Carcinogenesis vol.28 no.7 pp.1408–1417, 2007
doi:10.1093/carcin/bgm058
Advance Access publication March 19, 2007

EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes

Michael V.Grandal[†], Roza Zandi[1,†], Mikkel W.Pedersen[1], Berthe M.Willumsen[2], Bo van Deurs and Hans S.Poulsen[1,*]

Department of Cellular and Molecular Medicine, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark, [1]Department of Radiation Biology, Section 6321, Copenhagen University Hospital, DK-2100 Copenhagen, Denmark and [2]Department of Molecular Biology, University of Copenhagen, DK-2200 Copenhagen, Denmark

[*]To whom correspondence should be addressed. Tel: +45 35 45 63 03; Fax: +45 35 45 63 01;
Email: skovgaard@rh.dk

EGFRvIII is a mutant variant of the epidermal growth factor receptor (EGFR) found exclusively in various cancer types. EGFRvIII lacks a large part of the extracellular domain and is unable to bind ligands; however, the receptor is constitutively phosphorylated and able to activate downstream signaling pathways. Failure to attenuate signaling by receptor down-regulation could be one of the major mechanisms by which EGFRvIII becomes oncogenic. Using a cell system expressing either EGFR or EGFRvIII with no expression of other EGFR family members and with endogenous levels of key degradation proteins, we have investigated the down-regulation of EGFRvIII and compared it to that of EGFR. We show that, in contrast to EGFR, EGFRvIII is inefficiently degraded. EGFRvIII is internalized, but the internalization rate of the mutated receptor is significantly less than that of unstimulated EGFR. Moreover, internalized EGFRvIII is recycled rather than delivered to lysosomes. EGFRvIII binds the ubiquitin ligase c-Cbl via Grb2, whereas binding via phosphorylated tyrosine residue 1045 seems to be limited. Despite c-Cbl binding, the receptor fails to become effectively ubiquitinylated. Thus, our results suggest that the long lifetime of EGFRvIII is caused by inefficient internalization and impaired sorting to lysosomes due to lack of effective ubiquitinylation.

Introduction

Over-expression or mutations of the epidermal growth factor receptor (EGFR) are common in many human cancer types. The best characterized and most common mutation in the *EGFR* gene results in the type III EGFR deletion mutant (EGFRvIII). EGFRvIII is found exclusively in human tumors, including those of the brain, lung, prostate and ovary (1–4). EGFRvIII transforms fibroblasts and enhances the tumorigenicity of cancer cells both *in vitro* and *in vivo*, suggesting that EGFRvIII plays an active role in tumorigenesis (5–8). The cancer specificity makes EGFRvIII an obvious target for anticancer therapy, and therefore an understanding of the function and endosomal trafficking of EGFRvIII is important for future efforts to target the receptor.

Structurally, the EGFRvIII is characterized by a deletion of the extracellular amino acids 6–273 that constitute the dimerization arm and an essential part of the ligand-binding pocket in EGFR rendering EGFRvIII incapable of binding ligands (9–11). However, a subpopulation of EGFRvIII is constitutively active and seems to be present primarily at the plasma membrane (12–15). Even though EGFRvIII lacks its dimerization arm, the receptor is able to dimerize both with EGFR and ErbB2 (16–20).

Termination of epidermal growth factor (EGF)-induced EGFR signaling is a tightly regulated process involving receptor internalization followed by degradation in lysosomes. A key component is the ubiquitin ligase, c-Cbl, which binds to activated EGFR through Grb2 or phosphorylated tyrosine residue 1045 (pTyr1045) and mediates receptor ubiquitinylation followed by internalization and lysosomal degradation (21–28). The fast internalization and degradation induced by ligand activation of EGFR occur on top of a basal rate of internalization followed by either recycling or degradation at a slow rate (29).

It has long been a dogma that EGFRvIII is not down-regulated. However, Davies *et al.* (30) recently showed that upon over-expression of ubiquitin and one of several Cbl proteins, EGFRvIII can be ubiquitinylated, internalized and degraded (30), suggesting that EGFRvIII can enter the degradation pathway. In contrast, using cell lines expressing ErbB2 and other EGFR family members Han *et al.* (31) recently reported that EGFRvIII is not down-regulated due to lack of ubiquitinylation and degradation as a consequence of hypophosphorylation at Tyr1045. However, expression of other EGFR family members, such as ErbB2, could influence the down-regulation of EGFRvIII, as ErbB2 has previously been shown to inhibit EGFR down-regulation (32). Thus, it remains unclear to what extent EGFRvIII becomes internalized and degraded when ubiquitin and Cbl proteins are expressed at endogenous levels and when other EGFR family members are not expressed and hence unable to affect EGFRvIII down-regulation.

We have examined EGFRvIII internalization and degradation in cells where EGFRvIII has been shown to have a tumorigenic effect (5,6). Furthermore, the cells we used expressed endogenous levels of ubiquitin and Cbl proteins and only the EGFR family member in question. We report that EGFRvIII, unlike EGFR, is inefficiently degraded both in the presence and absence of EGF. Even with endogenous levels of ubiquitin and Cbl proteins, EGFRvIII retains the ability to become internalized, but at a significantly lower rate than that of unstimulated EGFR. Furthermore, the small fraction of internalized EGFRvIII is recycled to the plasma membrane rather than delivered to lysosomes. The low rate of EGFRvIII down-regulation seems to be linked to restricted c-Cbl binding and inefficient ubiquitinylation.

Materials and methods

Cell culture

NR6, NR6M and NR6wtEGFR cell lines were provided by Dr Darell Bigner, Duke University, NC, and described previously (8). The human glioma cell line U87MGΔEGFR was provided by Dr Webster Cavenee, San Diego, CA. U87MGΔEGFR expresses EGFRvIII as well as low levels of endogenous EGFR (33). All cells were maintained as monolayer cultures in a humidified chamber with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 2 mM glutamine, 10 U/ml penicillin and 10 μg/ml streptomycin or alternatively DMEM containing glutamax, 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin (all reagents from Invitrogen, Carlsbad, California).

Transfection

Cells were transfected with FuGene 6 as described by the manufacturer (Roche, Basel, Switzerland). Two days before the immunoprecipitation experiment, cells were transfected with Ha-c-Cbl, Ha-N-Cbl and/or Myc-dominant negative (d.n.) Grb2. Ha-c-Cbl were co-transfected in one-tenth concentration of the Ha-N-Cbl and/or Myc-dn.n.Grb2 concentration. Ha-c-Cbl, Ha-N-Cbl and Myc-d.n.Grb2 were gifts from Inger Helene Madshus, University of Oslo, Norway. pcDNA3.1 was used for mock transfection (control) and to ensure similar plasmid concentration and total plasmid load.

Pulse–chase experiments

Serum-starved cells were pre-incubated for 30 min with methionine/cysteine-free DMEM (Invitrogen) supplemented with 1 mM sodium pyruvate (Invitrogen),

Abbreviations: BSA, bovine serum albumin; CHX , cycloheximide; DMEM, Dulbecco's modified Eagle's medium; d.n., dominant negative; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; RT, room temperature; TfR, transferrin receptor.

[†]These authors contributed equally to this work.

2 mM L-glutamine (Sigma-Aldrich, Broendby, Denmark) and penicillin–streptomycin (Invitrogen). Cells were labeled with 0.25 mCi/ml L-[^{35}S]-methionine/cysteine (Amersham Biosciences, Buckinghamshire, United Kingdom and ICN, Irvine, California) for 1 h (pulse period). After the pulse, cells were rinsed once and chased in DMEM with 0.5% fetal calf serum and penicillin–streptomycin in the presence or absence of 10 nM EGF (Calbiochem, San Diego, California) for 0–15 h (chase period). As the post-translational processing of receptor polypeptide was not complete until 3 h of chase, this time point was chosen as starting point for the analysis of the kinetics of receptor degradation.

At harvest, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped off with a rubber policeman in RIPA lysis buffer (50 mM Tris–HCL pH 7.4, 1% NP40, 0.25% Na-deoxylat, 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid) supplemented with Protease Inhibitor Cocktail Set II and Phosphatase Inhibitor Cocktail Set III (Calbiochem). Cell debris was removed, the lysates were pre-cleared and the receptors were immunoprecipitated with mouse anti-EGFR antibody (Ab-1, Calbiochem), which binds to the extracellular domain of both EGFR and EGFRvIII. The immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and EGFR and EGFRvIII bands visualized both by PhosphorImaging and autoradiography. The receptor bands were quantified using the ImageQuant software program (Molecular Dynamics, Inc., Sunnyvale,California, USA) and the amount of radioactivity in each receptor band was determined after background subtraction and normalized to the 3 h time point (starting point). The kinetics of receptor degradation was analyzed by plotting % receptor as a function of chase time on a semi-logarithmical scale.

