confocal microscopy. Insets show selected areas of the images enlarged (~3×). Bar, 5 μm. (B) The percentage of FGF1-positive structures within transfected cells that colocalized with EEA1 after 2 h at 37°C was quantitated as described in *Materials and Methods*. The bars represent the mean of three independent experiments (5 cells counted for each construct in each experiment), and error bars denote the SE (n = 3). (C) The percentage of FGF1-positive structures within transfected cells that colocalized with EGF, Tf, neither EGF nor Tf, or both EGF and Tf was quantitated as described in *Materials and Methods*. The bars represent the mean of three independent experiments (5 cells counted for each construct in each experiment), and error bars denote the SE (n = 3).

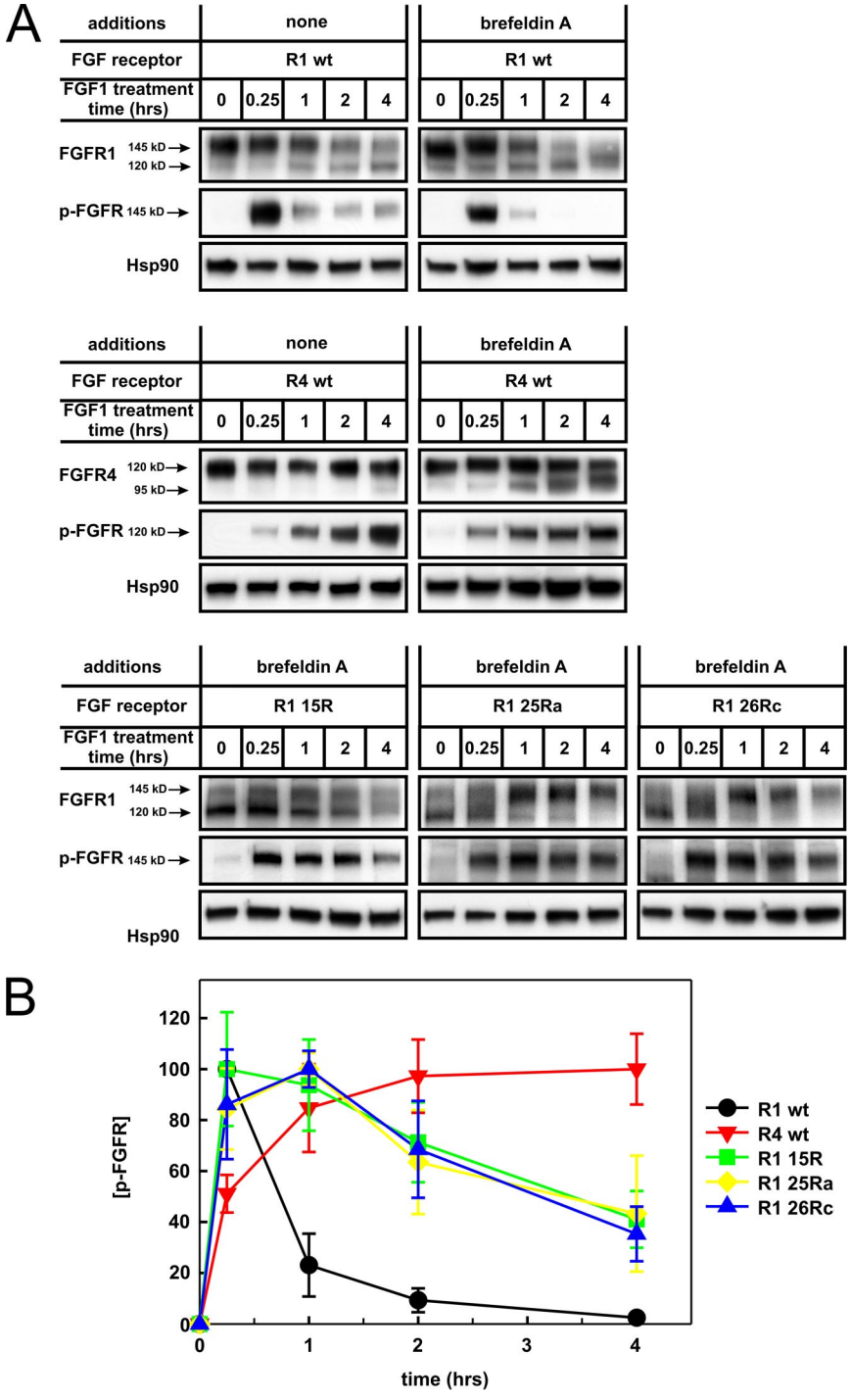

Figure 9. Half-life of phosphorylated FGFR in the presence of brefeldin A. (A) U2OS cells, stably expressing FGFR as indicated, were serum starved for 24 h and then left untreated or treated with 100 ng/ml FGF1 and 10 U/ml heparin, in the presence or absence of 2 μg/ml brefeldin A. Cells were lysed, and the cellular material was analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. A p in front of the name of the antibody indicates that it recognizes the phosphorylated form of the protein. (B) The amounts of phosphorylated FGFR (145 kDa for R1, R1 15R, R1 25Ra, and R126Rc and 120 kDa for R4) normalized to the total Hsp90 were expressed as a percentage with the maximum for each receptor set to 100. The mean of four independent experiments are plotted in the graph. Error bars denote the SD.

in cells stably expressing FGFR1 15R, FGFR1 25Ra, or FGFR1 26Rc (Figure 9A, bottom). Both the bands corresponding to the FGFR and to the phosphorylated FGFR were more persistent for FGFR1 15R, FGFR1 25Ra, and FGFR1 26Rc than for wild-type FGFR1. Similar experiments were also performed in the presence of cycloheximide (which blocks the synthesis of new receptors) or in the presence of both brefeldin A and cycloheximide, and this gave similar results as in the presence of brefeldin A (Supplemental Figures 2 and 3).