Immunoprecipitation

Cell lysates were pre-cleared and equal amounts of protein (250–1500 μg) were immunoprecipitated with 5 μg mouse anti-EGFR antibody (Ab-1, Calbiochem), 3 μl sheep anti-EGFR antibody (20-ES04, Fitzgerald Industries International, Concord, Massachusetts, USA) or 5 μl rabbit anti-Grb2 antibody (Cell Signaling Technology, Inc., Danvers, Massachusetts, USA). The immunocomplexes were precipitated with protein G or A agarose beads (Pierce, Rockford, Illinois and Upstate, Charlotteville, Virginia), collected by centrifugation and washed with ice-cold PBS. Antibody control was prepared by incubating 1 ml of pre-cleared RIPA lysis buffer with 5 μg mouse anti-EGFR antibody followed by incubation with protein G agarose beads. Immunoprecipitated/co-immunoprecipitated proteins were analyzed by western blotting.

Phosphatase treatment

Immunoprecipitated EGFR/EGFRvIII were washed twice in New England Biolabs buffer 2 and left untreated or dephosphorylated by incubation in 2 U/μl shrimp alkaline phosphatase and 10 U/μl calf intestine phosphatase (all from New England Biolabs, Ipswich, Massachusetts) for 60 min at 37°C. The phosphorylation level was evaluated by western blotting.

Cycloheximide experiments

Serum-starved cells were pre-incubated with 3 μg/ml cycloheximide (CHX) (Sigma-Aldrich, St. Louis, Montana) for 5 min and then incubated with or without 10 nM EGF for 0–21 h. Following the incubations, cells were washed with ice-cold PBS and scraped off with a rubber policeman in RIPA lysis buffer supplemented with inhibitors as described above. Cell debris was removed and equal amounts of protein were subjected to western blotting. The receptor bands were quantified and normalized to the zero time point. The kinetics of receptor degradation was analyzed by plotting % receptor as a function of time on a semi-logarithmical scale.

Biotin internalization assay

Cells were plated in 6 cm petri dishes (Nunc, Rochester, New York), and the day before the experiment the medium was changed to growth medium without serum. After a wash with ice-cold PBS, 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) was added to the cells at 4°C. After 20 min, the cells were incubated with fresh 0.5 mg/ml sulfo-NHS-SS-biotin and incubated at 4°C for 20 min. Following a wash with ice-cold DMEM–*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) buffer with 1% bovine serum albumin (BSA) and 2 mM glutamine for 10 min at 4°C, cells were incubated with DMEM–HEPES containing 1% BSA and 2 mM glutamine with 10 μM monensin (Sigma–Aldrich, St. Louis, Montana), 500 nM bafilomycin A1 (Sigma–Aldrich) or nothing for 2, 5, 15, 30 or 60 min at 37°C. Control cells were incubated with DMEM–HEPES containing 1% BSA and 2 mM glutamine for 30 or 60 min at 4°C. The treatment was stopped by transferring the dishes to ice and washing the cells. The biotin on the plasma membrane surface was cleaved by incubating the cells in reducing solution [50 mM 2-sodium-2-mercaptoethanesulfonate (Sigma), 100 mM NaCl, 50 mM Tris–HCl, pH 8.7 and 2.5 mM $CaCl_2$] for 20 min at 4°C, which was repeated twice. Cells used to determine total surface biotinylation were incubated in a solution identical to the reducing solution except for the lack of the reducing agent, 2-sodium-2-mercaptoethanesulfonate. This was followed by washing with ice-cold PBS. Then, the cells were scraped off in lysis buffer [1% Triton X-100, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 10 mM NaF, 1 mM vanadate and phosphatase inhibitor cocktail 1:100 (Sigma)], lysed for 20 min at 4°C, sonicated and centrifuged for 10 min at 16 000*g* at 4°C. Protein levels were determined and the samples standardized. Thirty microliters of washed streptavidin coated beads (Sigma) were added to the samples overnight at 4°C. The cells were centrifuged for 30 s at 6000*g* at 4°C and the pellet was washed in lysis buffer. This was repeated four times. The pellet was subjected to western blotting. Quantification of receptor internalization in relation to biotinylated surface receptor was calculated. Internalization rates, measured as the slopes of regression lines, were tested for significant difference. Furthermore, the difference between untreated and monensin-treated cells (n = 3–6) or bafilomycin A1-treated cells (n = 2–6) was tested using two-sided t-test for comparing means of small samples. Quantification and statistical testing were done in Excel.

Fab fragment production and fluorophore conjugation

Fab fragments were produced from anti-EGFR (clone 528, Calbiochem) using Immunopure Fab kit as described by the manufacturer (Pierce). Fab fragments were labeled using Alexa Fluor 488 Monoclonal Antibody Labeling kit as described by the manufacturer (Molecular Probes, Invitrogen, Carlsbad, California).

Antibody activation

Cells were plated in six-well plates (Nunc), and the day before the experiment the medium was changed to growth medium without serum. The cells were incubated in DMEM–HEPES containing 1% BSA and 2 mM glutamine with or without anti-EGFR (clone 528, Calbiochem), fluorophore-conjugated fab fragment or 10 ng/ml EGF for 15 min at 37°C. After washing, cells were lysed, protein levels determined and samples were equaled to same protein levels with lysis buffer. Samples were subjected to western blotting.

Immunofluorescence microscopy

Cells were plated on eight-well chamber slices (Lab-tek, Nunc, Rochester, New York) and the day before the experiment the medium was changed to growth medium without serum. The cells were washed in ice-cold PBS before incubation in DMEM–HEPES containing 1% BSA and 2 mM glutamine with fab fragments conjugated to Alexa Fluor 488 (or EGFR conjugated to Alexa Fluor 488) and transferrin conjugated to Alexa Fluor 568 for 30 min at 4°C. In addition, 10 μM monensin, 500 nM bafilomycin A1 or nothing was added to the medium. Afterward, cells were transferred to a 37°C bath for 60 min. The cells were washed in ice-cold PBS and stripped for 5 min with acetic acid (0.2 M acetic acid, 0.5 mM NaCl, pH 2.4). Immediately after, cells were fixed in 2% paraformaldehyde in PBS for 10 min on ice followed by 10 min at room temperature (RT). Non-specific binding was blocked in blocking buffer [5% goat serum (Dako, Glostrup, Denmark) in PBS] for 20 min at RT. Cells were incubated with anti-EGFR antibody (clone 528, Calbiochem) in blocking buffer for 1 h at RT, washed with PBS, incubated with Alexa Fluor 633-labeled goat anti-mouse IgG2a (Molecular Probes) in blocking buffer for 30 min at RT, washed with PBS and mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, Alabama). The slides were examined with a Zeiss LSM 510 Meta confocal microscope, and the images were acquired as 1 μm thick slides with the LSM software from Carl Zeiss (Jena, Germany). Test of fab fragments was performed similarly, except for incubation for 10 min at 37°C: no acid wash, addition of 0.2% saponin to the blocking buffer and 30 min incubation with To-Pro-3 (Molecular Probes) instead of antibodies. Images were acquired as 2 μm thick slides.

Protein determination

Protein concentrations were determined by the Bio-Rad DC protein assay (Bio-Rad) or BSA protein assay kit (Pierce) as described by the manufacturers and with BSA as standard.

Western blotting

Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Invitrogen) or polyvinyldifluoriden membranes (Amersham Biosciences AB, Uppsala, Sweden). After blocking in 5% non-fat milk, membranes were incubated with primary antibody overnight at 4°C followed by washing and incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at RT. The HRP signal was detected using enhanced chemiluminescence plus western blotting detection system (Amersham Biosciences) in an Autochemi system (UVP, Upland, California) or autoradiography. The antibodies used were as

follows: anti-Myc, anti-HA (both monoclonal mouse antibodies; gifts from Klaus Hansen, Biotech Research and Innovation Centre (BRIC), University of Copenhagen, Denmark), anti-EGFR (20-ES04, Fitzgerald Industries International), anti-EGFR pTyr (PY-20, BD Transduction Laboratories, BD Biosciences, Broendby, Denmark), anti-EGFR pTyr1045 (Cell Signaling), anti-EGFR pTyr1173 (Biosource, Invitrogen, Carlsbad, California), anti-EGFR pTyr1173 (clone 9 H2, Upstate), anti-c-Cbl (sc-15, Santa Cruz Biotechnology, Inc", Santa Cruz, California), anti-Eps15 (H-896, Santa Cruz), anti-AP-2 (M-300, Santa Cruz), anti-ubiquitin (P4D1, Santa Cruz), anti-tubulin (Ab-4, Neomarkers, Lab Vision Corporation, Runcorn, Cheshire, United Kingdom), HRP-conjugated swine anti-rabbit (Dako), HRP-conjugated goat anti-mouse (Dako), HRP-conjugated rabbit anti-mouse (Dako) and HRP-conjugated rabbit anti-sheep (Dako).

Results

EGFRvIII is very slowly degraded

To investigate the down-regulation of EGFRvIII, we measured the rate of EGFRvIII degradation and compared it to that of EGFR. Initially, we used the U87MGΔEGFR cell line, which expresses low levels of endogenous EGFR in addition to relatively high levels of EGFRvIII. Total cellular levels of EGFR and EGFRvIII were evaluated by western blotting in the presence of the protein synthesis inhibitor CHX for up to 21 h. Quantification of the receptor bands showed that EGFRvIII is neither degraded in the presence nor in the absence of EGF during the time period studied (Figure 1A). In

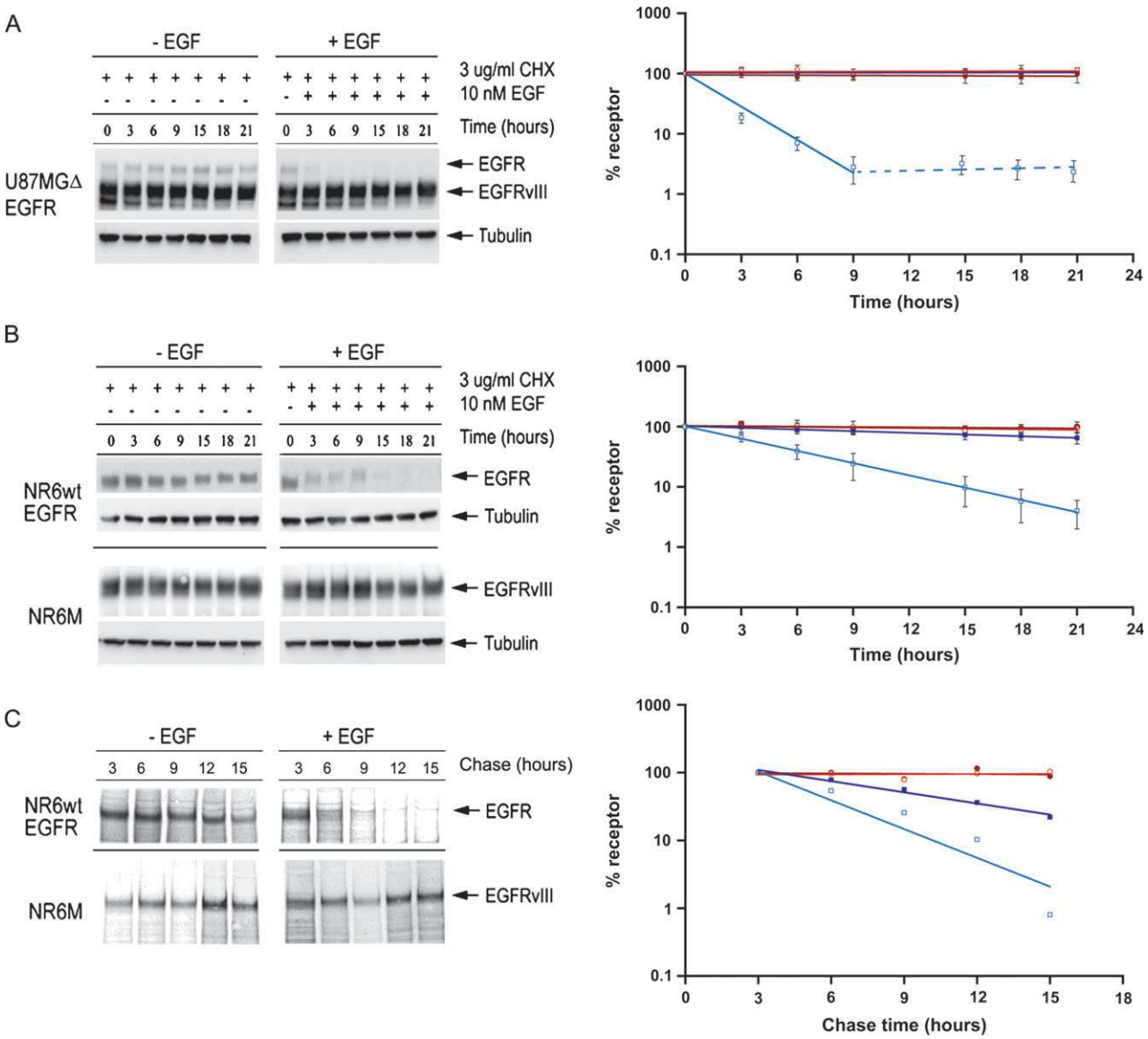

Fig. 1. EGFRvIII is very slowly degraded. (**A** and **B**) U87MGΔEGFR (A) and NR6M and NR6wtEGFR (B) cell lines were incubated with 3 μg/ml CHX in the presence (+) or absence (−) of 10 nM EGF for up to 21 h. Cells were lysed and equal amounts of protein subjected to western blotting. The EGFR and EGFRvIII were visualized by immunoblotting using an EGFR/EGFRvIII-specific antibody. The blots were stripped and reprobed with a tubulin-specific antibody for control of protein loading. The intensity of the EGFR/EGFRvIII bands was determined by densitometry and normalized to the zero time point. The amount of receptor (given as % receptor) was plotted as a function of time as following: EGFR (blue squares) and EGFRvIII (red circles) in the U87ΔEGFR, NR6wtEGFR and NR6M cell lines in the presence (light colored, open symbols) or absence (dark colored, closed symbols) of EGF. As EGFR is degraded immediately after EGF stimulation reaching a basal level after 9 h of stimulation in (A), a dot and dash line is drawn between 9 and 21 h data points to indicate EGFR basal level. Bars show standard deviation ($n = 3$). (C) Immunoprecipitated EGFR and EGFRvIII from [^{35}S]-methionine/cysteine-labeled NR6wtEGFR and NR6M cells chased various times in the presence (+) or absence (−) of 10 nM EGF. The amount of radioactivity in each receptor band in (C) was determined after background subtraction and normalized to the first time point. The amount of receptor (given as % receptor) was then plotted as a function of chase time as following: NR6wtEGFR (blue squares) and NR6M (red circles) cells chased in the presence (light colored, open symbols) or absence (dark colored, closed symbols) of EGF. One representative experiment of two is shown.

contrast, the level of EGFR decreased rapidly after EGF stimulation reaching a basal level after 9 h of stimulation (Figure 1A). This indicates that EGFRvIII might have an impaired degradation. However, the use of cell lines with other ErbB receptors than the one in question might influence the down-regulation of the receptor.

To ensure that this is not the case, we chose to study the down-regulation of EGFRvIII and EGFR in the NR6M and NR6wtEGFR cell lines, which express comparable levels of either EGFRvIII or EGFR, respectively. Investigating the degradation of EGFRvIII and EGFR in these cells in the presence of CHX, we again found that EGFRvIII was not degraded with or without EGF (Figure 1B). In contrast, the degradation of EGFR was accelerated upon EGF stimulation (Figure 1B). To further verify these results, NR6M and NR6wtEGFR were metabolically labeled and then chased for various periods in the presence or absence of EGF (Figure 1C). Quantification of the labeled receptors after background subtraction showed that EGFRvIII was not degraded within the time frame studied (Figure 1C), which is in agreement with the CHX experiments. Once again, the basal degradation rate on unstimulated EGFR was increased upon EGF stimulation. Taken together, these results suggest that EGFRvIII is very slowly degraded compared with EGFR.

EGFRvIII is internalized more slowly than EGFR

The lack of EGFRvIII degradation may be a result of either plasma membrane restrainment and/or internalization followed by efficient recycling. As receptor internalization is mediated by adaptor proteins such as AP-2 and Eps15, which bind to the activated EGFR and initiate internalization through clathrin-coated pits (34–37), we initially tested the interaction of EGFRvIII with these proteins. Both AP-2 and Eps15 were co-immunoprecipitated with EGFRvIII independently of EGF stimulation, whereas EGFR showed an EGF stimulation-dependent association with the adaptor proteins (Figure 2A). As phosphorylation of Eps15 by EGFR plays an important role for EGF-stimulated EGFR internalization (35), we also tested whether Eps15 was phosphorylated in NR6wtEGFR and NR6M cells. In contrast to EGF-stimulated EGFR, but similar to unstimulated EGFR, EGFRvIII did not induce phosphorylation of Eps15 (Figure 2B). Thus, EGFRvIII seems to retain the ability to associate with proteins involved in clathrin-dependent receptor internalization, but it does not efficiently phosphorylate Eps15. This probably affects the internalization rate of EGFRvIII.

To directly investigate internalization of EGFRvIII in relation to unstimulated EGFR, we used a biotin internalization assay. Plasma membrane proteins were biotinylated at 4°C, and the cells were then incubated at 37°C for various periods to allow internalization. Afterward, biotin bound to non-internalized surface proteins were removed so only internalized receptors were detected. Internalized receptors were quantified by calculating the percentage of internalized receptor in relation to total surface receptor. Using this assay, we showed that both unstimulated EGFR and EGFRvIII were internalized in a time-dependent manner (Figure 2C and D and supplementary figure 1, available at *Carcinogenesis* Online). Quantification of internalization up to 60 min indicated that a lower fraction of surface EGFRvIII was internalized than seen for unstimulated EGFR (Figure 2E), but this was not significant. However, if internalized receptor is recycled or degraded, it will not be detected. Therefore, recycling was blocked with the recycling inhibitor monensin (38,39) (Figure 2D and supplementary figure 1, available at *Carcinogenesis* Online), and quantification now showed that the internalization rate of unstimulated EGFR, as measured by the slope of the line, was significantly higher than that of EGFRvIII ($P < 0.01$) (Figure 2F). Furthermore, there was a significant difference in the intracellular fraction of biotinylated EGFR between untreated and monensin-treated NR6wtEGFR cells at 30 and 60 min ($P < 0.05$) (Figure 2E and F). The biotinylated, intracellular