The data in Figure 9A were quantified, by measuring the intensities of the bands. The mean of four independent experiments are presented in the graph in Figure 9B. The quantification revealed that the amount of phosphorylated FGFR1 was rapidly down-regulated to ~25% after 1 h and that it had almost disappeared at 2 h after stimulation by FGF1. In contrast, the signal from FGFR4 increased rapidly during the first hour, and it persisted over the next 3 h. The duration of signaling of the different multilysine mutants was intermediate to that of the FGFR4 and FGFR1 signal. Thus, the recycling FGFR1 lysine mutants retained their signaling activity longer than FGFR1 wild type. Together, the data indicate that the duration of signaling from FGFR1

seems to be regulated by ubiquitination and subsequent sorting to the lysosomes for degradation.

DISCUSSION

In this article, we present evidence that although ubiquitination of FGFR1 is required for sorting to lysosomes and subsequent degradation, it is not required for endocytosis. By substituting lysines for arginines to eliminate possible ubiquitination sites in the cytoplasmic part of FGFR1, we generated several FGFR1 constructs that were poorly, or not at all ubiquitinated. These mutants were used to study the role of ubiquitination in internalization and sorting. Wild-type FGFR1 was found to be sorted to lysosomes, whereas lysine mutants escaped into the recycling pathway. The altered sorting of the lysine mutants increased the duration of their signaling activity. Moreover, loss of ubiquitination did not alter the rate of internalization. It therefore seems that ubiquitination of the cytoplasmic part of the receptor acts as a signal for sorting to lysosomes.

Ubiquitination is thought to play a role in endocytosis and sorting of many tyrosine kinase receptors (Marmor and Yarden, 2004; Mukhopadhyay and Riezman, 2007). Despite intensive investigation, it has proven difficult to directly demonstrate a role for ubiquitination of the receptors in these processes. First, ubiquitination sites have not been mapped in most of the ubiquitinated proteins. Second, it is not clear whether an eliminated ubiquitination-site can be replaced by another site that is not normally ubiquitinated. Third, knockdown, overexpression, or mutational studies of ubiquitin ligases do not necessarily reflect the importance of ubiquitination of the receptor but rather the importance of ubiquitination in general. Also, in these types of experiments it is not known whether one ligase can substitute for the function of another. Therefore, to obtain a tool to study the role of ubiquitination in endocytosis and sorting more directly, we mutated possible ubiquitination sites (29 altogether) in the intracellular part of FGFR1.

Our conclusion that ubiquitination is not required for endocytosis is based on the similar rate of endocytosis of radiolabeled FGF1 in cells expressing wild-type FGFR1 and in cells expressing FGFR1 25Ra and FGFR1 26Rc, that lack 25 or 26 of 29 lysine residues in the cytoplasmic part. These mutants are not completely devoid of possible ubiquitination sites because some lysines required for receptor kinase activity are still present in their intracellular domain. According to structure analysis performed by Mohammadi *et al*. (1996), these lysines have a low FSA, and they are therefore not likely to be accessible for ubiquitination in the correctly folded receptor. Furthermore, substitution of the remaining lysines in FGFR1 25Ra or 26Rc for arginines, an amino acid with similar structure and properties as lysine, inactivated the receptor kinase. Considering the importance of these lysines in the structure of the kinase, the conjugation of an 8-kDa ubiquitin moiety to these remaining lysines would probably also destroy the kinase activity of these mutant receptors. Because kinase activity is required for internalization and because the FGFR1 25Ra and FGFR1 26Rc are internalized similarly to wild-type FGFR1, it is unlikely that these receptors are ubiquitinated at the cell surface.

In multilysine mutants, the lower band in Figure 9 representing the 120-kDa FGFR, which probably corresponds to a non- or not fully glycosylated FGFR, is stronger than the upper band, which represents fully glycosylated FGFR. In the wild-type FGFR1, this lower band is barely detectable in unstimulated cells, but it increases in strength as new receptors are synthesized after 4 h of FGF1 treatment (Figure 9, top left). It is possible that the mutant receptors are more slowly folded, and therefore they are retained longer in their unglycosylated form in the endoplasmic reticulum (ER). It is also possible that the mutant receptors have impaired stability; therefore, misfolded receptors accumulate in the ER, because their proteasomal degradation is inhibited as a result of their insufficient ubiquitination due to lower lysine content. Another possibility is that they are processed more slowly through the Golgi apparatus than the wild type. This has been observed for FGFR3 with mutations associated with skeletal disorders (Lievens *et al*., 2004; Bonaventure *et al*., 2007). Surprisingly, maturation of the mutants seemed to continue after addition of brefeldin A. It may be that the mutants mature in a possible brefeldin A-insensitive part of the Golgi or more likely, in the ER because brefeldin A causes accumulation of Golgi enzymes in the ER compartment. Although changing a lysine to an arginine is considered to be a quite conservative mutation, it may not be surprising if multilysine mutants are somewhat more unstable or less active than the wild-type receptor (Figure 3). Even so, they are active enough to stimulate downstream effectors; and importantly, they were endocytosed at the same rate as FGFR1. Furthermore, internalization of Cy3-FGF1 in U2OS cells expressing the wild type or the multilysine mutants is dependent on clathrin and partially dependent on Grb2 (Figures 6 and 7). The reduction in Cy3-FGF1 internalization was similar for wild type and multilysine mutants. Thus, it seems as both wild-type FGFR1 and the multilysine mutants use the same pathway for internalization.

Fusing an ubiquitin moiety in-frame to the cytoplasmic tail of the internalization-defective mutant FGFR1 29R did not induce its internalization (Figure 5E). However, fusion of ubiquitin to internalization-defective mutants of EGFR led to their internalization (Haglund *et al*., 2003; Mosesson *et al*., 2003). This difference could be a result of different experimental conditions or a real difference in the requirement for ubiquitination in endocytosis of the two receptors. However, an ubiquitination-deficient mutant of EGFR internalized normally, indicating that although ubiquitination can drive endocytosis of internalization-defective mutants, it is not required for endocytosis (Huang *et al*., 2006, 2007). This is in agreement with the findings for FGFR1 presented in this article.