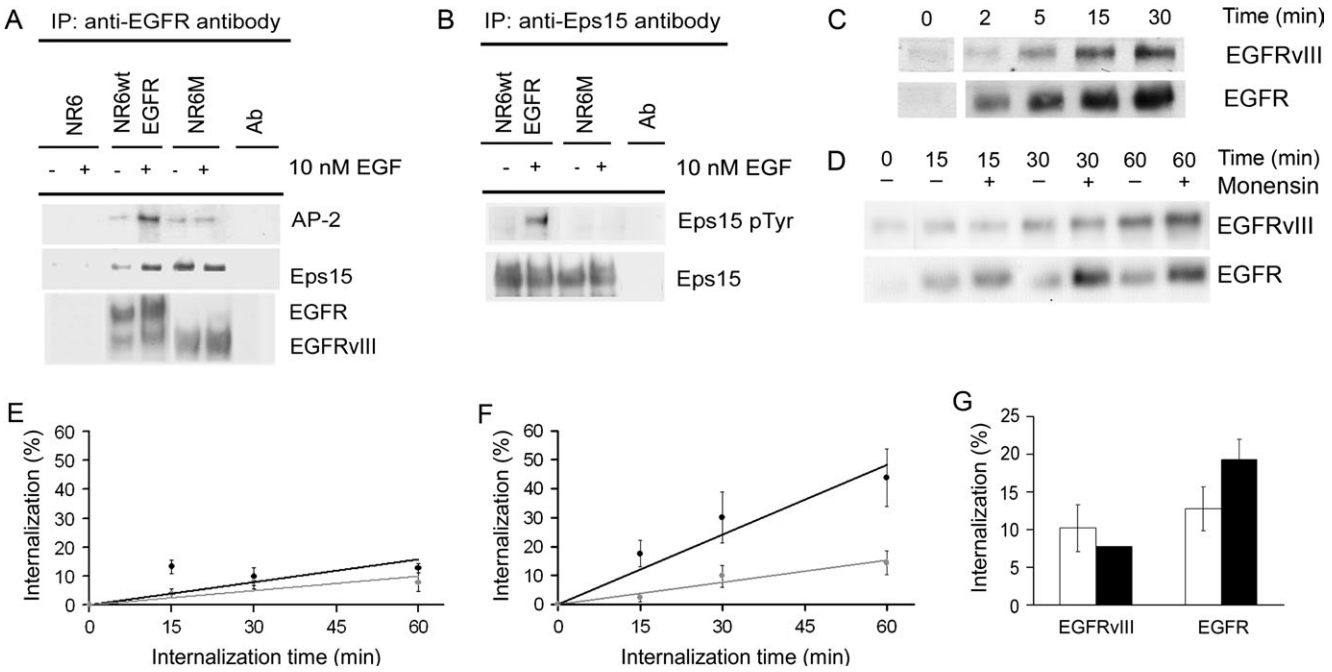

Fig. 2. EGFRvIII is internalized more slowly than EGFR. (**A** and **B**) Cell lines were left untreated (−) or treated (+) with 10 nM EGF for 15 min. Cells were lysed and equal amounts of protein immunoprecipitated with an EGFR/EGFRvIII- or Eps15-specific antibody and subjected to western blotting with specific antibodies against AP-2, Eps15, EGFR/EGFRvIII and total tyrosine phosphorylation. The antibody control (Ab) was included to detect unspecific binding. (**C**–**G**) Cells were incubated with a cleavable biotinylation reagent at 4°C followed by incubation at 4 or 37°C with or without 10 μM monensin (D and F) or 500 nM bafilomycin (G) for the indicated periods of time. Surface biotinylation reagent was then cleaved of the plasma membrane by incubation with a reducing solution, leaving only internalized receptors biotinylated. Following cell lysis, biotinylated proteins were precipitated with streptavidin, and the precipitate was analyzed by western blotting. (C) Western blots indicating internalization of both unstimulated EGFR and EGFRvIII. (D) Western blots showing the effect of monensin on unstimulated EGFR and EGFRvIII. (E and F) Quantification of internalization of unstimulated EGFR (black) and EGFRvIII (gray) without (E) or with 10 μM monensin (F). Bars show standard error of mean ($n = 3$–6). (G) Quantification of internalization of unstimulated EGFR and EGFRvIII with (black columns) or without (white columns) 500 nM bafilomycin after 60 min. Bars show standard error of mean ($n = 2$–6).

fraction of EGFRvIII was slightly increased by monensin treatment, but not enough to be significant in this assay (Figure 2E and F). Thus, unstimulated EGFR was internalized, but efficiently recycled, giving the impression of a low level of receptor internalization. In contrast, EGFRvIII was internalized very slowly. As expected from the degradation experiments, bafilomycin, which inhibits the vacuolar H^+-ATPase and transfer of cargo from late endosomes to lysosomes (40,41), did not significantly affect the internalized pool of unstimulated EGFR and EGFRvIII after 60 min (Figure 2G). This demonstrates that lysosomal degradation does not affect the internalized pool of the receptors during the time period of the experiment.

Internalized EGFRvIII is found in recycling compartments

The biotin internalization assay is suited for quantitative measurements of internalization, but it gives little information on the intracellular distribution of the receptor. To test the distribution of internalized EGFR and EGFRvIII, we used confocal microscopy. Since EGFRvIII does not bind any ligands, the only way to detect the receptor is with antibodies. However, it is well established that intact antibodies can induce clustering, activation and internalization of plasma membrane receptors (42–45). In line with this, incubation of NR6M cells with a monoclonal antibody (clone 528) against an extracellular epitope of EGFRvIII resulted in an increase in the phosphorylation of the receptor (Figure 3A). We therefore produced monovalent fab fragments from clone 528, and directly conjugated them to the fluorophore Alexa Fluor 488 (fab-488). The fab fragments did not induce phosphorylation (Figure 3A) and are therefore better suited to label live cells. In contrast to NR6M cells, NR6 cells, which lack EGFR and EGFRvIII expression, were not labeled by fab-488 (Figure 3B and C).

To test the internalization of EGFRvIII and EGFR, cells were incubated with fab-488 (green) and transferrin conjugated to Alexa Fluor 568 (Tf-568) (red) at 4 or 37°C. Transferrin binds to the transferrin receptor (TfR) which is a well-established marker of early sorting and recycling endosomes (39,46,47). After internalization of the two fluorescent probes, surface-bound fab-488 and TfR-568 were stripped off for better visualization of internalized probes. Finally, fixed and non-permeabilized cells were stained for surface EGFR or EGFRvIII to mark the cell surface (blue). No internalization was seen in control cells, which were incubated at 4°C, but residual, unstripped fab-488 and Tf-568 co-localized at the plasma membrane (seen as white dots in the merged picture; note that staining was accentuated to show lack of internalization) (Figure 4A). In contrast, both EGFRvIII and TfR were present in intracellular compartments after 1 h of incubation at 37°C, presumably the peripheral sorting endosomes and the perinuclear recycling endosomes (Figure 4B) (47). The low degree of co-localization of fab-488 and Tf-568 with the surface stain documents that EGFRvIII and transferrin are indeed internalized.

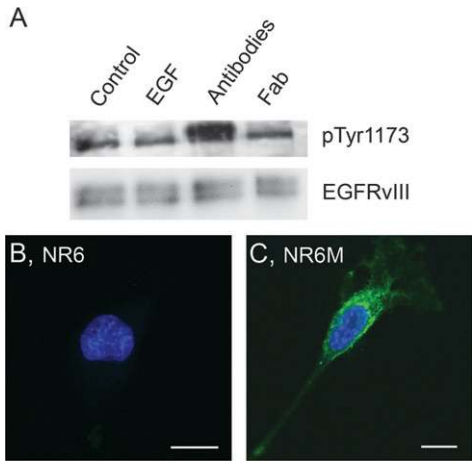

Fig. 3. Antibodies, but not fab fragments, induce phosphorylation of EGFRvIII. (**A**) NR6M cells were incubated for 15 min at 37°C with 10 ng/ml EGF, anti-EGFR/EGFRvIII antibodies or anti-EGFR/EGFRvIII fab fragments conjugated to Alexa Fluor 488 (fab-488) or left untreated (control). This was followed by western blotting with antibody against phosphorylated Tyr1173 of EGFR/EGFRvIII; membrane stripping; and antibody against EGFR/EGFRvIII. (**B** and **C**) NR6 and NR6M cells were incubated with fab-488 (green) for 30 min at 4°C followed by 10 min at 37°C. After fixation, nuclei were stained with To-Pro-3 (blue) and cells examined in a confocal microscope. Only NR6M cells expressing EGFRvIII show staining with fab-488. Bars, 10 μM.

Even though EGFRvIII was primarily seen in transferrin-positive sorting and recycling endosomes, we wished to verify that EGFRvIII is recycled. To this end, cells were stained as described above but additionally treated with monensin during the incubation with fab-488 and Tf-568. This resulted in a relocalization of internalized EGFRvIII and TfR from a scattered peripheral and perinuclear distribution to a dense, perinuclear conglomeration, and the two receptors showed almost complete co-localization (Figure 4C). This change in intracellular localization indicates that internalized EGFRvIII, along with internalized TfR, is concentrated in recycling endosomes when the cells are treated with monensin. The monensin effect was more pronounced using confocal microscopy than seen with the biotin internalization assay; however, in both assays monensin increased the perinuclear or intracellular localization, respectively.