The observation that the ubiquitin-deficient receptors are endocytosed at a similar rate as the wild-type receptor made it easier to study the role of ubiquitination in the subsequent sorting of the receptors. The receptors reach the sorting endosomes at the same time, which facilitates a proper spatiotemporal study. The data presented in this article are in accordance with previous data concerning the trafficking of FGFRs. FGFR1 has been found to be sorted to lysosomes, whereas the FGFR4 accumulated in the endocytic recycling compartment (Citores *et al*., 1999; Haugsten *et al*., 2005). Additionally, FGFR1 is degraded faster than FGFR4. The rate of degradation and the duration of signaling from the different multilysine mutants were intermediate to those of the FGFR4 and FGFR1. It might seem a bit surprising that FGFR4, which contains 16 lysines and is significantly ubiquitinated (Haugsten *et al*., 2005), is degraded more slowly than the FGFR1 25Ra and -26Rc mutants, which are hardly ubiquitinated. An explanation might be that FGFR4 contains additional signals for efficient recycling that are absent in FGFR1.

Little is known about receptor ubiquitination and its role in internalization and sorting of the FGFRs. It has been reported that FRS2α can recruit the ubiquitin ligase Cbl to

the FGFR through Grb2 in an FGF1-dependent manner and that this recruitment induced ubiquitination of FRS2α and FGFR (Wong *et al*., 2002). Furthermore, cells deficient in FRS2α internalized FGF1 more slowly than cells expressing wild-type FRS2α, and the half-life of FGFR and the duration of its signaling were extended. This is in accordance with the data presented in this article. We have shown that overexpression of a dominant-negative construct of Grb2 partially inhibited FGF1 uptake. The reduced endocytosis in the FRS2α-deficient cells or d.n.Grb2-expressing cells could result from low levels of ubiquitination of the machinery responsible for endocytosis. It is also possible that the reduced FGFR signaling capacity in FRS2α-deficient cells or d.n.Grb2-expressing cells slows down its endocytosis. It is known (Sorokin *et al*., 1994; this study) that kinase activity of the receptor is necessary for internalization. Conversely, we did not detect any difference in the uptake of FGF1 when cells were simultaneously depleted for c-Cbl and Cbl-b neither in U2OS cells expressing the wild-type FGFR1 nor in cells expressing the multilysine mutant (Supplemental Figure 1). It is possible that small amounts of Cbl remaining in the cells are sufficient to support FGFR internalization. Alternatively, even though Cbl is recruited to the receptors via FRS2α and Grb2 and overexpression of Cbl increases ubiquitination of the receptor, it is possible that Cbl is not a crucial member of the FGFR internalization machinery and that Cbl-independent mechanisms of FGFR internalization through clathrin coated pits exist.

Moreover, it has been shown that mutations in FGFR3 responsible for achondroplasia and related dwarfing conditions in humans disrupt c-Cbl–mediated ubiquitination of the receptor resulting in receptor recycling (Cho *et al*., 2004). The pathogenesis involves constitutive activation of FGFR3, and Cho *et al*. (2004) suggest that the defective lysosomal targeting of the activated receptors contributes to the molecular pathogenesis of achondroplasia and represents a potential target for therapeutic approaches.

Constitutively active FGFRs have also been associated with poor prognosis in several forms of cancer (Grose and Dickson, 2005). We have provided evidence that deficient FGFR1 down-regulation, resulting in recycling of the receptor, prolongs its signaling. Furthermore, the number of lysines influences the efficiency of lysosomal sorting of the receptor. FGFR1 15R is less efficiently sorted to the lysosomes than wild-type FGFR1 but more efficiently sorted than FGFR1 25Ra and FGFR1 26Rc (Figure 8). Thus, the number of lysines and the level of ubiquitination can fine-tune receptor down-regulation. Exact down-regulation of activated receptors is important to prevent excessive signaling. It is therefore possible that a defective ubiquitination system for down-regulation could result in aberrant signaling and growth in FGFR-expressing cells, leading to, in some cases, enhanced cancer progression.

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