When transfer of proteins to lysosomes was inhibited by incubation with bafilomycin (Figure 4D), a distribution of internalized EGFRvIII and TfR similar to non-treated cells was seen (Figure 4B). This again supports the finding that EGFRvIII is not degraded.

As expected, a pattern of staining much like that of EGFRvIII was seen for unstimulated EGFR (supplementary figure 2, available at *Carcinogenesis* Online). However, EGFR stimulated with EGF conjugated to Alexa Fluor 488 (EGF-488) was internalized, and in contrast to TfR, the distribution of stimulated EGFR was affected by bafilomycin, but only slightly by monensin (supplementary figure 2, available at *Carcinogenesis* Online). A similar effect of monensin on the intracellular distribution of TfR and unstimulated EGFR has previously been reported (32).

EGFRvIII binds c-Cbl but is not ubiquitinylated

To investigate the molecular mechanisms underlying the impairment of EGFRvIII internalization and degradation, we determined receptor phosphorylation, association with c-Cbl and Grb2 and receptor ubiquitinylation and compared the results to that of EGFR.

The level of total EGFR tyrosine phosphorylation and phosphorylation on tyrosine residue (Tyr) 992, Tyr1045 and Tyr1173 in whole-cell lysates and/or EGFR immunoprecipitations of the NR6wtEGFR cell line were low in the absence of EGF but increased upon EGF stimulation (Figure 5A and B). In the NR6M cell line, EGFRvIII was tyrosine phosphorylated independently of EGF stimulation; however, both total phosphorylation and phosphorylation on Tyr992 and Tyr1173 were lower than that of ligand-activated EGFR (Figure 5A and B). Although EGFRvIII was tyrosine phosphorylated, phosphorylation on Tyr1045 was barely detectable (Figure 5A and B and supplementary figure 3, available at *Carcinogenesis* Online). Despite Tyr1045 hypophosphorylation, c-Cbl bound EGFRvIII, but in contrast to activated EGFR, EGFRvIII failed to be ubiquitinylated (Figure 5C and D). To investigate alternative modes of EGFRvIII association with c-Cbl, and since Grb2 is known to mediate binding of c-Cbl to EGFR (23,28), co-precipitation of receptors and c-Cbl were investigated in Grb2 immunoprecipitations (Figure 6A and B). In the NR6wtEGFR cell line, binding of Grb2 to EGFR was increased in the presence of EGF, whereas in the NR6M cell line the interaction between Grb2 and EGFRvIII was independent of EGF incubation (Figure 6A). Furthermore, c-Cbl bound Grb2 in the NR6wtEGFR cell line and the binding was elevated upon EGF stimulation. An interaction between Grb2 and c-Cbl was also observed in the NR6M cell line independent of EGF stimulation. These data indicate that c-Cbl binds EGFRvIII primarily via Grb2 (Figure 6A and B). To further study the interaction between c-Cbl and EGFRvIII, NR6M cells were transfected with N-Cbl and/or d.n.Grb2. N-Cbl is a 50 kDa-truncated mutant of c-Cbl which upon

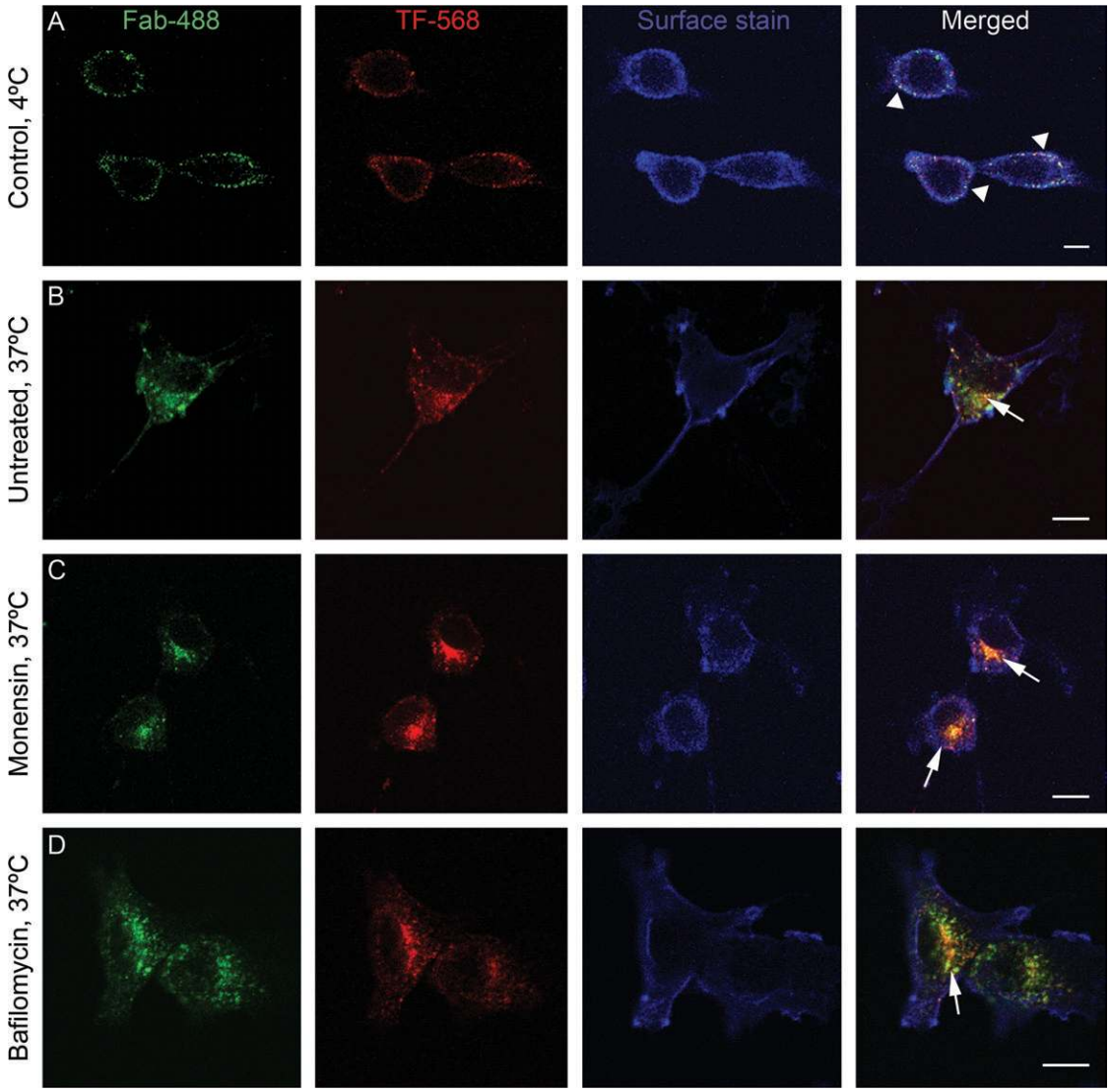

Fig. 4. EGFRvIII is internalized into the TfR-positive recycling compartments. Confocal images of cells incubated with fab-488 (green) and transferrin conjugated to Alexa Fluor 568 (Tf-568) (red) at 4°C for 30 min followed by 1 h at 4 or 37°C. Surface-bound fab-488 and Tf-568 were stripped off with acetic acid (pH 2.4). After fixation, cell surfaces were labeled with anti-EGFR/EGFRvIII antibody (blue). (**A**) NR6M incubated at 4°C. Co-localization of fab-488 and Tf-568 at the plasma membrane is seen as white dots in the merged picture (arrow heads, staining was accentuated to show lack of internalization). (**B**) NR6M incubated at 37°C. Partial co-localization of internalized fab-488 and Tf-568 in peripheral and perinuclear compartments is seen (arrow). (**C**) NR6M incubated with 10 μM monensin at 37°C. Incubation with monensin increases the localization of both internalized fab-488 and internalized Tf-568 in the perinuclear compartment (arrows). (**D**) NR6M incubated with 500 nM bafilomycin at 37°C. Incubation with bafilomycin does not change the distribution of internalized fab-488 and Tf-568 as compared with untreated cells (B). Bars, 10 μM.

over-expression will displace endogenous c-Cbl binding via pTyr1045 but not via Grb2 (26). d.n.Grb2 has both of its SH3 domains mutated so it is not able to bind c-Cbl (21). This gave only inconclusive results on the binding of endogenous c-Cbl, probably due to a relatively low transfection efficiency (data not shown). To avoid this problem, we co-transfected c-Cbl in a relative small concentration with N-Cbl and/or d.n.Grb2. Thus, exogenous c-Cbl would almost exclusively be expressed in cells also expressing N-Cbl and/or d.n.Grb2; however, exogenous c-Cbl would dominate the total c-Cbl pool. In these settings, NR6M cells transfected with d.n.Grb2 showed an almost complete block in c-Cbl binding (Figure 6C). This was also seen when N-Cbl and d.n.Grb2 were co-transfected into NR6M (Figure 6C). In contrast, transfection of N-Cbl alone did not affect the c-Cbl binding to EGFRvIII (Figure 6C), indicating that phosphorylated Tyr1045 is not important for the observed c-Cbl–EGFRvIII interaction. Furthermore, only a very weak binding of N-Cbl to EGFRvIII was observed (Figure 6C), supporting that Tyr1045 is very poorly phosphorylated in EGFRvIII. We therefore conclude that the interaction between c-Cbl and EGFRvIII is primarily via Grb2.

Discussion

EGFRvIII was described for the first time in 1990 (11); however, insight into the degradation and internalization of the receptor has long been elusive. Two recent papers have shed light on EGFRvIII down-regulation (30,31), but important questions has until now not been answered. Most important is whether EGFRvIII is down-regulated when the receptor is expressed with endogenous levels of key degradation proteins and with no other EGFR family members present. Furthermore, the extent of EGFRvIII degradation has only been tested for short periods (31), and as EGFRvIII has a low constitutive activity this might lead to a slow, but significant, degradation, which possibly will not be detected in the short periods investigated. Here, we report that EGFRvIII, independently of EGF, is not

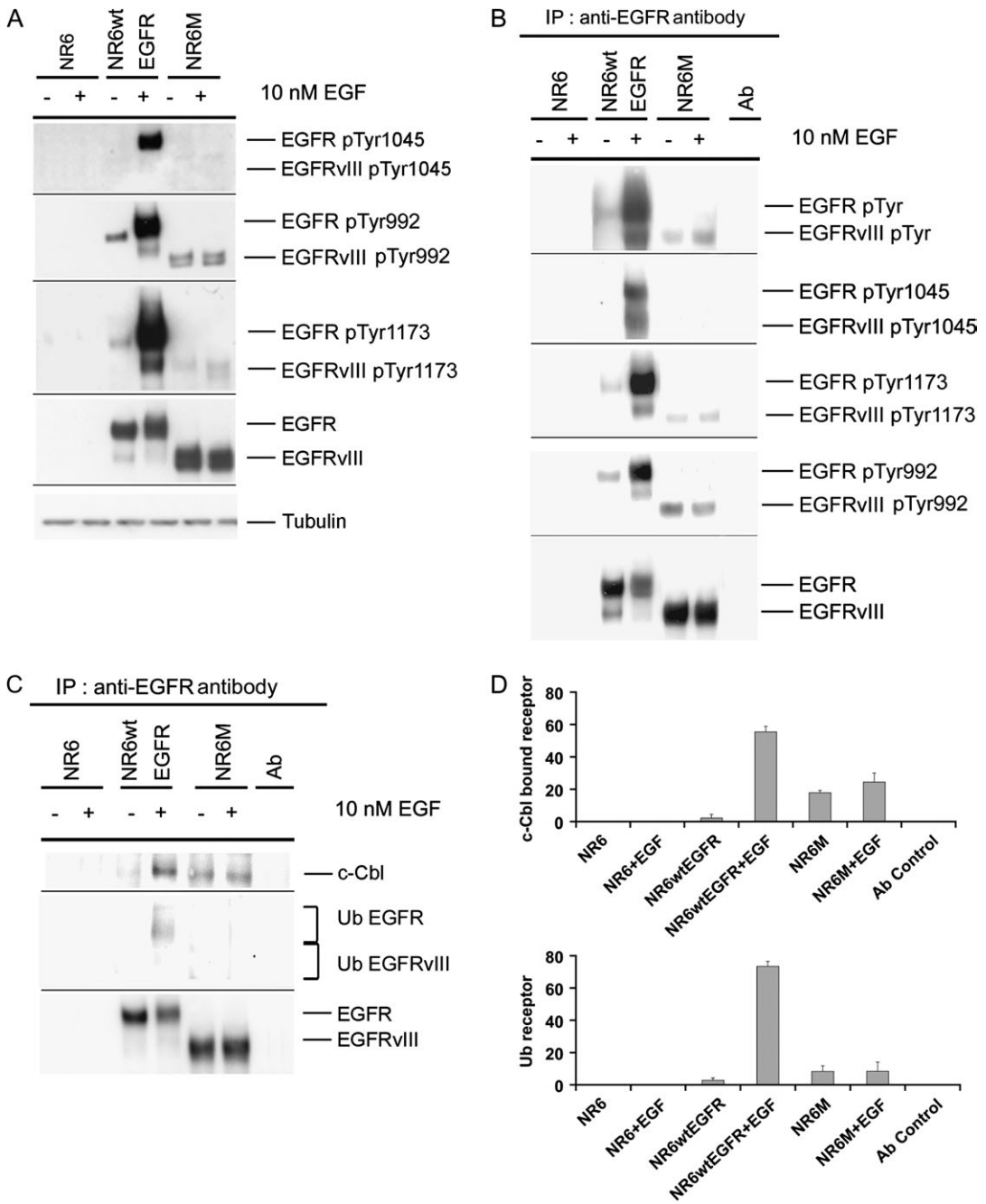

Fig. 5. EGFRvIII binds c-Cbl, but is not ubiquitinylated. (**A**) Cell lines were incubated with (+) or without (−) 10 nM EGF for 15 min. Cells were lysed and equal amounts of protein were subjected to western blotting. Receptors and the phosphorylated amino acid residues Tyr1045, Tyr992 and Tyr1173 on the receptors were detected by sequential immunoblotting and stripping. The stripped blots were reprobed with a tubulin-specific antibody for control of protein loading. (**B** and **C**) Cell lines were treated as described in (A). Cells were lysed and equal amounts of protein were subjected to immunoprecipitation with an EGFR/EGFRvIII-specific antibody. Immunoprecipitated receptors, phosphorylated sites, c-Cbl and ubiquitinylated receptors were detected by sequential immunoblotting and stripping of blots. The antibody control (Ab) was included to detect unspecific binding. Ub indicates ubiquitinylated. (**D**) Quantification of co-immunoprecipitated c-Cbl and level of receptor ubiquitinylation relative to immunoprecipitated receptor measured in arbitrary units from western blots like the one in (C); $n = 3$, bars show standard deviation.

detectably degraded for more than a cell generation time. In contrast, EGFR is degraded and the degradation rate is accelerated upon EGF stimulation. Thus, according to the results presented by Han *et al.* (31) and here, EGFRvIII is very slowly degraded.

The impaired degradation of EGFRvIII could be due to a low level of receptor internalization and/or efficient recycling of internalized receptor, which thereby avoids lysosomal proteolysis. According to Huang *et al.* (48), the internalization of EGFRvIII is less than that of EGF-stimulated EGFR and comparable with that of unstimulated EGFR. However, the antibody used in that study has been shown to activate EGFR (45). It is well established that antibodies can lead to cross-linking, activation and internalization of receptors including the EGFR (42–45). Thus, it is possible that the demonstrated internalization of EGFRvIII following antibody binding is antibody induced. Using a biotin internalization assay with no cross-linking of receptors, we found that EGFRvIII was internalized, but at a rate significantly less than unstimulated EGFR. Furthermore, internalized EGFRvIII was found in recycling compartments as evaluated with confocal

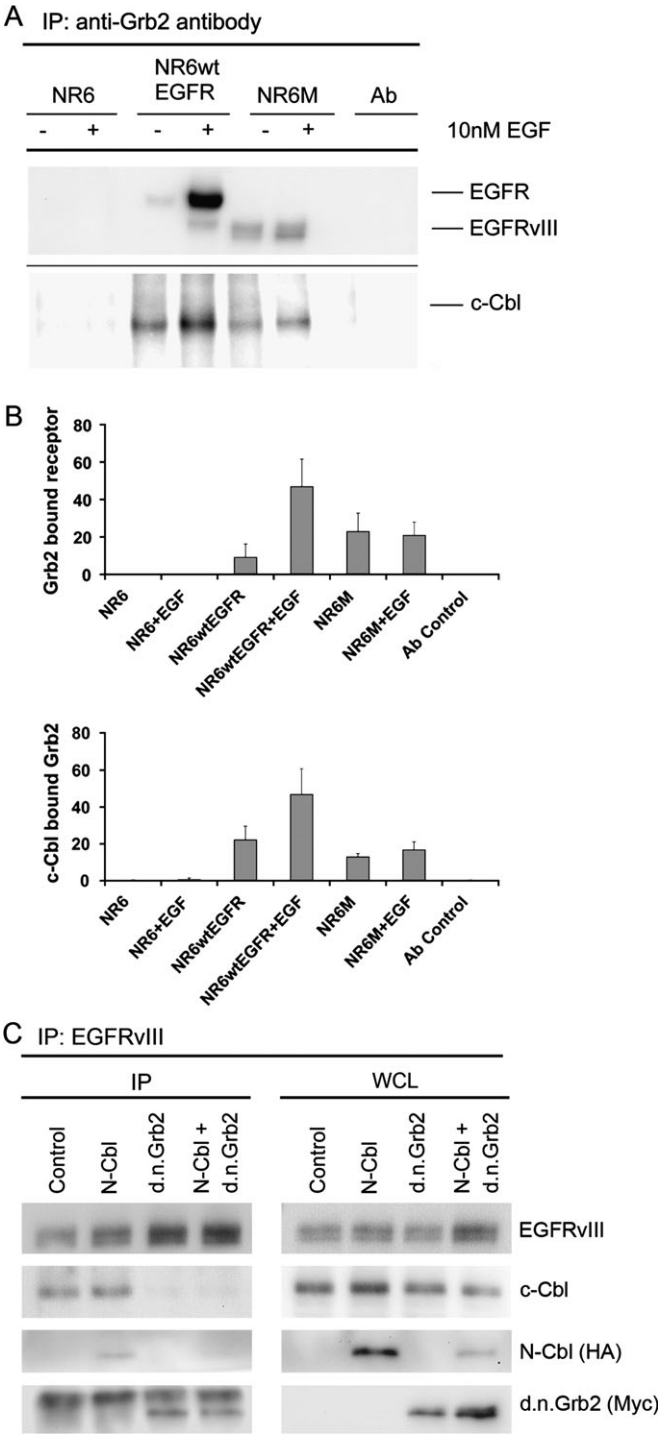

Fig. 6. c-Cbl binds EGFRvIII via Grb2. (**A**) Cells were left untreated (−) or treated (+) with 10 nM EGF for 15 min and then lysed. Grb2 was immunoprecipitated from equal amounts of protein and the receptors and c-Cbl were detected with an EGFR/EGFRvIII- and c-Cbl-specific antibody, respectively. (**B**) Quantification of co-immunoprecipitated EGFR/EGFRvIII and c-Cbl measured in arbitrary units from western blots like the one in (A); $n = 3$, bars show standard deviation. (**C**) NR6M cells were transfected with low concentration of Ha-c-Cbl and co-transfected with either control plasmid, Ha-N-Cbl and/or Myc-d.n.Grb2. After 2 days, cells were lysed and EGFRvIII immunoprecipitated from equal amounts of protein. Whole-cell lysates (WCL) and immunoprecipitated pellets were subjected to western blotting and EGFRvIII, c-Cbl, Ha-N-Cbl and Myc-d.n.Grb2 detected with specific antibodies. Note that the upper band in the immunoprecipitation (IP) d.n.Grb2 blot is antibody light chain.

microscopy. Consequently, we propose that the combination of inefficient internalization together with recycling protects the constitutively active EGFRvIII from being degraded. Such receptor trafficking will most probably not revealed by the FACS assay used by Han *et al.* (31), as this assay detects the level of surface receptor. A steady-state situation where constitutive internalization is followed by recycling will not be detected.

AP-2 and Eps15 are both involved in the internalization of TfR and EGF-stimulated EGFR through clathrin-coated pits (35,37). However, while tyrosine phosphorylation of Eps15 is required for EGF-stimulated EGFR internalization, it does not seem to be necessary for the constitutive internalization of TfR (35). In the present study, both AP-2 and Eps15 were found to bind EGFRvIII, but in contrast to EGF-stimulated EGFR, EGFRvIII did not phosphorylate Eps15, which could contribute to the slow internalization of EGFRvIII.

Even though EGFRvIII was tyrosine phosphorylated, the receptor seemed to be hypophosphorylated at Tyr1045. This is in agreement with previous reports showing that EGFRvIII phosphorylation on Tyr1045 is negligible compared with other tyrosine residues (8,31,48). However, we found c-Cbl to bind EGFRvIII and this interaction could potentially be mediated through Grb2, as we and others find this adaptor protein to be strongly associated with the receptor (18,49,50). Concordantly, we found that Grb2 was bound to c-Cbl in NR6M cells, and transfection with a d.n.Grb2 abolished the c-Cbl–EGFRvIII interaction, suggesting that c-Cbl binds EGFRvIII primarily through Grb2.

Using either mutated c-Cbl or a mutant EGFR, in which Tyr1045 was substituted with phenylalanine, it has been shown that direct binding of c-Cbl to EGFR is necessary for ubiquitinylation and degradation of the receptor (21,28,51,52). However, others have demonstrated that also indirect binding of c-Cbl through Grb2 can lead to ubiquitinylation (21,28). Davies *et al.* (30) found that over-expressed Cbl-b can bind to EGFRvIII, thereby showing that Cbl proteins are indeed capable of associating with EGFRvIII. In concordance with this, we observed an interaction between c-Cbl and EGFRvIII, whereas Schmidt *et al.* (53) and Han *et al.* (31) did not. However, in agreement with our results, they did not detect EGFRvIII ubiquitinylation (31,53). Despite the differences in c-Cbl binding, results presented by Schmidt *et al.* (53), Han *et al.* (31) and here indicate that restricted and inefficient binding of c-Cbl to EGFRvIII leads to defective receptor ubiquitinylation and degradation.

The lack of receptor ubiquitinylation may also explain the impaired internalization and recycling of EGFRvIII. c-Cbl-dependent ubiquitinylation has been suggested to serve as an internalization signal for EGFR and seems to be required for the translocation of EGFR to clathrin-coated pits (27). Furthermore, Waterman *et al.* (28) have found that defective phosphorylation on Tyr1045 of EGFR results in less internalization and more recycling of the activated receptor. Others have shown that transport of EGFR to lysosomes is dependent on ubiquitinylation and phosphorylated Tyr1045, whereas receptor internalization is not (21,26). As for EGFRvIII, our data suggest that the restricted c-Cbl binding and low ubiquitinylation lead to less receptor internalization followed by recycling of internalized receptors.

Han *et al.* (31) and Davies *et al.* (30) recently published conflicting observations on EGFRvIII down-regulation. Han *et al.* (31) used cell lines that express other EGFR family members, including ErbB2. ErbB2 is actively restrained from internalization under normal conditions (46,54–56), and the restrain is transferred to EGFR upon heterodimerization of the two receptors (32). This shows that the EGFR family members can negatively affect the down-regulation of each other. Davies *et al.* (30) used cells expressing no endogenous EGFR family members, but they over-expressed Cbl proteins and ubiquitin, which are key proteins in EGFR degradation. Thus, the difference between Han *et al.* (31) and Davis *et al.* (30) might very well be due the different expression levels of EGFR family members, and to the equilibrium shift for the ubiquitin association with EGFRvIII that happens when ubiquitin and Cbl proteins are over-expressed. To avoid these potential problems, we have used a cell system with no expression of other EGFR family members and with endogenous levels of degradation proteins.

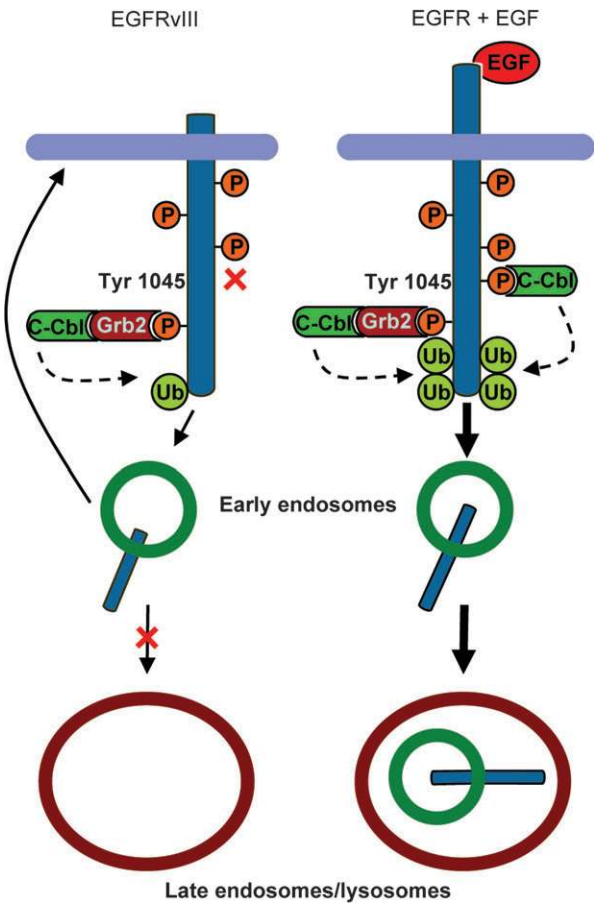

Fig. 7. Suggested model for the impaired down-regulation of EGFRvIII. EGFRvIII avoids degradation due to low or no c-Cbl-mediated ubiquitinylation. See Discussion for details. P and Ub indicate phosphorylation and ubiquitinylation, respectively. Red crosses indicate low or no binding/transport.

It has been hypothesized that the lack of EGFRvIII down-regulation is due to a general phosphorylation level below a threshold necessary for signal attenuation (53). Data presented here support this hypothesis, and in agreement with previous reports (8,31,48), our results indicate that the impaired down-regulation could be due to a lower level of phosphorylation at the specific site Tyr1045. However, it seems that the threshold necessary for signal attenuation can be circumvented by increased Cbl binding and enhanced ubiquitinylation when Cbl and ubiquitin are over-expressed (30).

In summary, we propose that c-Cbl binds to EGFRvIII primarily via Grb2, but this does not lead to effective receptor ubiquitinylation. Together with low phosphorylation of adaptor proteins, this results in impaired internalization. Lack of ubiquitinylation most probably prevents transport of internalized EGFRvIII from endosomes to lysosomes, and the receptor is recycled to the plasma membrane. Low internalization and recycling of internalized receptor leads to the low level of EGFRvIII degradation (illustrated in Figure 7).

Supplementary material

Supplementary figures 1–3 can be found at http://carcin.oxfordjournals.org/

Acknowledgements

We greatly appreciate the skilled work of Mette Ohlsen, Ulla Hjortenberg and Izabella Rasmussen. We thank Nina Pedersen, Mads Lerdrup, Anette Hommelgaard, Kirstine Roepstorff, Silas Bruun and Frederik Vilhardt for valuable discussions. This study was supported by grants from the Danish Cancer Society, the Danish Medical Research Council, the Novo Nordic Foundation, the John and Birthe Meyer Foundation, the Illum Foundation and the Danish Cancer Research Foundation

Conflict of Interest Statement: None declared.

References

1. Moscatello,D.K. *et al*. (1995) Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res.*, **55**, 5536–5539.
2. Olapade-Olaopa,E.O. *et al*. (2000) Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. *Br. J. Cancer*, **82**, 186–194.
3. Wikstrand,C.J. *et al*. (1998) The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *J. Neurovirol.*, **4**, 148–158.
4. Okamoto,I. *et al*. (2003) Expression of constitutively activated EGFRvIII in non-small cell lung cancer. *Cancer Sci.*, **94**, 50–56.
5. Batra,S. *et al*. (1995) Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Diff.*, **6**, 1251–1259.
6. Pedersen,M.W. *et al*. (2004) Expression of a naturally occurring constitutively active variant of the epidermal growth factor receptor in mouse fibroblasts increases motility. *Int. J. Cancer*, **108**, 643–653.
7. Pedersen,M.W. *et al*. (2005) Analysis of the epidermal growth factor receptor specific transcriptome: effect of receptor expression level and an activating mutation. *J. Cell. Biochem.*, **96**, 412–427.
8. Pedersen,M.W. *et al*. (2005) Differential response to gefitinib of cells expressing normal EGFR and the mutant EGFRvIII. *Br. J. Cancer*, **93**, 915–923.
9. Burgess,A.W. *et al*. (2003) An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Mol. Cell*, **12**, 541–552.
10. Kuan,C.T. *et al*. (2001) EGF mutant receptor vIII as a molecular target in cancer therapy. *Endocr. relat. cancer*, **8**, 83–96.
11. Sugawa,N. *et al*. (1990) Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *PNAS*, **87**, 8602–8606.
12. Ekstrand,A.J. *et al*. (1994) Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. *Oncogene*, **9**, 2313–2320.
13. Huang,F. *et al*. (2005) Growth factor receptor binding protein 2-mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis. *Mol. Biol. Cell*, **16**, 1268–1281.
14. Damstrup,L. *et al*. (2002) Epidermal growth factor receptor mutation type III transfected into a small cell lung cancer cell line is predominantly localized at the cell surface and enhances the malignant phenotype. *Int. J. Cancer*, **97**, 7–14.
15. Wikstrand,C.J. *et al*. (1997) Cell surface localization and density of the tumor-associated variant of the epidermal growth factor receptor, EGFRvIII. *Cancer Res.*, **57**, 4130–4140.
16. Fernandes,H. *et al*. (2001) Glycosylation-induced conformational modification positively regulates receptor-receptor association. A study with an aberrant epidermal growth factor receptor (EGFRvIII/Delta EGFR) expressed in cancer cells. *J. Biol. Chem.*, **276**, 5375–5383.
17. Luwor,R.B. *et al*. (2004) The tumor-specific de2-7 epidermal growth factor receptor (EGFR) promotes cells survival and heterodimerizes with the wild-type EGFR. *Oncogene*, **23**, 6095–6104.
18. Montgomery,R.B. (2002) Antagonistic and agonistic effects of quinazoline tyrosine kinase inhibitors on mutant EGF receptor function. *Int. J. Cancer*, **101**, 111–117.
19. O'Rourke,D.M. *et al*. (1998) Inhibition of a naturally occurring EGFR oncoprotein by the p185neu ectodomain: implications for subdomain contributions to receptor assembly. *Oncogene*, **16**, 1197–1207.
20. Tang,C.K. *et al*. (2000) Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer. *Cancer Res.*, **60**, 3081–3087.
21. Grovdal,L.M. *et al*. (2004) Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. *Exp. Cell Res.*, **300**, 388–395.
22. Jiang,X. *et al*. (2003) Epidermal growth factor receptor internalization through clathrin-coated pits requires Cbl RING finger and proline-rich domains but not receptor polyubiquitylation. *Traffic*, **4**, 529–543.

23. Jiang,X. *et al*. (2003) Grb2 regulates internalization of EGF receptors through clathrin-coated pits. *Mol. Biol. Cell*, **14**, 858–870.
24. Levkowitz,G. *et al*. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.*, **12**, 3663–3674.
25. Levkowitz,G. *et al*. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell*, **4**, 1029–1040.
26. Longva,K.E. *et al*. (2002) Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *J. Cell Biol.*, **156**, 843–854.
27. Stang,E. *et al*. (2004) Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. *Mol. Biol. Cell*, **15**, 3591–3604.
28. Waterman,H. *et al*. (2002) A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. *EMBO J.*, **21**, 303–313.
29. Wiley,H.S. *et al*. (1991) The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.*, **266**, 11083–11094.
30. Davies,G.C. *et al*. (2006) EGFRvIII undergoes activation-dependent downregulation mediated by the Cbl proteins. *Oncogene*, **25**, 6497–6509.
31. Han,W. *et al*. (2006) Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII. *Cancer Biol. Ther.*, **5**, 1361–1368.
32. Haslekas,C. *et al*. (2005) The inhibitory effect of ErbB2 on epidermal growth factor-induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane. *Mol. Biol. Cell*, **16**, 5832–5842.
33. Abulrob,A. *et al*. (2004) Interactions of EGFR and caveolin-1 in human glioblastoma cells: evidence that tyrosine phosphorylation regulates EGFR association with caveolae. *Oncogene*, **23**, 6967–6979.
34. Delft,S.V. *et al*. (1997) Association and colocalization of Eps15 with adaptor protein-2 and clathrin. *J. Cell Biol.*, **136**, 811–821.
35. Confalonieri,S. *et al*. (2000) Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. *J. Cell Biol.*, **150**, 905–912.
36. Sorkina,T. *et al*. (1999) Clathrin, adaptors and eps15 in endosomes containing activated epidermal growth factor receptors. *J. Cell Sci.*, **112**, 317–327.
37. Huang,F. *et al*. (2004) Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J. Biol. Chem.*, **279**, 16657–16661.
38. Basu,S.K. *et al*. (1981) Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts. *Cell*, **24**, 493–502.
39. Stein,B.S. *et al*. (1984) Complete inhibition of transferrin recycling by monensin in K562 cells. *J. Biol. Chem.*, **259**, 14762–14772.
40. Drose,S. *et al*. (1997) Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J. Exp. Biol.*, **200**, 1–8.
41. van Deurs,B. *et al*. (1996) Inhibition of the vacuolar H(+)-ATPase with bafilomycin reduces delivery of internalized molecules from mature multivesicular endosomes to lysosomes in HEp-2 cells. *Eur. J. Cell Biol.*, **69**, 343–350.
42. Huet,C. *et al*. (1980) The antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. *Cell*, **21**, 429–438.
43. Schreiber,A.B. *et al*. (1983) Biological role of epidermal growth factor-receptor clustering. Investigation with monoclonal anti-receptor antibodies. *J. Biol. Chem.*, **258**, 846–853.
44. Schwartz,A.L. *et al*. (1986) Antibody-induced receptor loss. Different fates for asialoglycoproteins and the asialoglycoprotein receptor in HepG2 cells. *J. Biol. Chem.*, **261**, 15225–15232.
45. Winkler,M.E. *et al*. (1989) Epidermal growth factor and transforming growth factor alpha bind differently to the epidermal growth factor receptor. *Biochemistry*, **28**, 6373–6378.
46. Hommelgaard,A.M. *et al*. (2004) Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. *Mol. Biol. Cell*, **15**, 1557–1567.
47. Maxfield,F.R. *et al*. (2004) Endocytic recycling. *Nat. Rev. Mol. Cell Biol.*, **5**, 121–132.
48. Huang,H.-J.S. *et al*. (1997) The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J. Biol. Chem.*, **272**, 2927–2935.
49. Moscatello,D.K. *et al*. (1996) Transformational and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene*, **13**, 85–96.
50. Prigent,S.A. *et al*. (1996) Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway. *J. Biol. Chem.*, **271**, 25639–25645.
51. Ravid,T. *et al*. (2004) c-Cbl-mediated ubiquitinylation is required for epidermal growth factor receptor exit from the early endosomes. *J. Biol. Chem.*, **279**, 37153–37162.
52. de Melker,A.A. *et al*. (2004) Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. *J. Biol. Chem.*, **279**, 55465–55473.
53. Schmidt,M.H. *et al*. (2003) Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. *Proc. Natl Acad. Sci. USA*, **100**, 6505–6510.
54. Baulida,J. *et al*. (1996) All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J. Biol. Chem.*, **271**, 5251–5257.
55. Lerdrup,M. *et al*. (2006) Geldanamycin stimulates internalization of ErbB2 in a proteasome-dependent way. *J. Cell Sci.*, **119**, 85–95.
56. Sorkin,A. *et al*. (1993) The carboxyl terminus of epidermal growth factor receptor/erbB-2 chimerae is internalization impaired. *Oncogene*, **8**, 3021–3028.

Received October 10, 2006; revised March 2, 2007; accepted March 9, 2007